STUDY REPORT

IN VITRO ASSESSMENT OF THE ACUTE EXPOSURE OF ORGANOTYPIC HUMAN BRONCHIAL TISSUE CULTURES TO AEROSOL PRODUCED BY THE TOBACCO HEATING SYSTEM 2.2 (STUDY NUMBER S1709)

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1 APPROVAL

1.1 Study Director's Statement of Compliance

The study director was accountable for this study.
The study director takes responsibility for the study having being executed as defined in the study plan.
Each laboratory head and supervisor is accountable for the data generated in his or her laboratory.

Name	Date / Signature
Study Director Carole Mathis	Not available. On maternity leave. Julia Hoeng
Laboratory Head - Cellular Research Stefan Frentzel	01-Jul-2016 Stefan Frentzel
Laboratory Head - Proteomics Ashraf Elamin	01-Jul-2016
Supervisor - Gene Expression Emmanuel Guedj	01.Jul.2016
Supervisor - Tissue Research Keyur Trivedi	01-JUL-2016 K.A. Trivedi
Statistician Patrice Leroy	01-JUL-2016
Computational Scientist Yang Xiang	01-Jul-2016 Yang Xiang
Program Manager Julia Hoeng	7-July-2016 Julia Hoeng

1.2 Quality Assurance Statement

Not applicable. This study was not subject to Good Laboratory Practice regulations.

2 GENERAL INFORMATION

2.1 Names and Addresses

Sponsor	Manuel C. Peitsch Philip Morris Products S.A Research & Development Biological System Research Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Philip Morris Products S.A Research & Development PMI Product Testing Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Former Study Director(s)	Experimental Repetition 1 (also known as Main Phase 2): Carole Mathis Experimental Repetition 2 (also known as Main Phase 3): Carole Mathis Experimental Repetition 3 (also known as Main Phase 4): Anita Iskandar Experimental Repetition 4 (also known as Main Phase 6): Anita Iskandar Experimental Repetition 5 (also known as Main Phase 7): Anita Iskandar Experimental Repetition 6 (also known as Main Phase 8): Carole Mathis Dose Range Assessment (also known as Dose Range Finding): Carole Mathis

Study Director Deputy(s)	Experimental Repetition 1 (also known as Main Phase 2): Stefan Frentzel Experimental Repetition 2 (also known as Main Phase 3): Stefan Frentzel Experimental Repetition 3 (also known as Main Phase 4): Carole Mathis Experimental Repetition 4 (also known as Main Phase 6): Carole Mathis Experimental Repetition 5 (also known as Main Phase 7): Carole Mathis Experimental Repetition 6 (also known as Main Phase 8): NA (not available) Dose Range Assessment (also known as Dose Range Finding): Anita Iskandar
Other Scientists/Technicians Involved in the Study (in alphabetical order)	Philippe Alexandre Guy, Martin Almstetter, Camille Basile, Karine Baumer, Abdelkader Benyagoub, Flavia Buchler, Maica Corciulo, Remi Dulize, Lola Hamied, Stephanie Johne, Samuel Kleinhans, Diana Kuehn, Shoaib Majeed, Florian Martin, Maude Mayer, Celine Merg, Dariusz Peric, Carine Poussin, Sophie Scheuner, Alain Sewer, Fabio Talamo, Laura Ortega Torres, Gregory Vuillaume, Filippo Zanetti.

2.2 Study Schedule

Experimental Start Date:	27.11.2013
Experimental Completion Date:	04.11.2015

2.3 Test Guidelines

- Health Canada Intense (HCI) Smoking Regimen, Official Method T-115, Determination of "Tar", Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, December 31, 1999 (adapted to meet the special conditions used in the Vitrocell® system)
- International Organization for Standardization: International Standard ISO 3402, Tobacco and Tobacco Products – Atmosphere for Conditioning and Testing, 4th ed., 1999.

3 DEVIATIONS FROM THE STUDY PLAN

The study plan can be accessed at: 1709_Organotypic_Bronch_SP
The deviations that were made from the study S1709 plan are outlined below.

Table 1. Deviations from the Study Plan

Number	Description	Justification of impact on the study data and the data integrity
1	**Concerning the Test Item: Batch Number** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.1.1) by updating the batch information for test items used in experimental repetitions as appropriate when new batches were used. This change was made to ensure that tobacco sticks were used before their expiry dates.	The batch certificates were used to ensure a comparable quality of the test items used. Therefore, it was considered that the deviations would not have a negative impact on data integrity.
2	**Concerning the Test System: Human Bronchial Tissue Culture** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.2.1) by replacing the EpiAirway-FT™ (with fibroblasts) test system (from Mattek), which was used in one experiment (not reported here), with the EpiAirway™ (without fibroblasts) test system in experiments 1 and 2 and with the bronchial Mucilair™ test system (from Epithelix) in experiments 3–6 and in the dose range assessment (DRA). The co-culture model was changed to the mono-culture model because of a tissue integrity problem linked to shrinkage of the fibroblast layer. The EpiAirway™ system was abandoned and the MucilAir™ system used because the MucilAir™ model had better morphology, because of the absence of vacuoles.	No negative impact on data integrity.
3	**Concerning the Experimental Design: Concentrations of the Reference and Test Items** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.1.4.2) by expressing the concentrations of the reference and test items in terms of the nicotine concentration in whole smoke/aerosol, rather than percent of smoke (based on EXtrelut® measurements, described in the study plan 1709_Organotypic_Bronch_SP in section 4.1.4.1 c *Nicotine Determination*). An additional test item concentration, at a high nicotine concentration, was tested in additional experimental repetitions. This change was made to: (i) allow different batches of reference and test item sticks to be standardized in terms of the nicotine concentrations in the smoke and aerosol produced; (ii) allow the nicotine concentrations in different reference and test items to be compared straightforwardly; and (iii) allow the impacts of a higher test item concentration to be assessed. **Concerning the Experimental Design: Number of Exposure Runs per Experimental Repetition** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.3). Three inserts were used for each endpoint for each exposure condition and each post-exposure time point in each smoking run in each experimental repetition in experiments 1 and 2. This was changed to one insert in each	No negative impact on data integrity, as only units for the applied smoke/ aerosol concentration were changed in data tables and figures. The experimental unit was changed to smoke exposure run, rather than tissue insert replicate of a single run, to account for run-to-run variation has the highest effect size, shown in recent experiments (Majeed 2014).

Table continues

	smoking run and three smoking runs in each experimental repetition in experiments 3–6 and the dose range assessment. This change was made to allow variability in the exposure runs to be assessed.	
4	**Concerning Reagents: Negative Controls** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.1.2), in which it was specified that controls treated with phosphate buffered saline (PBS) should be used as negative controls for both the adenylate kinase (AK) assay and the pro-inflammatory markers release (MAP) assay. Future experimental repetitions will have a different negative control for each assay, i.e., untreated controls will be used as negative controls for the AK assay and PBS-treated controls will be used as negative controls for the MAP assay. This change was made to allow a specific and appropriate negative control to be used for each assay. **Concerning Reagents: AK Cytotoxicity Assay Kits** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.5.5.2), in which the Bioluminescence Cytotoxicity Assay (BioVision) kit was specified to be used. The ToxLight™ Bioassay Kit (Lonza) was also used in the additional experimental repetitions. The specific kits that were used were recorded. This change was made to allow an alternative kit to be used if problems were found with a batch of the specified kit.	The changes regarding the reagents used for negative controls were done to have more appropriate controls (more compatible to be compared to their positive controls). Moreover, although two different kits were used for the AK assay, only one specific brand of the kit were used within a particular experimental repetition, and the cytotoxicity levels were always normalized to their positive and negative controls within the specific experimental repetition. Therefore, it was considered that these deviations would not have a negative impact on data integrity
5	**Concerning Procedures: Total Particulate Matter** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.5.11) by, in additional experimental repetitions, measuring the deposition of particles in real time using a microbalance while the reference item 3R4F was exposed for 28 min rather than while the test item was exposed. This change was made because the high liquid droplet content of the test item aerosol caused the microbalance measurements to be inaccurate.	No negative impact on data integrity
6	**Concerning the Study Timelines** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 6.1) because more experimental repetitions being conducted. The experiment completion and study reporting dates were later than 21 November 2013. This change was made to allow experimental repetitions to be performed to decrease the probability that the observed effects were caused by experimental variability (e.g., caused by variations over time, between tissue batches, and between different technicians) thereby gaining increased statistical power.	No negative impact on data integrity, as experimental conditions remained unchanged.
7	**Concerning Pro-inflammatory Marker Release Measurements** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.5.7) by using slightly different lists of Milliplex kits to measure the release of pro-inflammatory markers in experiments 1 and 2 and in experiments 3–6 (as shown in Table 9). This change was made to limit the measurement of analytes to those showing a clear response and whose basal levels are above the limits of detection. CCL11/Eotaxin, EGF, IL10, and IL13 were not measured in experiments 3–6.	No negative impact on data integrity, as experimental conditions remained unchanged.

Table continues

8	**Concerning the Experimental Groups: CYP Assays** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.3, Table 5) by only measuring the combined CYP1A1 and CYP1B1 activity at 24 h, 48 h, and 72 h post-exposure time points in experiment 2 and only measuring the combined CYP1A1 and CYP1B1 activity 48 h after exposure in experiments 3–6. This change was made to allow the results to be focused on only one post-exposure time point. Note: Measurements of the CYP1A1 and CYP1B1 protein concentrations were only part of the method development stage, and the results are therefore not reported here.	No negative impact on data integrity, as experimental conditions remained unchanged.
9	**Concerning the Experimental Groups: Trans-epithelial Electrical Resistance** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.3) in the experimental repetitions. The trans-epithelial electrical resistance was measured only as a quality control check (for randomly selected inserts) on the day a batch of tissue was delivered to confirm that the information provided in the Certificate of Analysis for the batch was correct.	No negative impact on data integrity, as experimental conditions remained unchanged.
10	**Concerning the Experimental Groups: New Method Development** Deviation from the study plan 1709_Organotypic_Bronch_SP (section 4.3) in the experiment 4 (a.k.a. Main Phase 6), experiment 5 (a.k.a. Main Phase 7), and experiment 7 (a.k.a. Main Phase 9), new methods/assays for proteomics analysis, mucociliary clearance, and enzyme-lined lectin assays were developed. However, because these measurements were in the developmental phase, the endpoint measurements were not reported here and considered pilot assays.	No negative impact on data integrity, as experimental conditions remained unchanged.

4 ABSTRACT/ EXECUTIVE SUMMARY

Human organotypic bronchial epithelial tissue culture is a relevant *in vitro* model to study the impact of inhaled toxicants such as whole cigarette smoke. This *in vitro* model is composed of basal cells, mucus-releasing cells and ciliated cells and has the capacity to secrete inflammatory markers and to undergo adaptive morphological changes upon challenge. The objective of the study was to assess and compare the impact of an acute exposure to combustible cigarette smoke (3R4F) and the aerosol from THS 2.2. Various concentrations of THS 2.2 aerosol were administered for 28 minutes at the air-liquid interface of human organotypic bronchial cultures and compared with cultures exposed to either fresh air or smoke from the reference cigarette 3R4F for up to 72 h post-exposure. To generate robust data, the experiments have been repeated multiple times, using different batches of bronchial organotypic epithelium from two different adult non-smoking donors. Overall, the impact of THS2.2 exposure was more comparable to air-exposed bronchial tissue cultures than to 3R4F-exposed tissue at comparable nicotine concentrations. This was observed for endpoints such as cytotoxicity, CYP1A1/1B1 activity, the release of pro-inflammatory mediators and tissue histology (higher number of apoptotic cells, epithelial atrophy, lower goblet cell abundance and loss of cilia/ciliated cells). By using systems toxicology-based risk assessment approaches combining computable biological network models and gene expression changes, the molecular perturbations in response to 3R4F smoke and THS 2.2 aerosol exposure were compared. The transcriptomics changes induced after 3R4F exposure indicated significant perturbations at all post-exposure time points in the cell fate networks (Senescence, Necroptosis, Apoptosis), in the cell stress networks (Xenobiotic Metabolism Response, Oxidative stress, Osmotic stress, NFE2L2 signaling), in the cell proliferation networks (Wnt signalling, nuclear receptors, Mapk and Jak Stat signaling, growth factors pathways) and in the inflammatory processes networks (Tissue damage, Epithelial Mucus Hypersecretion, Epithelial Innate Immune Activation).. At a comparable nicotine concentration, the exposure to THS2.2 showed almost the same perturbed networks, but with a much lower level compared with 3R4F exposure. Moreover, the response was mainly transient observed only at the early post-exposure time point. The overall assessment results support a reduced impact of an acute exposure of the aerosol generated from THS2.2 product on human bronchial epithelial tissue culture compared to combustible cigarette smoke.

5 INTRODUCTION

Cigarette smoke (CS) with more than 4000 compounds and 10^{14} oxidants per puff, is the major cause of two common lung disorders, chronic obstructive pulmonary disease (COPD) and bronchogenic carcinoma (Harvey 2007, Wang 2015). In parallel, the growing development of novel tobacco products and their increasing popularity raise the need of reliable toxicity assessment. CS is linked to oxidative stress, inflammation, genetic and epigenetic changes in the respiratory tract (Goldkorn 2014). The target tissue for CS is the epithelium lining the upper and lower airways (Wolz 2002). Airway epithelial cells as first point of contact for inhaled agents play critical roles in the defence against airborne pollutants. (Proud 2011). Accordingly, airway epithelial cells represent an important type of cells for studying the pathological effects of CS. These cells secrete a variety of cytokines, growth factors, and chemokines following exposure to stimuli that have pro-inflammatory effects in the epithelium and infiltration of immune cells (Baxter 2015, Garcia-Canton 2013). All these factors have significant roles in the pathogenesis of inflammatory diseases and may cause cell damage, altered mucin production, impaired barrier defense, and airway remodeling to quote only the best known hallmarks of respiratory diseases following CS exposure (Crotty Alexander 2015).

Bronchial epithelial cells have been used to study the biological activity of airborne substances (Gabrielson 1994). Mostly two-dimensional (2D) monolayer culture models have been widely used in lung toxicity studies and are typically based on lung-derived epithelial cells. Although they are readily available and frequently used, they have a number of limitations, including (i) their inherent tumorigenicity (e.g., the human bronchial epithelial cells immortalized using the hybrid virus of adenovirus 12 and simian virus 40 BEAS-2B cell line and the type-2 alveolar cell-like adenocarcinoma-derived tumor A549 cell line) (Frappart 2008); (ii) their ability to only partially mimic normal airway epithelial cell behavior (Singh 2006); (iii) the lack of multiple cell types (Rothen-Rutishauser 2008); and (iv) the inability to achieve organotypic differentiation (Constant 2014). Various more complex models of the respiratory epithelium have been developed, especially air–liquid interface (ALI) three-dimensional (3D) organotypic culture models (Duell 2011). Among these 3D organotypic culture models the EpiAirway™ and MucilAir™ tissue models are increasingly being used as an alternative *in vitro* test system in inhalation toxicity studies (BeruBe 2009, Constant 2014, Nichols 2014).

Human bronchial 3D organotypic culture models comprise a fully differentiated and polarized epithelium. They are composed of differentiated bronchial epithelial cells and have '*in vivo* like' features in terms of both morphology and gene expression. Moreover global gene expression profiles correlated between 3D organotypic tissues cultured at the ALI and bronchial epithelial cells collected directly *in vivo*, albeit with some differences (Mathis 2013, Pezzulo 2011). ALI cells expressed higher levels of basal cell-related genes, and brushed cells expressed higher levels of cilia-related genes. This correlates well with the higher percentage of basal cells in ALI cultures and higher percentage of ciliated cells in brushed cells (Dvorak 2011). The epithelial cells forming 3D organotypic culture models are arranged in a pseudo-stratified pattern. This epithelial morphology (all cells in contact with the basement membrane but only some reaching the luminal surface) is found in tissues lining the trachea and in the upper respiratory tract. These intact epithelia form a biological barrier by expressing tight junctions important in *in vivo* situations. The tight junctions hold the cells together and maintain the polarities of the cells, allowing two distinct compartments to be defined: the apical compartment (the air side in an *in vitro* culture) and basolateral compartment (the medium side in an

in vitro culture). This compartmentalization closely resembles the situation for *in vivo* tissue, i.e., the airway epithelial cells are the front line against exposure to toxic stimuli in ambient air and cigarette smoke. By ensuring the polarity of the cells, which prevents extracellular lateral diffusion processes (i.e. for most materials entering of the cell is required), osmotic balance and transcellular transport processes are maintained. This polarity allows, for example, receptor-mediated endocytosis at the apical surface to be distinguished from exocytosis at the basolateral surface. The transcriptional profile of such *in vitro* cultures recapitulated that of the *in vivo* airway epithelial tissues (Mathis 2013, Pezzulo 2011).

These 3D organotypic culture models have the potential to increase the robustness and relevancy of the evaluation of CS smoke toxicity (Paur 2011, Rothen-Rutishauser 2008). First, they resemble the *in vivo* exposure conditions of the relevant target tissue as they are cultured at the ALI (Baxter 2015, Paur 2011). Second, studies have reported that the ALI culture set-up allows the bronchial epithelial cells to differentiate and exhibit normal characteristics similar to the *in vivo* airway epithelium. Third, these cultures resemble the human respiratory tract epithelium in terms of their morphological and molecular properties (Aufderheide 2015, Iskandar 2013, Iskandar 2015).

Several studies have been conducted in which organotypic lung culture models were used to investigate the molecular and physiological effects of CS on respiratory cells. They consistently showed that CS up-regulated the activities of several CYP isoforms, especially CYP1A1 and CYP1B1. Moreover CS was associated with marked changes in gene expression profiles as determined by gene set enrichment (Hoeng 2014, Iskandar 2013, Iskandar 2015). It has been shown that differential expression in response to cigarette smoke can be assigned to functional categories and mapped to signaling pathways, such as xenobiotic metabolism, the oxidant/antioxidant balance, and DNA damage and repair (Mathis 2015, Maunders 2007). The changes in gene expression caused by CS exposure on organotypic lung epithelial cells were also consistent with those observed in human smokers (Iskandar 2013, Iskandar 2015).

3D organotypic culture models offer broad potential to study the toxicity of aerosols and the underlying pathophysiological mechanisms *in vitro*. Therefore are key *in vitro* models for systems toxicology assessments of our modified risk tobacco products in line with 3R strategy and the goals of the 21st Century Toxicology framework.

In this study, human bronchial organotypic cultures were exposed for 28 min to diluted whole 3R4F smoke and THS2.2 aerosol at two matching nicotine concentrations. The aim of the study was to assess the biological impact of exposure to a candidate of modified risk tobacco products (MRTPs), the Tobacco Heating System (THS)2.2, on human organotypic bronchial cultures, as compared with that of smoke from reference cigarette 3R4F at comparable nicotine concentrations, using biological endpoint measurements (cytotoxicity, histology, CYP1A1/1B1 activity, pro-inflammatory markers release, and gene expression) and systems toxicology approaches.

6 OBJECTIVES

The objectives of the study were:

To determine the biological impact of THS2.2 aerosol exposure on a human organotypic 3D bronchial culture model, as compared with that of 3R4F smoke. The biological impact assessment was done by combining results obtained from analyses using well known cellular and functional assays, and using systems biology approaches (leveraging mRNA and miRNA profile, as well as computational biology analyses).

The biological impact of THS2.2 aerosol and 3R4F smoke exposure on the test system human 3D bronchial cultures were explored in terms of:

- Dose-dependent responses of the test system after exposure
- Time-dependent responses of the test system after exposure (whether the impact transient or sustained over the post-exposure time)
- The possible biological pathway/processes perturbed in the test system after exposure

Moreover, the dose-range assessment of a broader concentrations of THS2.2 aerosol exposure was done to assess the concentration of THS2.2 aerosol at which overt cytotoxicity and morphological alterations of the human 3D bronchial cultures were observed.

This is an exploratory study. All the above objectives were exploratory.

7 EXPERIMENTAL DESIGN

7.1 Test and Reference Items

7.1.1 Identification and Description

The test item was the THS2.2 product specified in Table 2, and the reference item was the combustible reference cigarette 3R4F.

Table 2. Summary of the Test and Reference Items

Short Name	Description	Item Type
THS2.2	ZRH/DDA1/C3/CAST LEAF – CL/Flavour/Reynaldo	Test
3R4F	Conventional Standard Reference Cigarette	Reference

Information on the characteristics of the test item, including the composition, stability, and homogeneity of the product, was provided by the sponsor. The 3R4F reference cigarettes were purchased from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Kentucky Tobacco Research & Development Center). Information on the test and reference item batches used in the experiments is given in Table 3.

Table 3. Specifications of the Reference and Test Items Used in Each Experimental Repetition

	Experimental Phase						
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	DRA
	Dec 2013	Feb 2014	Mar 2015	Jun 2015	Jul 2015	Sep 2015	Apr 2015
Reference Item (3R4F)							
Stick Information on 3R4F							
Pack No.	V347Y61B5	V347Y61B5	V350Y61B5	V350Y61B5	V350Y61B5	V350Y61B5	NA
Date Received	Jul 2012	Jul 2012	Dec 2014	Dec 2014	Dec 2014	Dec 2014	NA
Expiry Date	Dec 2014	Dec 2014	May 2017	May 2017	May 2017	May 2017	NA
Test Item (THS2.2)							
Stick Information on THS2.2							
Stick Batch No.	THS2.2 Batch B-05771	THS2.2 Batch B-08163	THS2.2 Batch B-13055	THS2.2 Batch B-13055	THS2.2 Batch B-13055	THS2.2 Batch B-13055	THS2.2 Batch B-13055
Date Received	Jun 2013	Jan 2014	Dec 2014	Dec 2014	Dec 2014	Dec 2014	Dec 2014
Expiry Date	Jan 2014	Oct 2014	Jan 2016	Jan 2016	Jan 2016	Jan 2016	Jan 2016
Device Information on THS2.2							
Product Code	Device THS2.2 B-06050	Device THS2.2 B-06050	Device THS2.2 B-18731	Device THS2.2 B-18731	Device THS2.2 B-18731	Device THS2.2 B-18731	Device THS2.2 B-18731
Date Received	04 Sep 2013	04 Sep 2013	27 Feb 2015	27 Feb 2015	27 Feb 2015	27 Feb 2015	27 Feb 2015
Specific Description	Aerosol generation	Aerosol generation	Aerosol generation	Aerosol generation	Aerosol generation	Aerosol generation	Aerosol generation
Batch Description	DC.000067.RD(1)/ZRH/FPD4.2/3.2.2/C28/FDP 4.2 Cigarette Holder/3.2.2 software upgrade/Configuration without forced-cleaning feature.	DC.000067.RD(1)/ZRH/FPD4.2/3.2.2/C28/FDP 4.2 Cigarette Holder/3.2.2 software upgrade/Configuration without forced-cleaning feature. Aerosol generation.					

Abbreviations: DRA, dose range assessment; Exp., experiment; Month, month the experiment was performed in; NA, not available (i.e., not tested); THS, tobacco heating system. Information on the 3R4F reference item was provided by the supplier (Kentucky Tobacco Research & Development Center).

7.1.2 Item Storage

Once received, all of the items were stored in their original packaging at 5 ± 3 °C in a refrigerator. The humidity of the atmosphere in the refrigerator was not controlled.

7.1.3 Item Conditioning

Before use, each test and reference item was conditioned for between 7 d and 21 d at 22 ± 1 °C and a relative humidity of 60% ± 3%, as specified in International Organization for Standardization guidelines.

7.1.4 Carbonyl Levels Deposited in the Cultivation Base Module of the Vitrocell® 24/48 exposure System following 28 min Exposure to Reference Smoke and Test Aerosol

Figure 1 shows the amount of various carbonyls deposited in Base Module of the Vitrocell® 24/48 exposure system (filled with phosphate-buffered saline (PBS)) following 28 min of exposure to reference 3R4F smoke and test item aerosols (see section 7.4.3 for details) at the corresponding target nicotine concentrations used in this study.

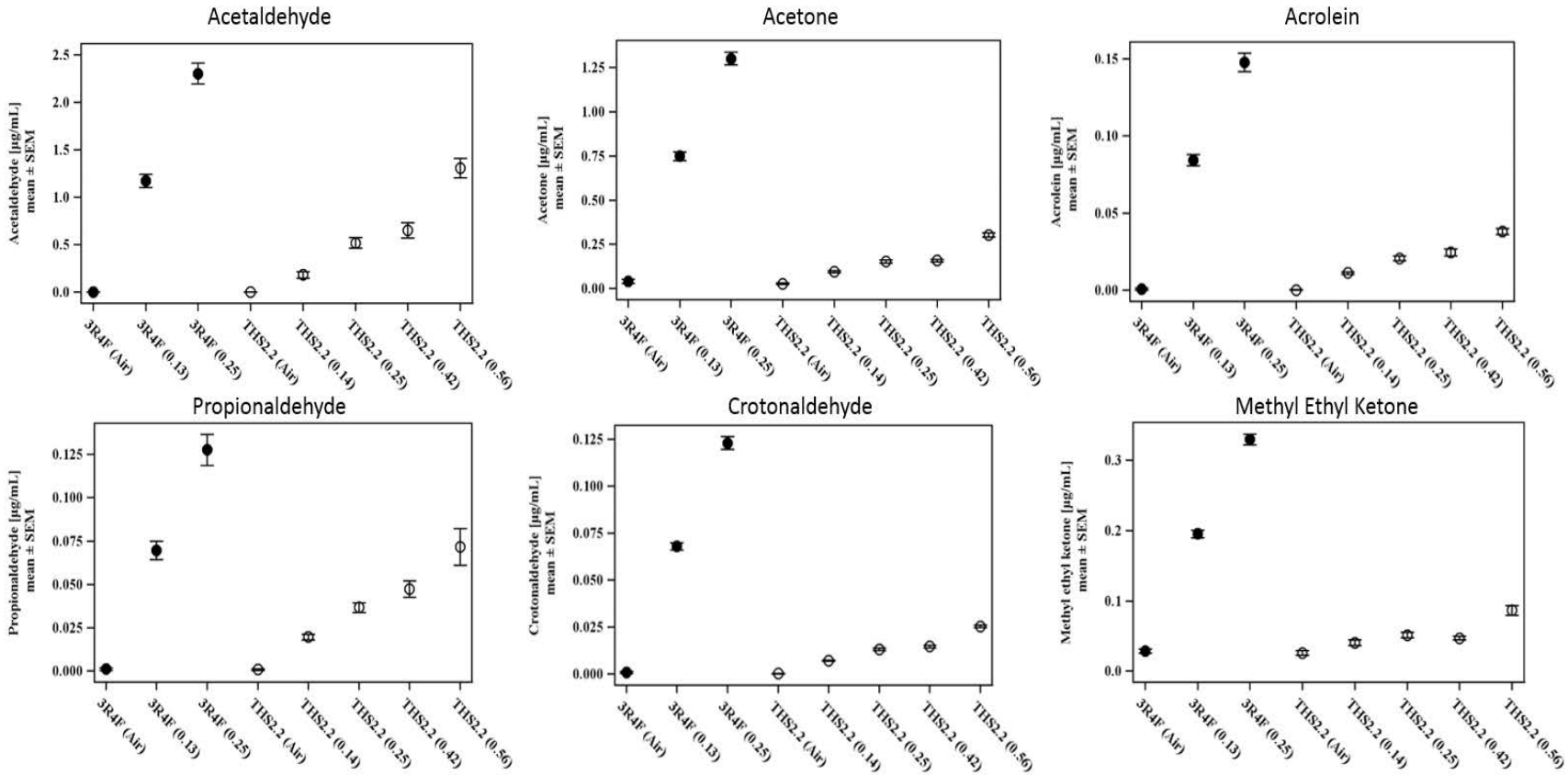


Figure 1. Concentrations of representative carbonyls deposited in the Cultivation Base Module of Vitrocell® 24/48 exposure system following exposures to the reference cigarette smoke and test item aerosol.

The mean concentration (in µg/mL of phosphate buffered saline) ± the standard error of the mean (N = 10 samplings during the study period) is shown for each carbonyl in each test. Descriptive statistics, including the concentrations found in each set of samples, are given in Supplementary Table 1-Supplementary Table 8.
Abbreviations: N, number of sample; SEM, standard error of the mean; THS, tobacco heating system.

7.2 Test System

Human organotypic bronchial epithelial tissue cultures were purchased from two different suppliers. Cultures purchased from MatTek (Ashland, MA, USA) were used in experiments 1 and 2, and cultures purchased from Epithelix (Geneva, Switzerland) were used in experiments 3–6 and the dose range assessment. The specifications of the cultures used in the experiments are shown in Table 4.

Table 4. Specifications of the Different Batches of Bronchial Tissue Culture Used in the Experimental Repetitions

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	DRA
Month	Dec 2013	Feb 2014	Mar 2015	Jun 2015	Jul 2015	Sep 2015	Apr 2015
Tissue Delivery Date	27 Nov 2013	12 Feb 2014	13 Mar 2015	26 Jun 2015	03 Jul 2015	28 Aug 2015	24 Apr 2015
Supplier	MatTek	MatTek	Epithelix	Epithelix	Epithelix	Epithelix	Epithelix
Bronchial Model	EpiAirway	EpiAirway	MucilAir	MucilAir	MucilAir	MucilAir	MucilAir
Lot Number	Lot 19434	Lot 19480	ND	ND	ND	ND	ND
Donor ID	ND	ND	MD050401	MD050401	MD050401	MD050401	MD050401
Batch ID (Seeding Date)	ND	ND	5 Feb 2015	21 May 2015	26 May 2015	24 Jul 2015	16 Mar 2015
Cell Type	hAEC/ bronchial	hAEC/ bronchial	hAEC/ bronchial	hAEC/ bronchial	hAEC/ bronchial	hAEC/ bronchial	hAEC/ bronchial
Age of Donor	23	23	28	28	28	28	28
Sex of Donor	Male	Male	Male	Male	Male	Male	Male
Origin of Donor	ND	ND	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Smoking Status of Donor	Non-smoker	Non-smoker	Non-smoker	Non-smoker	Non-smoker	Non-smoker	Non-smoker
Pathology Status of Donor	None reported	None reported	None reported	None reported	None reported	None reported	None reported
Sterile	See notes	See notes	Yes	Yes	Yes	Yes	Yes
Mycoplasma	See note	See note	Negative	Negative	Negative	Negative	Negative
TEER[1] (Ω cm^2)	ND	ND	379 ± 12	395 ± 11	386 ± 13	401 ± 16	314 ± 9
CBF[1] (Hz)	ND	ND	7.4 ± 0.2	7.7 ± 0.1	7.2 ± 0.1	7.8 ± 0.1	7.1 ± 0.2
Morphology	ND	ND	Normal	Normal	Normal	Normal	Normal
Mucus	ND	ND	Present	Present	Present	Present	Present
HIV-1 and HIV-2	See note	See note	Negative	Negative	Negative	Negative	Negative
Hepatitis B	See note	See note	Negative	Negative	Negative	Negative	Negative
Hepatitis C	See note	See note	Negative	Negative	Negative	Negative	Negative
Certificate of Analysis	See note	See note	CoA_seeded_05-Feb-2015	CoA_seeded_21-May-2015	CoA_seeded_26-May-2015	CoA_seeded_24-Jul-2015	CoA_seeded_16-Mar-2016

Note: Batches received from MatTek were not accompanied by certificates of analysis, but each batch of Epithelix MucilAir™ inserts was accompanied by a certificate of analysis. However, MatTek provided a general "tissue and cell source declaration", in which it was stated that the cells used to produce MatTek cell and tissue model products are screened for the potential biological contaminants HIV-1 viruses, hepatitis B viruses, hepatitis C viruses, bacteria, yeasts, and fungi.

[1] Each transepithelial electrical resistance (TEER) and cilia beating frequency (CBF) is given as the mean ± the standard error of the mean (N = 12 tissue culture inserts). Abbreviations: CoA, certificate of analysis; DRA, dose range assessment; Exp., experiment; Month, month in which the experiment was performed; ND, not done or not communicated by the supplier.

7.3 Sample Generation

7.3.1 Experimental Repetitions: The Impact Assessment of THS2.2 Aerosol as Compared with 3R4F Smoke Exposure

Six experimental repetitions were performed. In each experiment, tissue cultures were exposed to smoke produced from the reference items and aerosol produced from the test items at similar nicotine concentrations. The target nicotine concentrations used in the experiments are shown in Table 5.
Smoke samples from the 3R4F reference items were generated using a SM 2000 30 port carousel smoking machine (Philip Morris International). Aerosol samples from the THS2.2 test items were generated using a SM 2000 ZRH 30 port carousel smoking machine (Philip Morris International). Each smoking machine was connected to a Vitrocell® 24/48 exposure system (Vitrocell Systems, Waldkirch, Germany), in which the tissue inserts were exposed.

Table 5. Target Nicotine Concentrations (mg/L) in the Smoke and Aerosol Used in the Experiments

	Experimental Month						
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	
	Dec 2013	Feb 2014	Mar 2015	Jun 2015	Jul 2015	Sep 2015	
Group Name of 3R4F-exposed Cultures	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**Smoking Machine**
Air Control (3R4F)	0.00	0.00	0.00	0.00	0.00	0.00	SM 2000
3R4F (0.13)	0.13	0.13	0.13	0.13	0.13	0.13	
3R4F (0.25)	0.25	0.25	0.25	0.25	0.25	0.25	
Group Name of THS2.2-exposed Cultures	**mg nicotine/L aerosol**	**mg nicotine/L aerosol**	**mg nicotine/L aerosol**	**mg nicotine/L aerosol**	**mg nicotine/L aerosol**	**mg nicotine/L aerosol**	**Smoking Machine**
Air Control (THS2.2)	0.00	0.00	0.00	0.00	0.00	NA	SM 2000 ZRH
THS2.2 (0.14)	0.14	0.14	0.14	0.14	0.14	NA	
THS2.2 (0.25)	0.25	0.25	0.25	0.25	0.25	NA	
THS2.2 (0.42)	NA	NA	0.42	0.42	0.42	NA	
THS2.2 (0.56)*	NA	0.56	NA	NA	NA	NA	

* The higher THS2.2 concentration (at a nicotine concentration of 0.56 mg/L) was tested in only one experiment, so the results for this concentration were omitted from the Results sections. Abbreviations: Exp., experiment; Month, the month in which the experiment was performed; NA, not available (not tested during the experiment).

The Vitrocell® 24/48 exposure system was equipped with a dilution system, which allows mixing of the smoke/aerosol with fresh air. To achieve the desired concentrations of nicotine, the smoke/aerosol stream was mixed with different rates of dilution airflow, while passing through the Vitrocell® 24/48 exposure system. Figure 2 illustrates the set-up of the exposure system, which consists of a Dilution/Distribution Module (the top part) and Cultivation Base Module (underneath). From the Dilution/Distribution Module, diluted smoke/aerosol is conducted by negative pressure into the Cultivation Base Module, which houses the tissue culture inserts during exposure.
The apparatus used in the 3R4F smoke and THS2.2 aerosol tests are shown schematically in Figure 2, and the target nicotine concentrations are shown in Table 5.

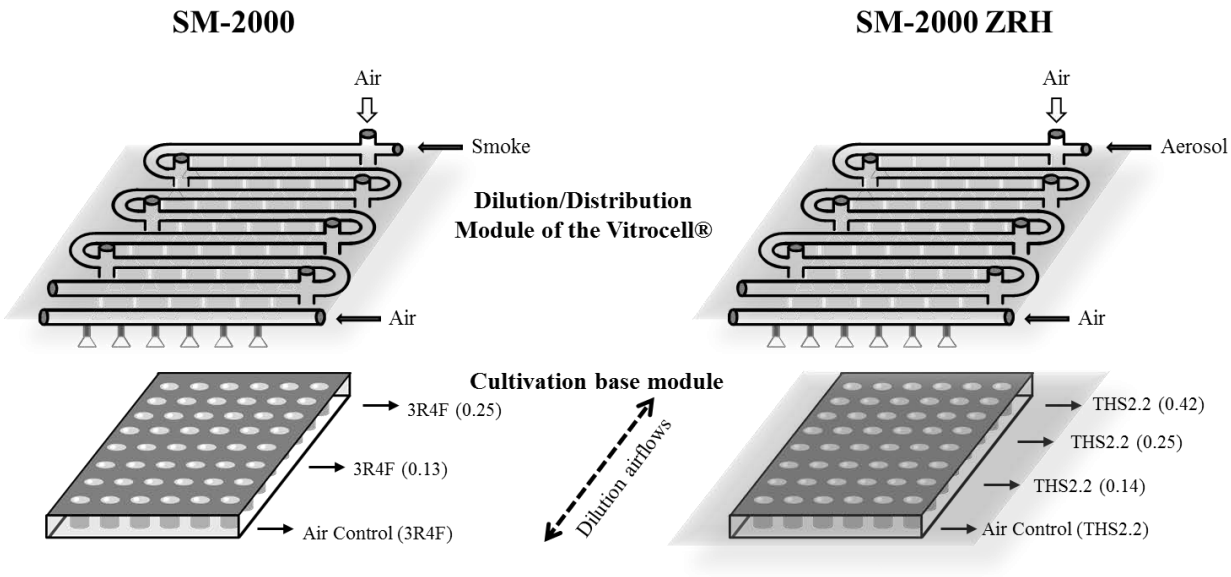


Figure 2. Schematic representation of the Vitrocell® 24/48 exposure system.
A Dilution/Distribution Module was located above a Cultivation Base Module. Up to 48 wells (tissue culture inserts) in the Cultivation Base Module could be simultaneously exposed in a single run. Diluted whole smoke or aerosol was delivered to each well through an individual trumpet. An exposure run included smoke and/or aerosol tests at the target concentration(s) (given in parenthesis for each test item) and an appropriate air control (N = 1 insert per run for any given endpoint and post-exposure time point).

Data for various endpoints were acquired in each experimental repetition, as shown in Table 6. The results presented in this document were calculated from the aggregated data obtained in experiments 1–6.

Table 6. List of Endpoints Used in Each Experimental Repetition and the Post-exposure Time Points

Experiment and Month		Cytotoxicity				Gene Expression Microarray				Inflammatory Mediators				CYP1A1/CYP1B1 Activity				Tissue Histology and IHC			
		Post-exposure time (h)				Post-exposure time (h)				Post-exposure time (h)				Post-exposure time (h)				Post-exposure time (h)			
		4	24	48	72	4	24	48	72	4	24	48	72	4	24	48	72	4	24	48	72
Exp. 1	Dec 2013	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	NA	NA	√	√
Exp. 2	Feb 2014	√	√	√	√	√	√	√	√	√	√	√	√	NA	√	√	√	NA	NA	√	√
Exp. 3	Mar 2015	√	√	√	√	√	√	√	√	√	√	√	√	NA	NA	√	NA	NA	NA	NA	√
Exp. 4	Jun 2015	√	√	√	√	√	√	√	√	√	√	√	√	NA	NA	√	NA	NA	NA	√	√
Exp. 5	Jul 2015	√	√	√	√	√	√	√	√	√	√	√	√	NA	NA	√	NA	NA	NA	√	√
Exp. 6	Sep 2015	√	√	√	√	√	√	√	√		√	√	√	NA	NA	√	NA	NA	NA	√	√

Abbreviations: CYP, cytochrome P450; Exp., experiment; IHC, immunohistochemistry; NA, not available (i.e., endpoint data were not generated or collected in the experimental repetition).

7.3.2 Dose Range Assessment of the THS2.2 Aerosol

The dose range of the aerosol produced by the test item was assessed using various concentrations of the diluted aerosol produced by the THS2.2 using a modified SM 2000 ZRH 30 port carousel smoking machine and the Vitrocell® 24/48 exposure system. The dilutions that were used and the endpoints that were measured are shown in Table 7. Three independent exposure runs were performed for each endpoint, and one replicate was performed in each exposure run.

Table 7. Concentrations of diluted Aerosol (%) Applied and Endpoints Assessed in the Dose Range Assessment of THS2.2

	Dose Range Assessment Experiment, Performed in April 2015			
Group	**Aerosol Concentrations in the Dilution/Distribution Module of the Vitrocell® 24/48 exposure system**	**Endpoints Measured 72 h after Exposure**		**Smoking Machine Used**
		Cytotoxicity (N)	**Tissue Histology (N)**	
Air Control (THS2.2)	-	√ (3)	√ (3)	SM 2000 ZRH
THS2.2 Dilution 1	13%	√ (3)	√ (3)	
THS2.2 Dilution 2	23%	√ (3)	√ (3)	
THS2.2 Dilution 3	31%	√ (3)	√ (3)	
THS2.2 Dilution 4	48%	√ (3)	√ (3)	
THS2.2 Dilution 5	60%	√ (3)	√ (3)	
THS2.2 Dilution 6	80%	√ (3)	√ (3)	
THS2.2 Dilution 7	100%	√ (3)	√ (2)*	

Abbreviations: N, number of replicates; THS, tobacco heating system. * One histology sample not reported (problem with the sample integrity).

7.4 Testing Procedure

7.4.1 Generation of Aerosol and Smoke and the *in Vitro* Exposure Regimens

Reference smoke from 3R4F reference cigarettes was generated using a SM 2000 30-port carousel smoking machine connected to a Vitrocell® 24/48 exposure system for inserts containing 24 wells. A SM 2000 ZRH 30 port carousel smoking machine was used to generate test aerosol from THS2.2 items. The 3R4F cigarettes and THS2.2 sticks were conditioned for at least 48 h (but up to 21 d) at 22 ± 1 °C at a relative humidity of 60% ± 3%, as required by the International Organization for Standardization standard ISO 3402 (International Organization for Standardization). In the exposure experiments, each 3R4F cigarette was smoked to a standard butt length of approximately 35 mm and each THS2.2 stick was used for up to 12 puffs per stick. During a 28 min exposure experiment, ten 3R4F cigarettes were smoked and ten THS2.2 sticks were aerosolized using the dedicated smoking machines, using a 55 mL puff lasting 2 s twice per minute and an 8 s pump exhaust time (following the Health Canada smoking regimen).

7.4.2 Determination of Nicotine in Whole Smoke and Whole Aerosol

The nicotine concentrations in whole smoke or test aerosol delivered to the *in vitro* exposure systems were routinely determined during each *in vitro* exposure experiment to ensure that the 3R4F smoke and THS2.2 aerosol the cultures were exposed to had the same nicotine concentrations. Diluted whole 3R4F smoke or THS2.2 aerosol at the exhaust of the dilution and distribution module of the Vitrocell® 24/48 system was trapped on an EXtrelut® 3NT column (Merck Millipore, Billerica, MA, USA) impregnated with 2 mL of 0.5 M H_2SO_4 (following work instruction (PMI_RD_WKI_001066). Whole smoke or aerosol produced from two sticks per test item (10 puffs per stick, with a trapping duration of 5 min) was trapped. Each stick was smoked or aerosolized using a modified Health Canada intense puffing protocol of a 55 mL puff lasting 2 s twice per minute with an 8 s pump exhaust time. The exposed EXtrelut® columns were either processed immediately or stored at 2–8 °C for a maximum of four weeks before being processed. When the columns were processed, nicotine was determined by gas chromatography-flame ionization detection (following PMI_RD_WKI_000409). Briefly, 250 µL of isoquinoline (4 mg/mL), which was used as an internal standard, was added to the top of the column and the trapped nicotine and the internal standard were eluted by adding 5 mL of a 5% vol/vol solution of triethylamine in N-butylacetate three times (giving a total volume of 15 mL). The extract was mixed well and then 1 mL of the extract was transferred to an amber glass vial for analysis. A 1 µL aliquot of the extract was injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), which was used in split mode with a split ratio of 1:20 and an injector temperature of 220 °C. Separation was performed in isothermal mode at an oven temperature of 140 °C (using a runtime of 6 min) using a 15 m DB-5 fused silica capillary column (Agilent Technologies) with an internal diameter of 0.25 mm and a film 0.25 µm thick. The carrier gas was helium, and the flow rate was 2.25 mL/min. The nicotine peak in a chromatogram was detected, integrated, and quantified (using an internal standard calibration curve) automatically using ChemStation software (Agilent Technologies).

7.4.3 Determination of Carbonyls in Whole Smoke or Aerosol

Carbonyls in PBS exposed to whole smoke or test aerosol (10–12 sticks per item, each used following a modified Health Canada intense puffing protocol of 55 mL puff over 2 s twice per minute with an 8 seconds pump exhaust time) (following PMI_RD_WKI_001049) were measured. Briefly, before exposure, each row in the Cultivation Base Module of the Vitrocell® 24/48 exposure system was filled with 18.5 mL of PBS. After the PBS had been exposed to smoke or aerosol, a 1.2 mL aliquot of a PBS sample from each row was mixed with 1.8 mL of a 2,4-dinitrophenylhydrazine solution (15 mM). Then, 150 µL of pyridine was added to quench the derivatization reaction. A 500 µL aliquot of the mixture was then placed in an amber glass liquid chromatography-mass spectrometry vial. After 30 min, 485 µL of acetonitrile and 15 µL of an internal standard mixture (24 µg/mL of acetone-d6 and 21 µg/mL of methyl ethyl ketone-d5) were added. Finally, 5 µL of the sample was injected into a high performance liquid chromatograph coupled to a tandem mass spectrometer, which was used to determine the concentrations of various carbonyls using an isotope dilution technique.

7.4.4 Maintenance of the Bronchial MucilAir™ 3D-organotypic Tissue Cultures (Air–Liquid Interface Cultures)

As soon as received, each human bronchial MucilAir™ (Epithelix) culture was placed in fresh culture medium (following PMI_RD_WKI_001065). To achieve this, 0.7 mL of MucilAir™ culture medium (Epithelix), pre-warmed to 37 °C, was placed in each well in 24-well culture plates. MucilAir™ inserts were then transferred from the shipping plates to the fresh plates using sterile tweezers. MucilAir™ cultures grown in 6.5 mm Transwell® inserts (Corning, Tewksbury, MA, USA) were used in each *in vitro* exposure study to allow the dimensions of the Vitrocell® 24/48 exposure system to be matched. The MucilAir™ cultures were maintained at 37 °C (in a 5% CO_2 atmosphere with 90% humidity), and the medium was changed every two days, as specified in the instructions provided by the supplier. The cultures were regularly inspected using a microscope throughout each study to check for potential morphological changes and for bacterial or fungal contamination.

7.4.5 Maintenance of EpiAirway™ 3D-organotypic Tissue Cultures (Air–Liquid Interface Cultures)

As soon as received, each human bronchial EpiAirway™ culture (MatTek) was placed in fresh culture medium (following PMI_RD_WKI_001064). To achieve this, 5.5 mL of medium, pre-warmed to 37 °C, was placed in each well in 12-well culture plates with modified lids (HNG-TOP-2x; MatTek). EpiAirway™ cultures grown in 6.5 mm Transwell® inserts (Corning) were used in each *in vitro* exposure study to allow the dimensions of the Vitrocell® 24/48 exposure system to be matched. The EpiAirway™ cultures were maintained at 37 °C (in a 5% CO_2 atmosphere with 90% humidity), and the medium was changed every 2–3 d, as specified in the instructions provided by the supplier. The cultures were regularly inspected using a microscope throughout each study to check for potential morphological changes and bacterial or fungal contamination.

7.4.6 Apical Washing of the MucilAir™ and EpiAirway™ 3D-organotypic Tissue Cultures (Air–Liquid Interface Cultures)

Three days before the MucilAir™ tissue inserts (Epithelix) or EpiAirway™ tissue inserts (MatTek) were exposed to smoke or aerosol, the apical sides of the inserts were gently washed by adding, dropwise, 200 µL of culture medium (see above) that had been pre-warmed to 37 °C. Not more than 12 tissue inserts at a time were washed in this way, to avoid the cells suffering prolonged stress. The medium was carefully aspirated using a Pasteur pipette connected to a vacuum pump. The apical wash removed accumulated mucus and minimized the risk of the apical sides of the tissue cultures having high mucus loads, which would interfere with the subsequent exposure tests. The apical wash procedure was performed following PMI_RD_WKI_001087.

7.4.7 Adenylate Kinase Cytotoxicity Assay

The AK activities in the basolateral media of 3D organotypic tissue cultures were measured using BioVision bioluminescence cytotoxicity assay kits (BioVision, Milpitas, CA, USA), following PMI_RD_WKI_001048. Briefly, 50 µL of a basolateral medium was transferred to a well in a luminescence-compatible 96-well microtiter plate and mixed with 50 µL of "reagent working solution". The mixture was incubated for 5 min, then the luminescence was measured using a FluoStar Omega reader (BMG Labtech, Ortenberg, Germany). Alternatively, a ToxiLight™ bioassay kit (Lonza, Rockland, MA, USA) was used to determine the cytotoxicity of an aerosol to 3D organotypic tissue cultures. This was achieved by transferring 20 µL of basolateral medium to a well in a luminescence-compatible 96-well microtiter plate and mixing it with 100 µL of AK detection reagent (Lonza). The mixture was incubated for 5 min, then the luminescence was measured using a FluoStar Omega reader. The AK assay results were normalized by treating the basolateral sides of 3D organotypic tissue inserts with Triton X100, at a final concentration of 1%, for 24 h to induce the complete lysis of the cells (i.e., 100% cytotoxicity). The kit used in each experiment is stated in Table 8.

Table 8. Cytotoxicity Kits Used in the Different Experiments

Experiment and Month		BioVision Kit	ToxiLight™ Kit
Experiment 1	Dec 2013	√	
Experiment 2	Feb 2014	√	
Experiment 3	Mar 2015		√
Experiment 4	Jun 2015		√
Experiment 5	Jul 2015		√
Experiment 6	Sep 2015		√
DRA	Apr 2015		√

Abbreviation: DRA, dose range assessment.

7.4.8 Measurements of Secreted Pro-inflammatory Mediators

Multi-analyte profiling of pro-inflammatory mediators (MAP analysis) was carried out by collecting 200 µL of basolateral medium from a 3D organotypic tissue culture 4 h, 24 h, and 48 h after the culture had been exposed to whole smoke or aerosol. The MAP analysis was performed following PMI_RD_SOP_000361, PMI_RD_WKI_001032, PMI_RD_WKI_001274, and PMI_RD_FOR_000803). The analysis was performed using the Luminex® xMAP® technique (Luminex, Austin, TX, USA), using commercially available assay panels (EMD Millipore, Schwalbach, Germany), as outlined in Table 9 and following the instructions provided by the manufacturer. A 25 µL aliquot of a diluted or undiluted sample was used in each analysis, and the analysis was performed using a Luminex® 200™ or FLEXMAP 3D® instrument equipped with xPONENT software v.4.2. (Luminex). The data were represented as median fluorescence intensities, and a five-parameter logistic or spline curve-fitting method was used to calculate the concentrations of the analytes in a sample. The positive controls were MucilAir™ cultures (N = 3) treated with TNFα and IL-1β for 24 h (following PMI_RD_FOR_000849). The TNFα and IL-1β were each added to the basolateral medium at a final concentration of 10 ng/mL. The TNFα and IL-1β were added to induce the majority, but not all, of the mediators targeted by the MAP analysis.

Table 9.Summary of the Milliplex Kits Used in the Experimental Repetitions

Experiment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
Month and Year	Dec 2013	Feb 2014	Mar 2015	Jun 2015	Jul 2015	Sep 2015
CCL11/Eotaxin	a	a	NA	NA	NA	NA
CSF3/G-CSF	a	a	d	d	d	d
CSF2/GM-CSF	a	a	d	d	d	d
IL-1a	a	a	d	d	d	d
IL-1b	a	a	d	d	d	d
CXCL10/IP10	a	a	d	d	d	d
CCL2/MCP1	a	a	d	d	d	d
CCL5/RANTES	a	a	d	d	d	d
VEGFA	a	a	d	d	d	d
IL-6	a	a	d	d	d	d
CSCL8/IL-8	a	a	d	d	d	d
EGF	b	b	NA	NA	NA	NA
CXCL1/GROalpha	b	b	d	d	d	d
TNFA	b	b	d	d	d	d
IL-10	b	b	NA	NA	NA	NA
IL-13	b	b	NA	NA	NA	NA
TIMP-1	b	b	c	c	c	c
TLSP	e	e	e	e	e	e
CCL20/MIP-3α	e	e	e	e	e	e
sICAM-1	e	e	e	e	e	e
MMP-1	f	f	f	f	f	f
MMP-9	f	f	f	f	f	f

Milliplex kits were purchased from EMD Millipore. The kits used were: a) human 11-plex, HCYTOMAG-60K-11; b) human 6-plex, HCYTOMAG-60K-6; c) human single-plex, HTMP2MAG-54K-1; d) human 12-plex, HCYTOMAG-60K-12; e) human 3-plex, custom panel SPR507; and f) human 2-plex, HMMP2MAG-55K-2. Abbreviations: CCL, chemokine (C–C motif) ligand; CSF, colony-stimulating factor; CXCL, chemokine (C–X–C motif) ligand; EGF, epidermal growth factor; Exp., experiment; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; GROalpha, growth related oncogene alpha; IL, interleukin; IP10, interferon gamma-induced protein 10; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; NA, not available (a different kit was used for the indicated analyte/mediator); RANTES, regulated on activation, normal T expressed and secreted; sICAM, soluble intercellular adhesion molecule; TIMP, tissue inhibitor of metalloproteinase; TLSP, thymic stromal lymphopoietin; TNFA, tumour necrosis factor alpha; VEGFA, vascular endothelial growth factor alpha.

7.4.9 Tissue Processing, Embedding, Sectioning, and Staining

Tissue inserts were washed three times with PBS and fixed for 2 h in freshly prepared 4% paraformaldehyde (following PMI_RD_WKI_001242). The fixative was then aspirated and the fixed organotypic cultures were washed three times, both apically and basally, with PBS at room temperature. Each fixed culture was then separated from the insert by detaching the membrane from the plastic using forceps. Each culture was then bisected at the mid-point and then processed using a Leica ASP300 S tissue processor (Leica Biosystems Nussloch, Nussloch, Germany) following PMI_RD_WKI_001243. Both pieces of each culture were then placed in a cassette and embedded in paraffin following PMI_RD_WKI_001260. Sections (5 µm thick) of each paraffin block were then prepared using a microtome (following PMI_RD_WKI_001262), and each section was mounted on a glass slide, The slides were transferred to a Leica ST5020 automated slide stainer (Leica Biosystems Nussloch) and stained with haematoxylin (Merck Millipore) and eosin (Sigma-Aldrich, St. Louis, MO, USA) and Alcian blue (Sigma-Aldrich) following PMI_RD_WKI_001266. Digital images of each slide (containing both bisected pieces of a culture sample) were generated using a Hamamatsu NanoZoomer 2.0 slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) following PMI_RD_WKI_001314.

7.4.10 Immunohistochemical Analysis

Automated immunostaining was performed using a Leica BOND-MAX autostainer (Leica Biosystems Nussloch) following PMI_RD_WKI_001309. The primary antibodies anti-Ki67 (PA0230; ready to use; Leica Biosystems Nussloch), anti-p63 (1:100 µL; ab735; Abcam, Cambridge, UK), and anti-FoxJ1 (1:2000 µL; ab40869; Abcam) were used. Digital images of each slide were generated using the Hamamatsu NanoZoomer slide scanner following PMI_RD_WKI_001314. The p63, ki67, and FoxJ1 were immunohistochemically quantified using Tissue Studio and Image Miner software (Definiens AG, Munich, Germany). The total numbers of positively stained and negatively stained nuclei were calculated as described next. Digitized slides were transferred to a workstation (Definiens Tissue Studio 2.0). A pre-processing step was performed first, to classify and segment the tissue and to identify and discriminate between the cellular compartments (nucleus, cilia, and cytoplasm) and the insert membrane. Positively and negatively stained areas were then detected and classified using a combination of color thresholds and morphological features. The algorithm that was used segmented the pictures iteratively, recognizing groups of pixels as objects. Furthermore, for each marker, the objects were classified (from the staining intensity) as weak, moderate, or strong staining. The total strong staining count was considered to be positive for Ki67 immunohistochemistry, the total strong and moderate staining count was considered to be positive for p63, and the total strong, moderate, and weak staining count was considered to be positive for FoxJ1. The processed data (including the total numbers of cells analyzed and the numbers of nuclei with each staining intensity) were exported into Excel spreadsheet files to allow statistical analyses to be performed. The relative proportion of positively stained cells for each specific marker relative to the total number of cells was calculated. Statistical analyses were performed and graphical representations of the data created using SAS v9.2 (SAS Institute, Cary, NC, USA).

7.4.11 CYP1A1/CYP1B1 Activity Assay

The combined CYP1A1/CYP1B1 activity was determined using the non-lytic P450-Glo™ assay (Promega, Madison, WI, USA), following the manufacturer's instructions (following PMI_RD_WKI_001049 and PMI_RD_FOR_000846). Briefly, the luminogenic P450-Glo™ substrate luciferin-6′-chloroethyl ether, which is a substrate for both CYP1A1 and CYP1B1, was added to the basolateral medium 24 h before a sample was collected. The luciferin product of the CYP reaction was detected by mixing 50 µL of the collected medium with 50 µL of luciferin detection reagent. The mixture was incubated for 30 min, then the luminescence was measured using a FluoStar Omega reader. As a positive control for CYP1A1/B1 induction, three replicates of 3D-organotypic culture inserts were treated for 48 hours with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Sigma-Aldrich), then added to a final concentration of 30 nM to the basolateral side. As a negative control, basolateral media from three replicates of untreated culture inserts were tested for CYP1A1/B1 activity.

7.4.12 RNA/microRNA Purification

Total RNA, including microRNA (miRNA), was isolated after washing both the basal and apical sides of 3D organotypic culture inserts twice with cold (4 °C) PBS. The cells were then disrupted in 700 µL of QIAzol™ lysis buffer (Qiagen, Hilden, Germany), then RNA was extracted using a Qiagen miRNeasy mini kit and a QIAcube robot (Qiagen) (following PMI_RD_WKI_001117). The quantity of purified RNA was determined using a NanoDrop™ ND8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the quality of the RNA was determined using an Agilent 2100 bioanalyser (Agilent Technologies) following PMI_RD_WKI_000978.

7.4.13 mRNA Profiling Analysis Using GeneChips

The target preparation workflow was performed using the Biomek FXP Target Prep Express liquid handling system (Beckman Coulter, Pasadena, CA, USA). Total RNA (100 ng) was reverse-transcribed to complementary DNA using an Affymetrix® HT 3′ IVT PLUS kit (Affymetrix, Santa Clara, CA, USA) following PMI_RD_WKI_001109. The complementary DNA was labelled and then amplified to cRNA (complementary RNA, which is also referred to as amplified RNA), and the cRNA was purified using magnetic beads to remove unincorporated nucleotide triphosphates, salts, enzymes, and inorganic phosphates (following PMI_RD_WKI_001109).

Purified cRNA (≥12 µg) was quantified, normalized, and quality-checked using a Fragment Analyzer™ (Advanced Analytical, Ames, IA, USA) following PMI_RD_WKI_001125. The cRNA was then chemically fragmented following PMI_RD_WKI_001109, and an Agilent 2100 bioanalyser was used to check that the fragmentation process was complete. Then, 29.4 µL of fragmented and labelled cRNA was added to 190.6 µL of "hybridization cocktail" (following PMI_RD_WKI_001126). The mixture was allowed to denature for 5 min at 95 °C and then 5 min at 45 °C, then the mixture was centrifuged at Vmax (16,000 × *g*) for 1 min, then 200 µL of the cRNA cocktail was hybridized on a GeneChip® Human Genome U133 Plus 2.0 array (Agilent Technologies) (following PMI_RD_WKI_001126). The arrays were incubated in a GeneChip® hybridization oven 645 (Affymetrix) for 16 h at 45 °C using a rotation speed of 60 rpm. The arrays were then washed and stained using a GeneChip® FS450 DX fluidics station (Affymetrix) using

Affymetrix® GeneChip® Command Console® software (AGCC) v 3.2, using protocol FS450_0001. Finally, the arrays were scanned using a GeneChip® 3000 7G scanner (Affymetrix). Raw images from the scanner were saved as DAT files. The AGCC software automatically gridded each DAT file image and extracted the probe cell intensities into a CEL file.

7.4.14 MicroRNA Profiling

A FlashTag™ biotin HSR kit (Affymetrix) was used to label the miRNA following PMI_RD_WKI_001123. Total RNA (200 ng) containing low molecular weight RNA was subjected to a brief tailing reaction, then the biotinylated signal molecule was ligated to the target RNA sample. The mixture was allowed to denature for 5 min at 99 °C and then 5 min at 45 °C, then the mixture was centrifuged at Vmax (16,000 × *g*) for 1 min, then 21.5 µL of the biotin-labelled sample was mixed with 110.5 µL of hybridization cocktail. The arrays (miRNA version 3.0) were incubated in a GeneChip® 645 hybridization oven for 16 h at 48 °C using a rotation speed of 60 rpm. The arrays were washed and stained using a GeneChip® FS450 DX fluidics station using AGCC software v 3.2, using protocol FS450_0002. Finally, the arrays were scanned using a GeneChip® 3000 7G scanner. Raw images from the scanner were saved as DAT files. The AGCC software automatically gridded each DAT file image and extracted the probe cell intensities into a CEL file.

8 STATISTICAL AND COMPUTATIONAL METHODS

8.1 Statistical Methods

Statistical analyses were performed and graphical representations of the data created using SAS v9.2.

8.1.1 Experimental Unit

For the exposure experiments using human organotypic bronchial culture models and the Vitrocell® 24/48 exposure system, the experimental unit was the smoke exposure run. All of the average values for an endpoint were calculated if that endpoint was measured several times using the same tissue culture insert or using different tissue culture inserts exposed to smoke or aerosol under the same Vitrocell® 24/48 exposure system, for each of the experimental conditions (e.g., a specific smoke or aerosol concentration at a specific post-exposure time point).

The experimental unit was the study phase (i.e., a single experimental repetition) for the controls (i.e., in runs in which the endpoints were measured in tissue inserts that had not been exposed to smoke or aerosol from the Vitrocell® 24/48 exposure system but had been treated with the other reagents and chemicals). The average value for an endpoint in the controls was calculated from the measurements performed on tissue inserts that were treated identically with a specific reagent or chemical.

8.1.2 Derived Variables

8.1.2.1 Normalized Cytotoxicity Levels

All values from the AK assay were normalized using the average of the positive controls (tissue inserts treated with Triton X-100) and negative controls (PBS-treated or untreated tissue inserts [unexposed, incubator controls]), and the percentage cytotoxicity was calculated as shown below.

$$Cytotoxicity\ (\%) = \frac{AK_{tissue} - AK_{Neg\ CTRL}}{AK_{Pos\ CTRL} - AK_{Neg\ CTRL}} X\ 100, \text{ where}$$

$$AK_{Neg\ CTRL} = \sum_{i=1}^{nbPhase} \frac{\sum_{j=1}^{nbCTRL^i} \frac{AK_{i,j}}{nbCTRL^i}}{nbPhase}$$

$$AK_{Pos\ CTRL} = \sum_{i=1}^{nbPhase} \frac{AK_{TX-100}}{nbPhase}$$

$$AK_{Tissue} = relative\ luminescence\ unit\ of\ a\ given\ sample$$
$$nbPhase = number\ of\ experimental\ phase$$
$$Neg = negative$$
$$Pos = positive$$
$$CTRL = control$$

8.1.2.2 Normalized CYP Activity Levels

All of the CYP activity measurements were normalized using the average of the positive controls (tissue inserts treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and negative controls (unexposed tissue inserts treated with luciferin substrate), as shown below.

$$Normalized\ CYP\ activity\ (\%) = \frac{CYP_{tissue} - CYP_{Neg\ CTRL}}{CYP_{Pos\ CTRL} - CYP_{Neg\ CTRL}}\ x\ 100, \text{ where}$$

$$CYP_{Neg\ CTRL} = \sum_{i=1}^{nbPhase} \frac{\sum_{j=1}^{nbCTRL^i} \frac{CYP_{i,j}}{nbCTRL^i}}{nbPhase}$$

$$CYP_{Pos\ CTRL} = \sum_{i=1}^{nbPhase} \frac{CYP_{TCDD}}{nbPhase}$$

$$Neg = negative$$
$$Pos = positive$$
$$CTRL = control$$

8.1.3 Data Transformation

All numerical values produced from the MAP analysis were logarithmically transformed (using the natural logarithm).

8.1.4 Statistical Comparisons

For continuous variables:

- an exposed sample was compared with the air control (i.e., paired samples from the same exposure run in a single experimental repetition were used) using a paired t-test; and
- samples exposed to 3R4F smoke were compared with samples exposed to THS2.2 aerosol, after appropriate air controls (i.e., using paired samples) had been subtracted, using a t-test corrected for non-equal variance (Satterthwaite correction).

P-values were not adjusted for multiple testing.

8.2 Computational Methods: Affymetrix Gene Expression Analysis

8.2.1 Processing and Quality Control of Raw CEL Files

Hybridization was achieved using an Affymetrix GeneChip® human genome U133 Plus 2.0 array, which probed the expression of thousands of genes simultaneously.
In accordance with PMI_RD_WKI_001228 and standard operating procedure PMI_RD_SOP_000346, the raw CEL files were background-corrected, normalized, and summarized using frozen robust microarray analysis (McCall 2010). The data from all of the arrays were background corrected and quantile normalized to generate microarray expression values that passed quality control checks, which were performed using the custom Chip Definition Files (CDF)environment HGU133Plus2_Hs_ENTREZG v16.0 (Dai 2005). A log-intensities plot, normalized-unscaled standard error (NUSE) plot, relative log expression (RLE) plot, median absolute value RLE (MARLE) plot, and pseudo as well as raw images were generated for quality checks using R packages (AffyPLM; Bioconductor, Seattle, WA, USA) (Bolstad 2005, Gautier 2004).
CEL files that fulfilled at least one of the quality metric rules described below were dropped from further analysis.

a) Pseudo-image displayed a spatial pattern covering approximately 10% of the pseudo-image.
b) Median NUSE >1.05
c) |Median RLE| >0.1
d) |(MARLE-median(MARLE))|/ (1.4826 *mad(MARLE)) >1/√0.01; (where mad is the median absolute deviation).

Subsequently, the RLE and NUSE-based metrics were recomputed, until no more CEL files were removed.

8.2.2 Gene-level Analysis

A model to estimate the effects of each treatment (i.e., using each experimental factor, item, concentration, and post-exposure time) was fitted using the "limma" package (Smyth 2004). The covariate smoking run was used as a blocking variable to take into account insert pairing during an exposure experiment (an exposure run was made up of samples exposed to a test item and controls exposed to air, see Figure 2). The *p*-values for the different effects were adjusted for the different genes using the Benjamini–Hochberg false discovery rate (FDR) method. The results were then plotted as volcano plots (in which the *x*-axis represented the estimated effect and the *y*-axis the $-\log_{10}$(FDR) value for each gene). Differentially expressed genes were defined as the genes with FDRs lower than 0.05.

The reproducibility was estimated by correlating the fold changes (in exposed samples relative to the controls) found in different experimental repetitions. We first combined the gene expression data from experiments 3 and 4 and calculated the average number of fold-changes for each gene in the dataset. The combined data from experiments 5 and 6 were used to calculate the average number of fold changes in a similar way. Correlation plots for the fold changes in all of the genes in the dataset were then generated for each contrast.

8.2.3 Statistical Analysis: Network-level Analysis

8.2.3.1 Network Perturbation Amplitude

The human network suite CBN v1.3 (Boue 2015) contained the causal biological networks that were used in the study. These networks ranged in size between a few dozen and two hundred nodes. The relevant network and subnetwork models that were considered are summarized in Table 10.

Table 10. List of Network and Subnetwork Models Used in the Analysis

No.	Abbreviated Network Name	Subnetwork Name
1	CFA	Apoptosis
2	CFA	Autophagy
3	CFA	Necroptosis
4	CFA	Response to DNA damage
5	CFA	Senescence
6	CPR	Calcium
7	CPR	Cell cycle
8	CPR	Cell interaction
9	CPR	Clock
10	CPR	Epigenetics
11	CPR	Growth factor
12	CPR	Hedgehog
13	CPR	Hox
14	CPR	JAK-STAT
15	CPR	MAPK
16	CPR	Mtor
17	CPR	Notch
18	CPR	Nuclear receptors
19	CPR	PGE2
20	CPR	Wnt
21	CST	Endoplasmic reticulum stress
22	CST	Hypoxic stress
23	CST	NFE2L2 signalling
24	CST	Osmotic stress
25	CST	Oxidative stress
26	CST	Xenobiotic metabolism response
27	IPN	Epithelial innate immune activation
28	IPN	Epithelial mucus hypersecretion
29	IPN	Tissue damage

Abbreviations: CFA, cell fate; CST, cell stress; CPR, cell proliferation; IPN, inflammatory process network; JAK-STAT, Janus kinase/signal transducers and activators of transcription; MAPK, mitogen-activated protein kinases; Mtor, mechanistic target of rapamycin; NFE2l2, nuclear factor, erythroid 2-like 2; PGE2, prostaglandin E2.

Network scoring exploits the connections between network backbone nodes and downstream mRNA-abundance-nodes based on the known relationships (i.e., relationships that have been described in the literature) between the backbone nodes and downstream mRNA-abundance-nodes. These relationships, in which a backbone node can increase or decrease the abundance of certain mRNAs, were used with transcriptomics data to determine whether a backbone node was activated. Not all backbone nodes have downstream mRNA nodes, so the network models had to be prepared for scoring in a way that allowed the overall NPA score to have good specificity and relevancy. Only backbone nodes on directed paths that started and ended at nodes with downstream mRNA nodes were considered. After the nodes that did not satisfy that condition had been removed, the largest connected component was retained. Finally, the "causesNoChange" edges were disregarded before the scoring process was performed.

The NPA methodology (Hoeng 2014, Martin 2014, Martin 2012) was aimed at contextualizing the high-dimensional transcriptomics data by combining gene expression ($\log_2$) fold changes (β) into a smaller number of differential node values (one value for each node in the network), f. The differential node values were determined using a fitting procedure that determined the values that best satisfied the directionality of the causal relationships (positive or negative signs) in the network model while being constrained by the experimental data (the gene expression $\log_2$ fold changes, which were described as being downstream effects of the network itself). The differential node values were determined using the equation

$$f = L_3^{-1} L_2^T \beta,$$

where L is the weighted Laplacian (positive or negative) of the network and the extra edges and nodes describing the downstream effects (on the gene expression nodes), L_3 is the sub-matrix of L for the nodes in the network, and L_2 is the sub-matrix corresponding to the edges connecting the network nodes to the downstream gene expression nodes. The differential node values were summarized as a single positive number, called the amplitude of perturbation (NPA score). The NPA score was calculated using the equation

$$NPA = \frac{1}{|E|} \sum_{e\ in\ E} (f(e_0) + \sigma(e) f(e_1))^2,$$

where E is the set of edges in the network, |E| is the size of E, and e_0 and e_1 are the start and the end, respectively, of edge e. The equation used to calculate the NPA score can be expressed as $f^T Q f$, where Q is the Laplacian (positive or negative) of the network when all of the edge signs had been reversed. The methodology is described in detail in a previous publication (Martin 2014).

A confidence interval taking into account experimental variation and the associated *p*-values was calculated for each NPA score. Companion statistics were derived to provide information on the specificity of the NPA score with respect to the biology described in the subnetwork model. The companion statistics were labelled *O and K* if the *p*-values were lower than the required significance level ($p = 0.05$). A network was considered to be significantly affected if all three values (the confidence interval, *O, and K*) were lower than 0.05.

8.2.3.2 Biological Impact Factor

The biological impact factor (BIF) method (Hoeng 2014, Thomson 2013) was used to provide a unified and coherent framework for investigating mechanistic effects at each level of granularity in the biological processes represented in the network models. The method used allowed the relative

BIF, a measure of the overall biological impact across all network models, to be derived. The BIFs allowed the effects of exposure to smoke or aerosol to be assessed in an objective, systematic, and quantifiable manner. The BIF was used as a systems-wide pan-mechanistic measure of the biological effects of a given substance, mixture, or test item.

The network models were divided into five major families representing global biological processes. These families were cell stress, cell proliferation, inflammation, tissue repair, and cell fate. Each family contained various subnetwork models representing specific processes within the network family.

An NPA aggregation should satisfy the following criteria:

1) *credibility*: the BIF should be an intuitive and logical aggregation scheme;
2) *fairness*: all network families are equally important;
3) *understandability*: each decision or step must have a defined rationale.

The NPA scores were aggregated using a weighted sum (because every NPA score was additive), fulfilling the first criterion. Only networks with the all three *O, *, K* statistic *p*-values lower than 0.05 were considered, and these networks were labelled N_i^*. The BIF was defined as shown in the equation

$$BIF = \sum_{\mathcal{N}_i^*} w_i NPA(\mathcal{N}_i) = \sum_{\mathcal{N}_i^*} w_i \sum_{e \in E(\mathcal{N}_i)} (f(e_0) + \sigma(e) f(e_1))^2$$

Where f is the function of the network nodes describing the differential node values, $E(N_i^*)$ are the network edges, and e_0 and e_1 are the start and end, respectively, of edge e.

In terms of the second criterion, a "fair" BIF would properly represent the equal importance of different biological processes (networks) in a given system (e.g., in 2D normal human bronchial epithelial cells, 3D organotypic tissue cultures, or lung tissue samples obtained in *in vivo* studies). All of the subnetworks were assumed to make equal contributions to the BIF if all of the subnetwork models perfectly fitted the gene expression data. In the method, therefore, it was assumed that all of the specific processes represented in the subnetwork models were equally involved in and important to the appropriate network family (e.g., cell stress). A perfect fit for a network would maximize the NPA value and be consistent over the whole network. Using the notation shown above, a "perfect" network response (differential node values) would maximize the f^TQf with $||f||_2 = 1$ and minimize the f^TL_3f (the NPA criterion (Martin 2014)). There was also the constraint that L_3f would be negligible for network nodes that did not have direct downstream gene expression nodes (Martin 2014, Martin 2012), such as those expected to be in the L^T_2 image. Such nodes were labelled *NH*. The optimization problem shown below could therefore be solved.

$$argmax_{f\ s.t.(L_3 f)|_{NH}=0} \frac{f^T Q f}{f^T L_3 f}$$

The network families were rebalanced taking the average values for the subnetworks into consideration.

For each edge, $e = (e_0, \sigma(e), e_1)$, the number of times the edge occurred (e) in the set of networks used in the BIF calculation was denoted o(e). The maximum occurrence-corrected amplitude of network N_i could therefore be defined as shown below.

$$NPA_{max}(\mathcal{N}_i) \doteq \sum_{e \in E(\mathcal{N}_i)} \frac{1}{o(e)} (f_{max}(e_0) + \sigma(e) f_{max}(e_1))^2$$

Finally, the NPAs were normalized using the correction factor $|E(N_i)|/NPA_{max}(N_i)$. Taking all of these factors into consideration, the BIF was defined as the weighted sum (F denotes the subnetwork families), as shown below.

$$\sum_{\mathcal{F}} \frac{1}{|\mathcal{F}|} \sum_{\mathcal{N}_i^* \in \mathcal{F}} \frac{1}{NPA_{max}(\mathcal{N}_i)} \sum_{e \in E(\mathcal{N}_i)} \frac{1}{o(e)} (f(e_0) + \sigma(e) f(e_1))^2$$

Therefore, the contribution of each subnetwork F was determined as shown below.

$$\frac{\frac{1}{|\mathcal{F}|} \sum_{\mathcal{N}_i^* \in \mathcal{F}} \frac{1}{NPA_{max}(\mathcal{N}_i)} \sum_{e \in E(\mathcal{N}_i)} \frac{1}{o(e)} (f(e_0) + \sigma(e) f(e_1))^2}{BIF}$$

8.2.4 Gene-set Analysis

Gene-set analysis (GSA) of transcriptomics data was performed using the *Piano* package in the R statistical environment (Varemo 2013). Pathway maps were obtained from the KEGG database (Kanehisa 2014) and exported as R objects into the package *graphite* (Sales 2012). Gene-set enrichment was assessed using over-representation analysis (ORA) and gene-set analysis. For the ORA, a one-tailed Fisher's exact test was employed. For the GSA, the fold-change was used as the gene-level statistic, the mean was used as the gene-set enrichment score and sample permutation (Q2) was used to assess statistical significance (Ackermann 2009). For each test, the *p*-value was adjusted using the Benjamini-Hochberg procedure. The gene-set enrichment are the gene-set statistics mentioned before.

8.3 Computational Methods: Affymetrix miRNA Expression Analysis

8.3.1 Processing and Quality Control of the Raw CEL Files

The arrays raw data for the arrays in the CEL files were pre-processed following a standard set of processes based on the generic PMI_RD_WKI_001228 and standard operating procedure PMI_RD_SOP_000346. The CEL files were read using the *read.celfiles* function of the *Oligo* package in the bioconductor suite of microarray analysis tools for the R statistical software environment (Carvalho 2010, Huber 2015, RCoreTeam 2013). The quality of the array data was controlled using the *Array quality metrics* package (Kauffmann 2009) and examined using the following four metrics:

a) the distances between the arrays at the raw data level;
b) the distances between the arrays at the normalized data level;
c) the normalized unscaled standard errors; and
d) the array intensity distributions.

Arrays that were found to be outliers in at least two of the metrics were discarded. The data quality was subsequently iteratively re-examined for the remaining arrays until all the remaining arrays were accepted. The quality control procedure described above was applied separately to each experimental repetition (also called "main phases" in the studies using organotypic tissue models), meaning that two experiments at a time were processed. This approach led to nine arrays out of 367 being discarded in a maximum of two iterations. The normalized probe-level data were subsequently obtained by

applying robust multi-array normalization, and the data were summarized using the median polish method at the probe set level (Bolstad 2003, Irizarry 2003).

8.3.2 Probe Set Filtering

Hybridization was achieved using Affymetrix GeneChip® miRNA 4.0 arrays. The system probed the expression of 30,424 mature miRNAs relevant to 203 species. Of these mature miRNAs, 2,578 miRNAs were relevant to humans. The information provided by Affymetrix and the latest miRNA nomenclature provided by miRBase (released on 21 June 2014) (Kozomara 2014) were used to retain only the probe sets relevant to humans in the expression matrix. Probe sets not available in the Affymetrix GeneChip® miRNA 3.0 arrays were discarded to ensure that the miRNA results for all of the organotypic tissues (bronchial, nasal, and oral) that were considered were comparable. The final expression matrix contained 1728 miRNA probe sets.

Only the miRNA probe sets with significantly higher intensities than the intensities of the matched background probes (based on the GC content and sequence length) were considered to be detected, as specified in the detection section of the instructions provided by Affymetrix, written in 2011. Wilcoxon tests were performed, and a *p*-value threshold of 0.01 was used to determine whether a probe was detected. Each miRNA probe set detected in more than 50% of the samples in at least one sample group was kept for further analysis, and the others were discarded. This pre-processing approach gave 548 miRNA probe sets in the final expression matrix, after the control probe sets had been excluded.

8.3.3 Determining a Differential miRNA Expression

An experimental unit consisted of a number of pairwise "treatment versus control" comparisons (i.e., an exposure run contained samples exposed to a test item and samples exposed to air (controls), as described in section 8.1.1). For each comparison, a submatrix was extracted from the global expression matrix, keeping only the samples belonging to the corresponding treatment or control groups and the miRNA probe sets that were detected in more than 50% of the samples in at least one group or the other. Linear models for differential expressions were applied to all of the resulting submatrices using moderated t statistics in the limma package (Ritchie 2015). Each model included an additional variable to take the exposure runs into account. This improved the fitting procedure in the limma package. Adjusted *p*-values were then obtained after a number of test corrections had been performed using the Benjamini–Hochberg FDR method (Benjamini 1995). Any miRNAs that were below the FDR threshold of 0.05 were considered to be differentially expressed.

The results for each comparison were plotted as a volcano plot, with the *x*-axis representing the estimated treatment effect (differential expression) of each miRNA and the *y*-axis representing the corresponding statistical significance, given by $-\log_{10}(\text{FDR})$.

For a given comparison, QQ plots were produced by first sorting into ascending order all of the miRNAs according to their unadjusted (raw) *p*-values. The *x*-axis represented the $-\log_{10}$ transformed raw *p*-values of the resulting quantiles (i.e., the rank divided by the total number of miRNAs) and the *y*-axis represented the corresponding $-\log_{10}$ transformed raw *p*-values. For a given statistical

significance threshold α, the Z-shaped boundaries of the plot corresponded to raw *p*-values below α (the lower horizontal line), a line parallel to the $x = y$ diagonal corresponded to FDR values below α, and the upper horizontal line corresponded to the Bonferroni adjusted *p*-value below α.

9 RESULTS

9.1 Nicotine Concentrations in the Smoke and Aerosol

The Vitrocell® 24/48 exposure system was equipped with a dilution system. The smoke or aerosol was diluted using the dilution system to give the target nicotine concentration in the smoke or aerosol. The actual nicotine concentration in each smoke or aerosol sample was measured after the smoke or aerosol had been trapped on an EXtrelut® 3NT column. At least three independent samples were collected during each experimental repetition (Table 11). Furthermore, according to the design of the study Table 5), at least two concentrations of the test aerosol THS2.2 would have comparable nicotine concentrations in the aerosol as compared with those of the reference 3R4F smoke.

It should be noted that the smoke or aerosol could not be diluted further while a sample was being collected on an EXtrelut® 3NT column, so a sample could not be collected in a smoking run in which tissue cultures were exposed to smoke or aerosol. However, samples for nicotine analysis were collected in the same experimental week as each exposure run was performed.

Dosing of 3R4F and THS2.2 was based on two equal (matching) nicotine concentrations in the smoke/aerosol, to allow a comparative analysis. The targeted smoke/aerosol nicotine concentrations in the exposure system were achieved by adjusting the dilution as shown in Table 11. The determined nicotine concentration from 3R4F smoke or THS2.2 aerosol clearly correlated with the applied dilution / % aerosol concentration, and the actual concentrations for a given dose level were consistent among the experimental repetitions. Table 11 illustrates that to reach the comparable nicotine levels, the concentration of the THS2.2 aerosol was approximately 1.65 times the 3R4F smoke concentration.

Table 11. Target and Actual Nicotine Concentrations in the Smoke and Aerosol

Group	Experiment / Month	Exp. 1 Dec 2013	Exp. 2 Feb 2014	Exp. 3 Mar 2015	Exp. 4 Jun 2015	Exp. 5 Jul 2015	Exp. 6 Sep 2015
3R4F Concentration Level 1	Target Nicotine Concentration (mg/L)	0.13	0.13	0.13	0.13	0.13	0.13
	Vitrocell Dilution (%)	8	8	7	7	7	7
	Actual Nicotine Concentration (mg/L)	0.148 ± 0.007 (N = 3)	0.103 ± 0.009 (N = 8)	0.151 ± 0.006 (N = 3)	0.137 ± 0.006 (N = 3)	0.144 ± 0.001 (N = 3)	NA
3R4F Concentration Level 2	Target Nicotine Concentration (mg/L)	0.25	0.25	0.25	0.25	0.25	0.25
	Vitrocell Dilution (%)	15	15	13	13	13	13
	Actual Nicotine Concentration (mg/L)	0.275 ± 0.007 (N = 3)	0.254 ± 0.034 (N = 8)	0.268 ± 0.012 (N = 4)	0.235 ± 0.005 (N = 3)	0.253 ± 0.007 (N = 3)	NA
THS2.2 Concentration Level 1	Target Nicotine Concentration (mg/L)	0.14	0.14	0.14	0.14	0.14	NA
	Vitrocell Dilution (%)	12	12	13	13	13	NA
	Actual Nicotine Concentration (mg/L)	0.140 ± 0.003 (N = 4)	0.139 ± 0.011 (N = 5)	0.144 ± 0.007 (N = 4)	0.137 ± 0.006 (N = 4)	0.137 ± 0.009 (N = 3)	NA
THS2.2 Concentration Level 2	Target Nicotine Concentration (mg/L)	0.25	0.25	0.25	0.25	0.25	NA
	Vitrocell Dilution (%)	23	22	24	24	24	NA
	Actual Nicotine Concentration (mg/L)	0.271 ± 0.010 (N = 4)	0.227 ± 0.010 (N = 5)	0.263 ± 0.008 (N = 4)	0.283 ± 0.020 (N = 4)	0.223 ± 0.007 (N = 3)	NA
THS2.2 Concentration Level 3	Target Nicotine Concentration (mg/L)	NA	NA	0.42	0.42	0.42	NA
	Vitrocell Dilution (%)	NA	NA	31	31	31	NA
	Actual Nicotine Concentration (mg/L)	NA	NA	0.495 ± 0.006 (N = 4)	0.393 ± 0.019 (N = 4)	0.369 ± 0.035 (N = 3)	NA

Actual nicotine concentrations in the smoke/aerosol were measured following trapping of smoke/aerosol in EXtrelut® 3NT columns; at least three independent samplings were done per experimental repetition; nicotine concentrations are expressed as mean ± SEM. Abbreviation: N, number of samples (trapped in the EXtrelut® 3NT columns); NA, Not available (the dose was not tested in the experimental repetition); SEM, Standard error of the mean; THS, Tobacco heating system.

9.2 Histological Evaluation

The effects of the exposure of a tissue culture to smoke or aerosol on the morphology of the tissue was evaluated by performing histological assessments and performing immunohistochemical analyses 48 h and 72 h after exposure. Only the results of the histological evaluations of the bronchial MucilAir™ tissue cultures used in experiments 3–6 are presented below.

9.2.1 Morphological Evaluation

Bronchial organotypic tissue cultures, 48 h and 72 h after being exposed to smoke or aerosol, were embedded in paraffin and then sectioned. The sections were then stained with haematoxylin and eosin and Alcian blue. Representative stained sections are shown in Figure 3. A comparable tissue morphology, showing the typical pseudostratified structure was observed at both 48 h and 72 h post-exposure time points for the air-exposed control cultures. Bronchial tissue cultures exposed to 3R4F smoke with a nicotine concentration of 0.25 mg/L were strongly damaged and had started to become detached from the membrane, so further histological evaluation was not possible. This observation was supported by the high cytotoxicity of the 3R4F smoke with a nicotine concentration of 0.25 mg/L that was found in the AK release assay (see Figure 6). After 3R4F (0.13) smoke exposure, bronchial epithelial tissue cultures were less damaged than after 3R4F (0.25) exposure, demonstrating that the tissue damage caused by 3R4F smoke is dose-dependent. Compared to air exposed tissues, less ciliation at the apical surface as well as a decrease of alcian blue stained goblet cells could be observed. Moreover, in tissues exposed to the low 3R4F concentration, a number of cells contained pyknotic (condensed) and karyorrhexic (fragmented) nuclei can be seen, indicative for apoptotic cell death. Also noticeable are holes within the epithelium suggesting either a loss of tight-junctional cell-cell adhesion or a loss of cells. In contrast, at comparable nicotine concentrations, the morphology of THS2.2 (0.14)-exposed or THS2.2 (0.25)-exposed tissues was similar to that of the air control. In fact, even after exposure to the highest concentration of THS2.2 (0.42 mg nicotine /L) aerosol, the morphology of the bronchial epithelial tissues was not substantially different from the corresponding air control tissues at both post-exposure time points.

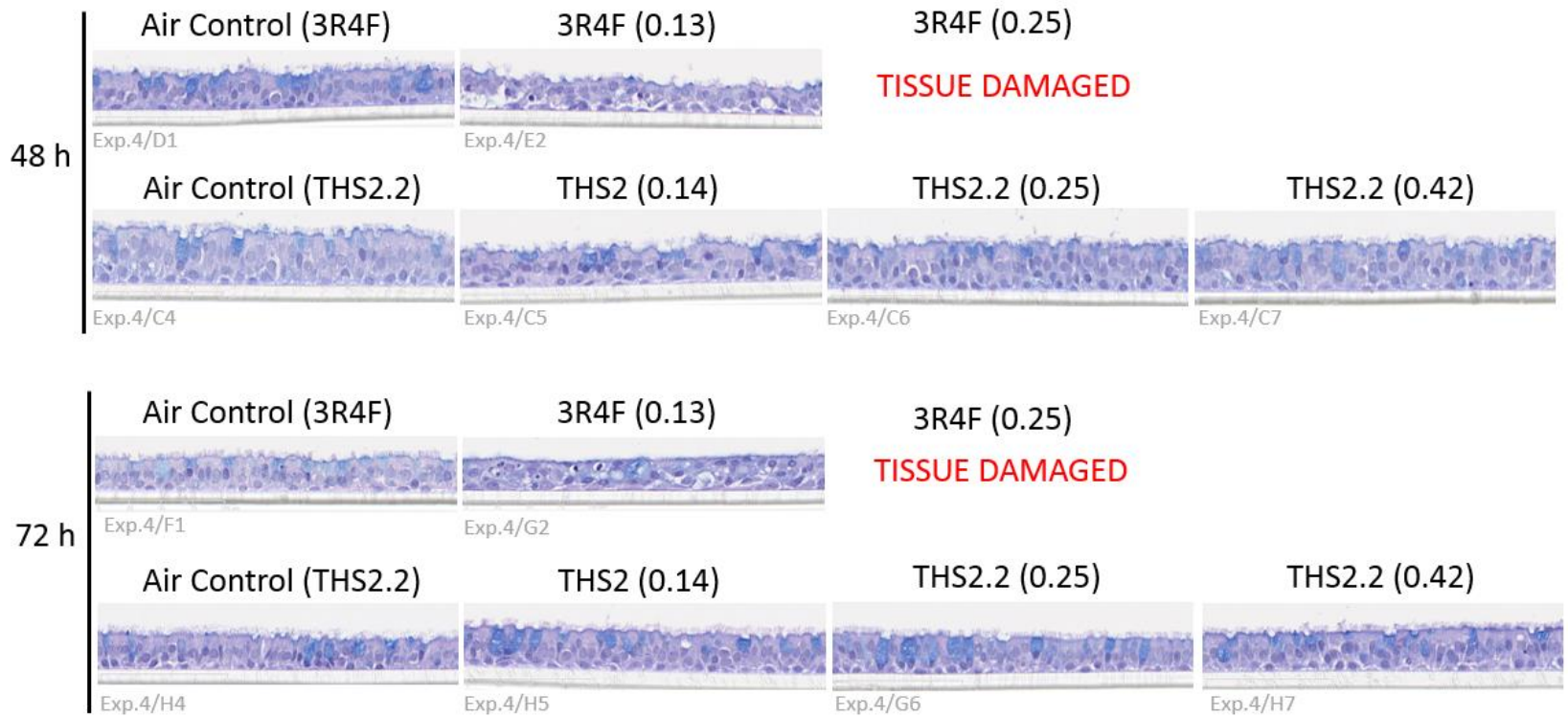


Figure 3. Histology of representative organotypic tissue culture samples that had been exposed to smoke or aerosol

Representative images (40× magnified) of bronchial organotypic tissue sections stained with haematoxylin and eosin and Alcian blue 48 h and 72 h after being exposed to 3R4F smoke or THS2.2 aerosol at the nicotine concentrations (mg/L) indicated in parentheses.

9.2.2 Immunohistochemical Analysis

Immunohistochemical analyses were performed to allow the expression of FoxJ1, Ki67, and p63 in tissue sections 48 h and 72 h after the tissues had been exposed smoke or aerosol to be examined. FoxJ1, Ki67, and p63 expression were used as markers of ciliated cells, proliferating cells, and basal cells, respectively. The markers were quantified, and the results were used to assess the effects of the exposure of the tissues to smoke or aerosol relative to the tissues exposed only to air (the control groups). Representative images of the stained sections of tissues 48 h and 72 h after the tissues had been exposed to smoke or aerosol are shown in Figure 4.

Using FoxJ1, p63 and Ki67 immunostaining we quantified the proportion of these cells within the exposed inserts. We found that (see Figure 5):

- The proportions of p63-positive basal cells and Ki67-proliferating cells were higher in inserts exposed to 3R4F smoke with a nicotine concentration of 0.13 mg/L than in the control inserts both 48 h and 72 h after exposure, but the increase was statistically significant only at 48 h after exposure.
- The proportions of FoxJ1-positive ciliated cells were lower (but not statistically significantly) in inserts exposed to 3R4F smoke with a nicotine concentration of 0.13 mg/L than in the control inserts both 48 h and 72 h after exposure.
- The proportions of p63-positive basal cells were not different in inserts exposed to THS2.2 aerosol at any concentration and in the control inserts at either 48 h or 72 h after exposure.
- The proportions of Ki67-proliferating cells were slightly lower in inserts exposed to THS2.2 aerosol, at any concentration, than in the control inserts 72 h after exposure. However, 48 h after exposure, the proportions of Ki67-proliferating cells were lower only in inserts exposed to THS2.2 aerosols with nicotine concentrations of 0.25 mg/L and 0.42 mg/L than in the control inserts.
- The proportions of FoxJ1-positive ciliated cells in inserts exposed to THS2.2 aerosol at any concentration 72 h after exposure or at nicotine concentrations of 0.25 mg/L and 0.42 mg/L 48 h after exposure were not different from the proportions of FoxJ1-positive ciliated cells in the control inserts. However, the proportions of FoxJ1-positive ciliated cells were lower in inserts exposed to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L 48 h after exposure than in the control inserts.

The descriptive statistics for these results are shown in Supplementary Table 9- Supplementary Table 11

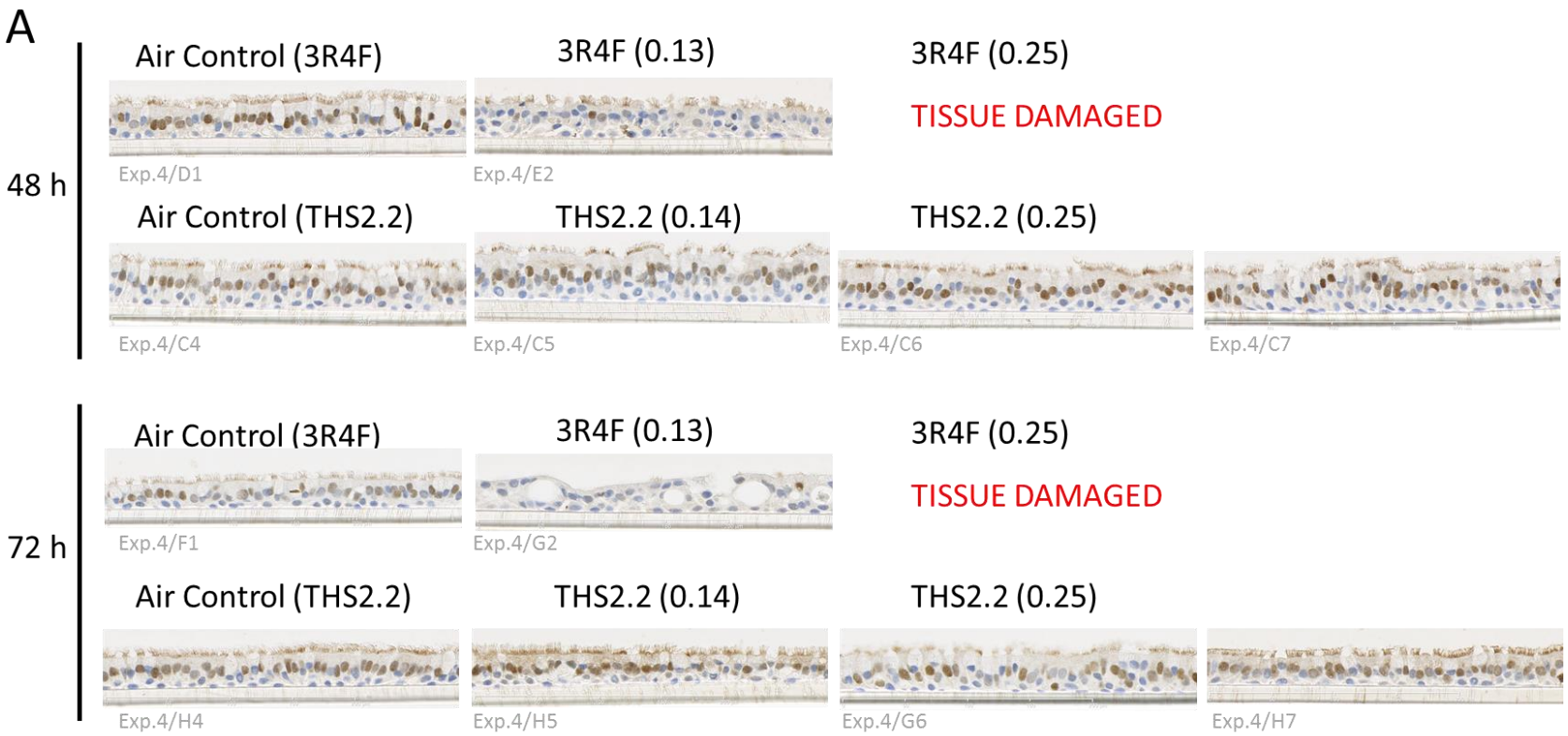


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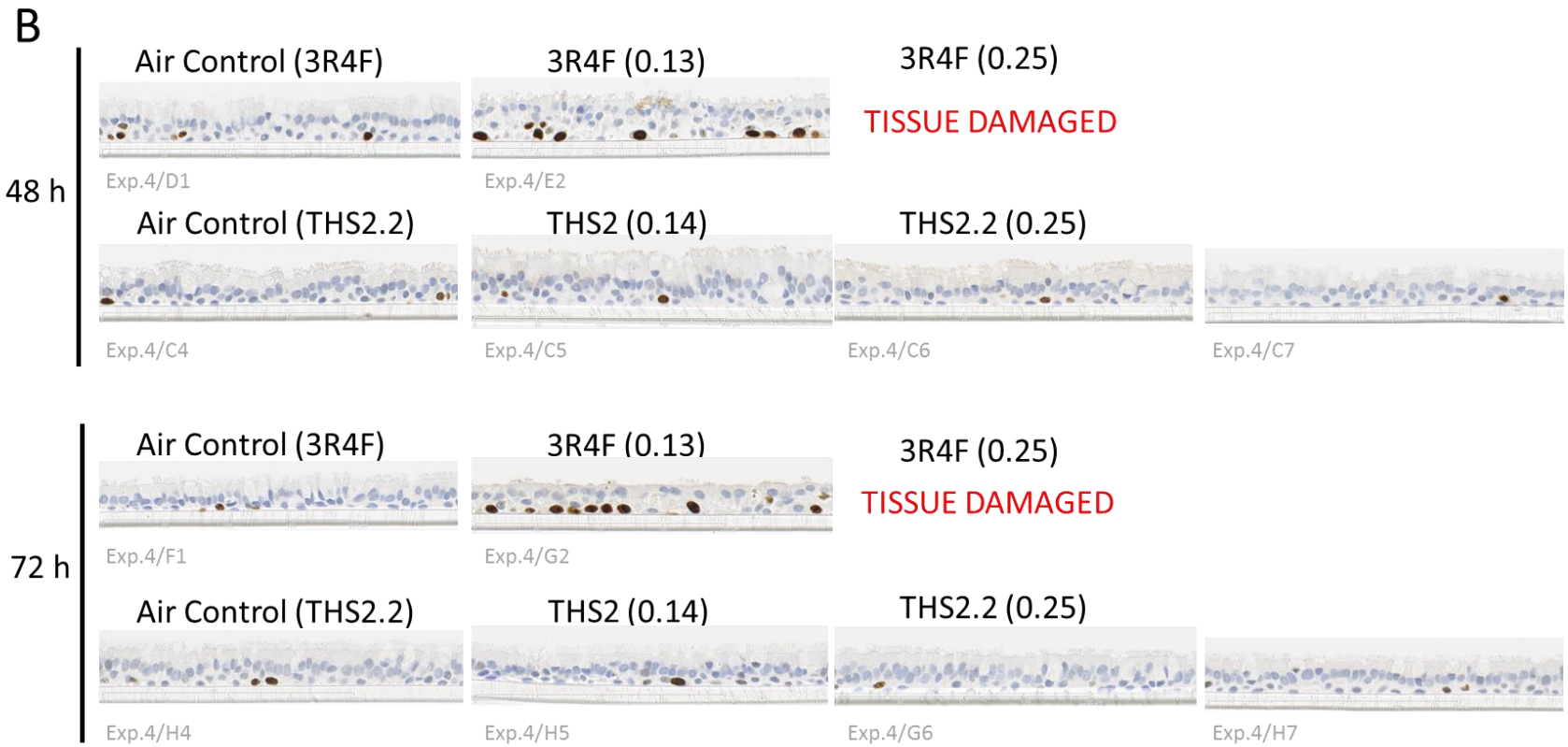


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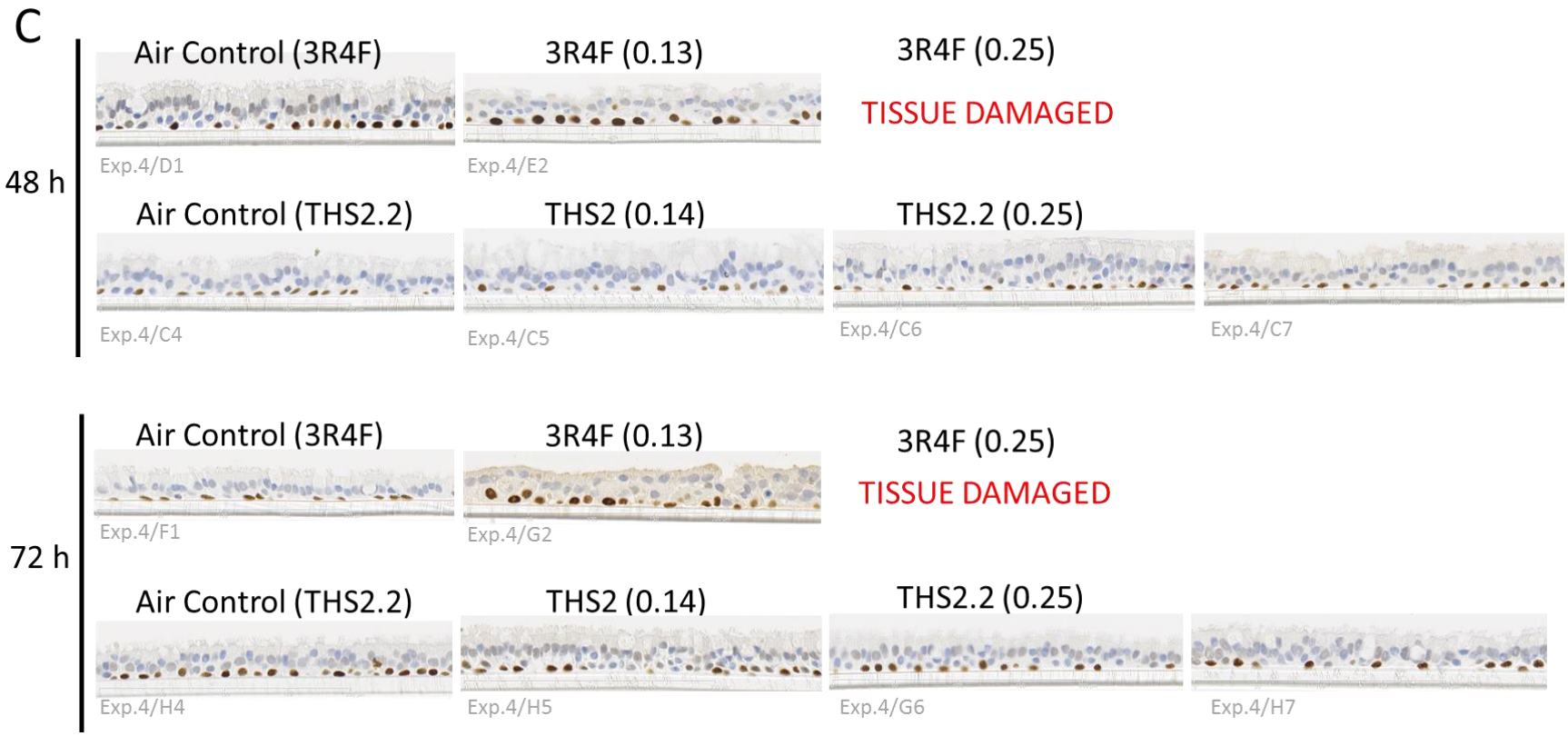


Figure 4. Images of (A) FoxJ1, (B) Ki67, and (C) p63 immunostained tissues

Representative pictures (40× magnified) of sections of bronchial organotypic tissues 48 h and 72 h after exposure to 3R4F smoke and THS2.2 aerosol at the nicotine concentrations (mg/L) indicated in parentheses. The sections were stained with anti-FoxJ1, anti-Ki67, and anti-p63 antibodies.

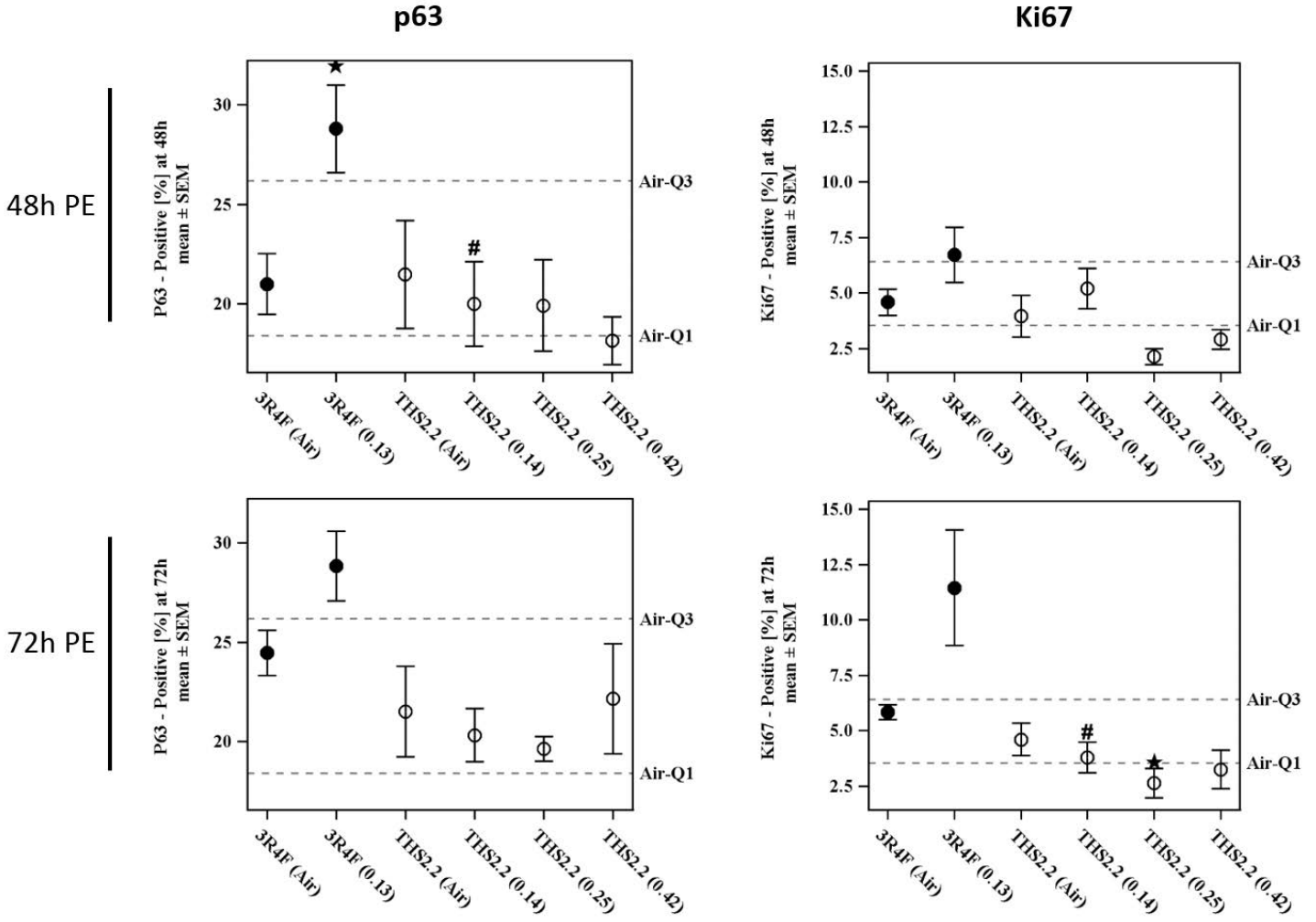


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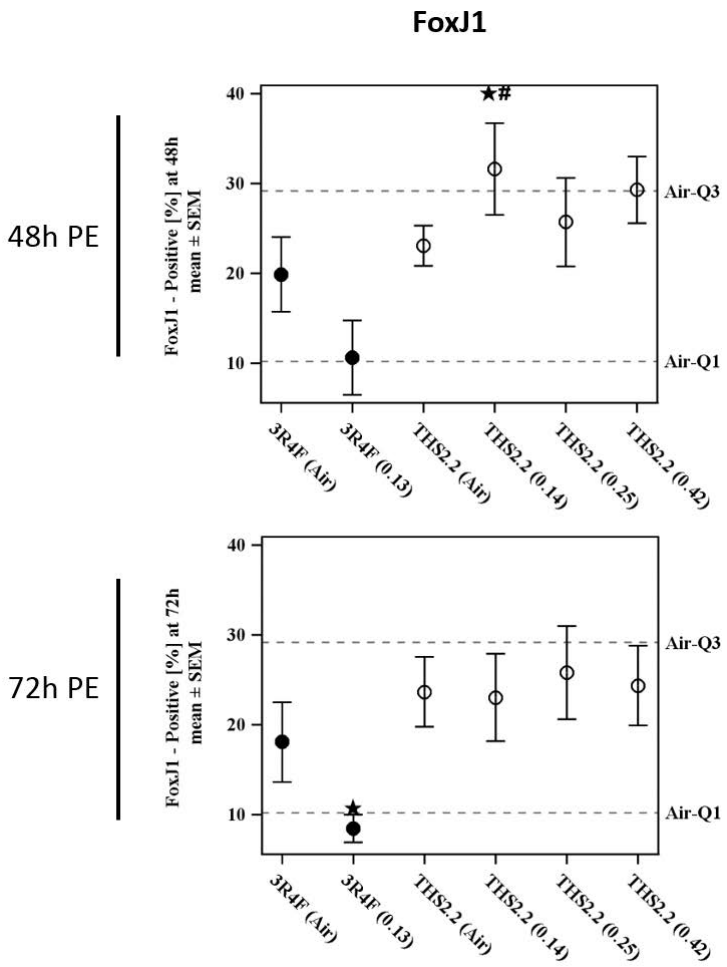


Figure 5. Results of the quantitative analyses of FoxJ1, Ki67, and p63 immunostaining tests of bronchial organotypic tissue cultures exposed to 3R4F smoke and THS2.2 aerosol

The mean ± the standard error of the mean proportions of immunopositive-stained cells found in sections of tissue cultures 48 h and 72 h after the tissues had been exposed for 28 min to 3R4F smoke or THS2.2 aerosol at the nicotine concentrations (in mg/L) indicated in parentheses are shown (N = 6 to 11). The results are for four experimental repetitions, with three exposure runs performed in each repetition. The staining was digitally quantified and expressed as the proportion of positive cells with respect to the total number of cells. The lower and upper dotted lines in each plot represent the first quartile (Q1) and third quartile (Q3), respectively, of the proportions of immunopositive-stained cells found in sections of the air-exposed control cultures after 48 h and 72 h (all combined). A ★ symbol indicates that the value was significantly different from the corresponding air controls ($p < 0.05$). A # symbol indicates that the value was significantly different from the value for cultures exposed to 3R4F smoke at a comparable nicotine concentration ($p < 0.05$).

9.3 Cytotoxicity

Cytotoxicity in a culture exposed to 3R4F smoke or THS2.2 aerosol was assessed by measuring the amount of AK released from the tissue cultures into the basolateral media. It can be seen from Figure 6 that:

- no cytotoxicity was detected 4 h after a culture had been exposed under any of the test conditions (Figure 6A);
- more cytotoxicity was found in cultures exposed to 3R4F smoke at a nicotine concentration of 0.25 mg/L (and this increased with time, from 14% more after 24 h to around 40% more after 48 h and 72 h) than in the air control cultures (Figure 6B, Figure 6C, and Figure 6D); and
- The cytotoxicity levels of all THS2.2-exposed groups were not significantly higher than that of the air controls at all post-exposure time points, and the statistical significance indicated for THS2.2 (0.42) appears to indicate different variabilities but not a relevant numerical increase in cytotoxicity (Figure 6).

The descriptive statistics for these results are shown in Supplementary Table 12.

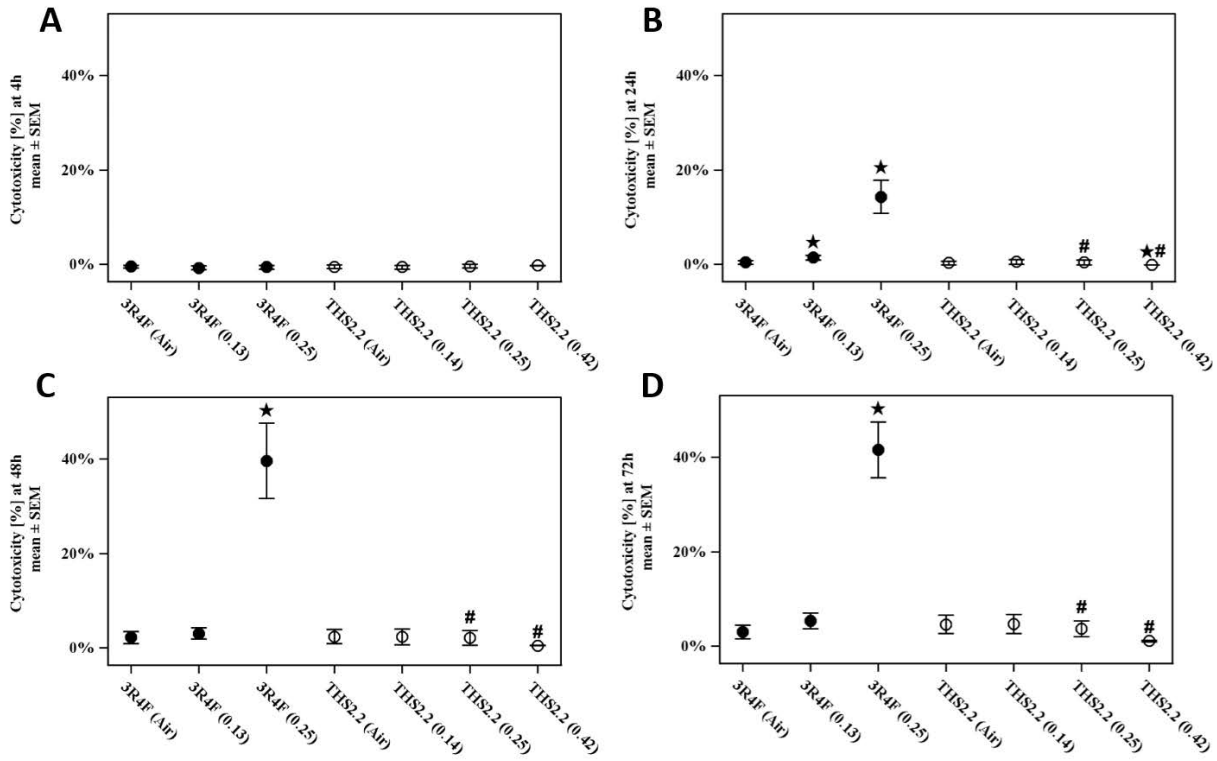


Figure 6. Cytotoxicity assessment results after organotypic tissue cultures had been exposed to 3R4F smoke or THS2.2 aerosol

The amount of AK in the basolateral medium of each tissue culture was measured (A) 4 h, (B) 24 h, (C) 48 h, and (D) 72 h after the culture had been exposed for 28 min to 3R4F smoke or THS2.2 aerosol at the nicotine concentration (mg/L) indicated in parentheses. The mean ± the standard error of the mean (N = 6 to 14 exposure runs) of the normalized cytotoxicity relative to the positive control (cultures treated with Triton X-100 were used to indicate 100% cytotoxicity) is shown for each test condition. A ★ symbol indicates that the value was significantly different from the corresponding air controls ($p < 0.05$). A # symbol indicates that the value was significantly different from the value for cultures exposed to 3R4F smoke at a comparable nicotine concentration ($p < 0.05$) except for the high THS2.2 aerosol dose (at a nicotine concentration of 0.42 mg/L), which was compared with exposure to 3R4F smoke at a nicotine concentration of 0.25 mg/L.

9.4 CYP1A1/CYP1B1 Activity

The CYP1A1/CYP1B1 activities found 48 h after cultures had been exposed to 3R4F smoke or THS2.2 aerosol are shown in Figure 7.The combined CYP1A1/CYP1B1 activity data for the EpiAirway™ and MucilAir™ cultures that were exposed to smoke or aerosol were not significantly different from the activities in the air control cultures, as is shown in Figure 7A. The CYP1A1/CYP1B1 activities in the EpiAirway™ cultures exposed to 3R4F smoke were higher (but not statistically significantly higher) than the activities in the air control cultures, and the CYP1A1/CYP1B1 activities in the EpiAirway™ cultures exposed to THS2.2 aerosol were the same as the activities in the air control cultures (Figure 7B). However, the CYP1A1/CYP1B1 activities were lower in the MucilAir™ inserts exposed to 3R4F smoke than in the air control cultures, but the CYP1A1/CYP1B1 activities in the MucilAir™ inserts exposed to THS2.2 aerosol were the same as the activities in the air control cultures (Figure 7C). The descriptive statistics for these results are shown in Supplementary Table 13.

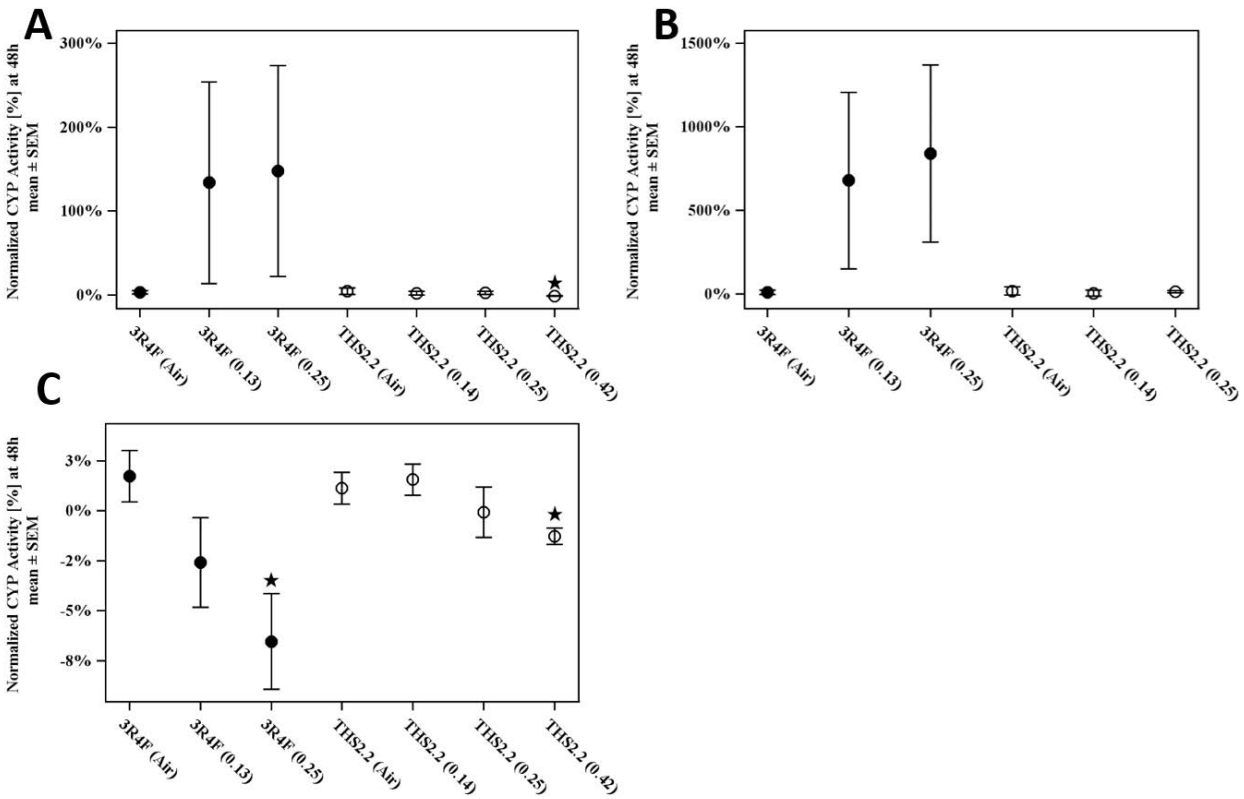


Figure 7. **CYP1A1/CYP1B1 activities in bronchial organotypic tissue cultures exposed to 3R4F smoke or THS2.2 aerosol**

The combined CYP1A1 and CYP1B1 activities were measured 48 h after the tissue cultures had been exposed for 28 min to 3R4F smoke or THS2.2 aerosol at the nicotine concentrations (mg/L) indicated in parentheses. (A) Aggregated results for all of the experimental repetitions. (B) Aggregated results for experiments 1 and 2, which were performed using EpiAirway™ tissue cultures. (C) Aggregated results for experiments 3–6, which were performed using MucilAir™ tissue cultures. The mean ± the standard error of the mean (for plot A, N = 9 to 11 in six experimental repetitions with three exposure runs per repetition; for plot B, N = 2 in two experimental repetitions with one exposure run per repetition; for plot C, N = 9 in four experimental repetitions with three exposure runs per repetition) of the activity normalized relative to the positive control (a culture treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was considered to be 100% induced). A ⋆ symbol indicates that the value was significantly different from the corresponding air controls ($p < 0.05$).

9.5 Secretion of Pro-inflammatory Mediators

The inflammatory response of the bronchial organotypic tissue cultures following exposure was assessed by measuring the mediators secreted into the basolateral media at 24, 48 and 72 h post-exposure (from different cultures as cross-sectional data). A higher number of pro-inflammatory mediators were significantly increased following 3R4F exposure than following THS2.2, at both concentrations tested (Figure 8). However, the upregulation of many of the mediators following the high concentration of 3R4F smoke exposure (0.25 mg nicotine/L) needs to be seen in the context of substantial tissue damage and tissue loss, which may have negatively impacted on the secretion of pro-inflammatory mediators. The reported mediator concentrations released in response to the high concentration of 3R4F smoke may therefore be dampened by overt cytotoxicity (see VEGFA and TIMP1). In both cases there seems to be a dose-effect, as more mediators were changed at the respective higher concentration of 3R4F smoke or THS2.2 aerosol exposure. The descriptive statistics for these results are shown in Supplementary Table 14-Supplementary Table 35, and the fold changes in each of the mediators are shown in scatter plots in Supplementary Figure 1.

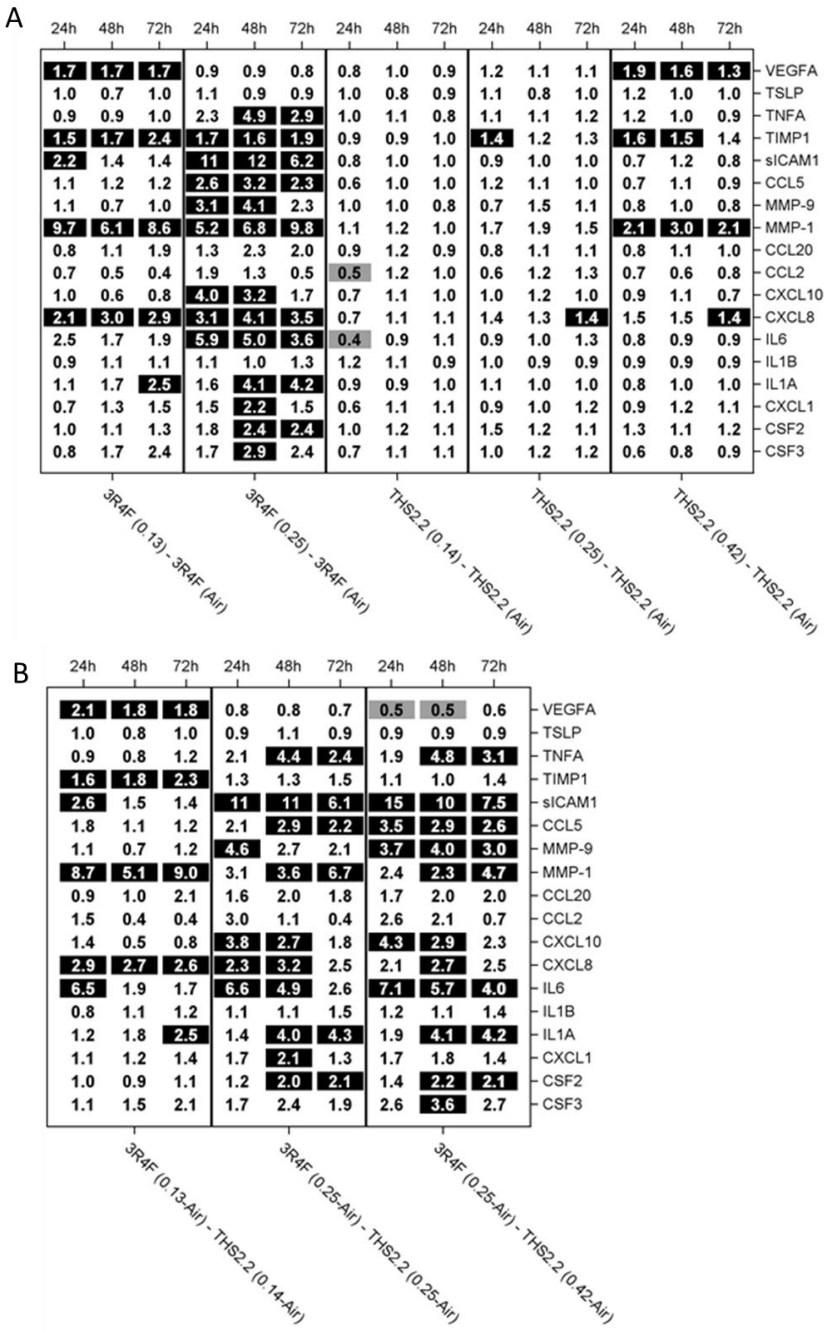


Figure 8. **Changes in inflammatory mediators in bronchial organotypic tissue cultures exposed to 3R4F smoke or THS2.2 aerosol**

Fold changes in the mediators listed on the *y*-axis in the basolateral media of tissue cultures were measured 24 h, 48 h, and 72 h after the cultures were exposed for 28 min to 3R4F smoke or THS2.2 aerosol at the nicotine concentrations (mg/L) indicated in parentheses. (A) The numbers of fold changes relative to the air control are shown (N = 6 to 12 exposure runs). (B) The differences between the mediator levels in cultures exposed to 3R4F smoke and THS2.2 aerosol are shown as the ratio between the numbers of fold changes in the cultures exposed to smoke and aerosol. Dark grey shading indicates a significant increase and light grey shading indicates a significant decrease relative to the contrasting culture set ($p < 0.05$).

It is important to note that, because of the experimental design that was used, the inflammatory mediators were not measured in the same tissue inserts at the different post-exposure time points. The study was not, therefore, a longitudinal study. Four inflammatory mediators (VEGFA, TIMP-1, MMP-1, and CXCL8) out of the 18 that were measured were significantly increased in the culture media in inserts exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L relative to the mediators in the air control inserts at all of the post-exposure time points. A higher concentration of 3R4F smoke induced larger amounts of additional mediators (including TNFA, sICAM1, CCL5, MMP-9, CXCL10, IL6, IL1A, and CSF2) to be released at either two or three of the of the post-exposure time points that were used, and a dose-dependent effect was found for CXCL8. In contrast, the amount of VEGFA was significantly higher than in the air control inserts only in inserts exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L and not in inserts exposed to 3R4F smoke at a nicotine concentration of 0.25 mg/L.

The amounts of some inflammatory mediators were significantly higher in the basolateral media of inserts exposed to THS2.2 aerosol, mostly for THS2.2 (0.42), where higher amounts of VEGFA, TIMP1, MMP-1 and CXCL8 were secreted at one or more post-exposure time points, qualitatively resembling the effect seen in 3R4F (0.13) though at a lower level.. At comparable nicotine concentrations, the effect of THS2.2 exposure on the release of pro-inflammatory markers was limited to a few sporadic changes: in THS2.2 (0.13), CCL2 and IL-6 were lower than the air controls at 24 h, and in THS2.2, TIMP1 was 1.4-fold higher at 24 h, and CXCL8 was 1.4-fold higher at 72 h only. To conclude, the exposure to THS2.2 aerosol had a lesser impact on the release of inflammatory mediators than the exposure to 3R4F smoke at comparable nicotine concentration and did not induce additional mediators. Even when comparing the highest concentration of THS2.2 (0.42) to the high concentration of 3R4F (0.25 mg/L nicotine), the increases were lower at all post-exposure time points, as shown in Figure 8B. These findings suggest that no new inflammatory mechanisms were activated by THS2.2 aerosol, and the conventional 3R4F smoke-related effects were markedly attenuated in response to THS2.2 aerosol exposure, probably reflecting the reduction in harmful constituents.

9.6 Transcriptomes of the Bronchial Epithelial Cultures

9.6.1 Global differential gene expression

The investigation of possible toxicity-specific mechanisms associated with 3R4F and THS2.2 exposure was conducted in tissue cultures that exhibited limited tissue damage. This choice was made to assess toxicity-related mechanisms in surviving cells, instead of reflecting morphological alterations associated only with severely damaged tissue (Haschek 2013) and (Davis 2013). Substantial cytotoxicity (Figure 6) and tissue damage (Figure 3) as observed in 3R4F (0.25)-exposed tissues, potentially showing gene expression profiles of acute damage and cell killing, without exhibiting compensatory gene transcription mechanisms consequently, gene expression data was evaluated from only the 3R4F (0.13), THS2.2 (0.14), THS2.2 (0.25), and THS2.2 (0.42) groups. The gene expression profiles of all these groups are reported in Supplementary Figure 2.

Changes in global gene expression in the tissues in the four selected exposure groups 4 h, 24 h, 48 h, and 72 h after the tissues had been exposed relative to gene expression in the corresponding air controls are shown in Figure 9. For all exposure conditions tested, the higher number of genes was differentially expressed at the 4-h post-exposure time point, compared to later time points, although the response to 3R4F remained similarly high over all post-exposure time points.

Exposure to 3R4F smoke at a nicotine concentration of 0.13 mg/L triggered more gene expression changes than exposure to THS2.2 aerosol at any of the test concentrations at all of the post-exposure time points. Interestingly, gene expression changes increased dose-dependently after exposure to THS2.2 aerosol, especially at the 4 h and 24 h post-exposure time points.

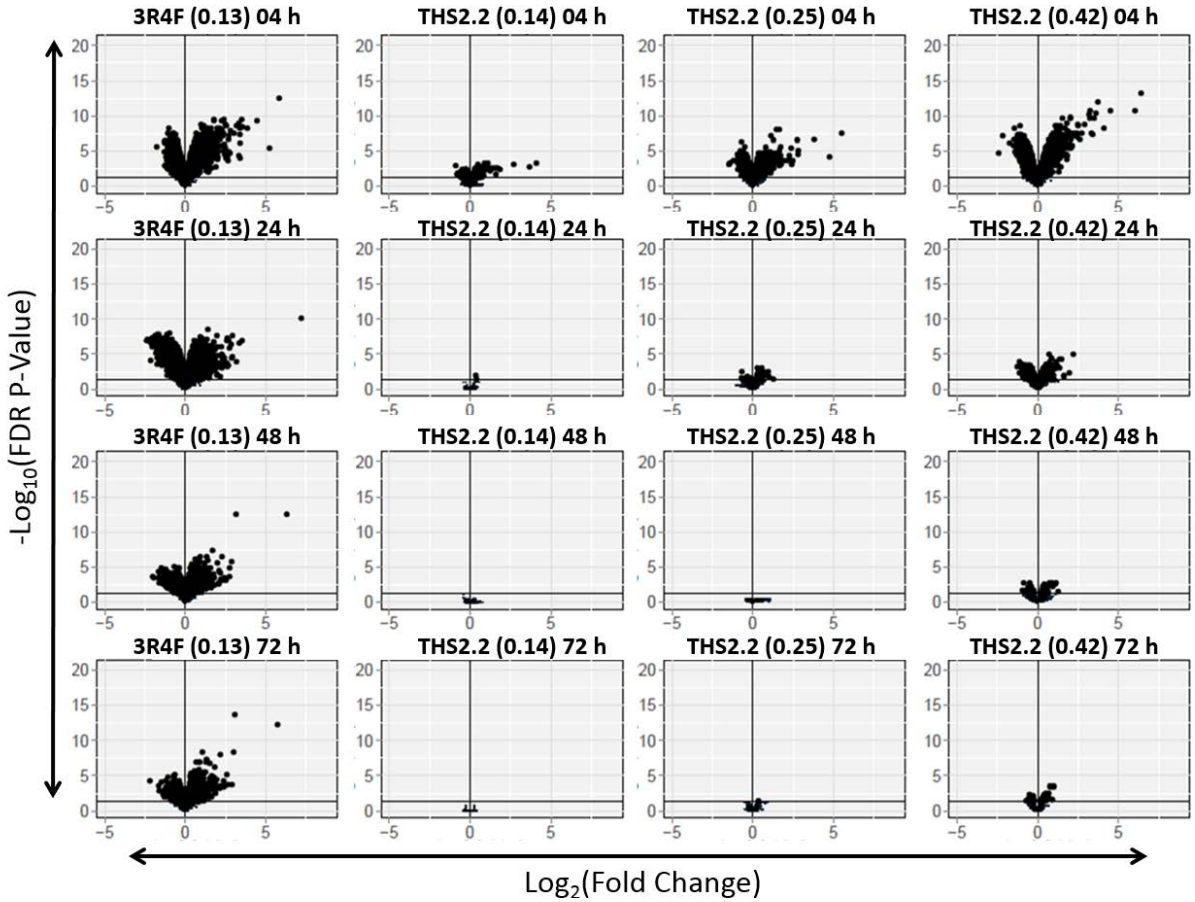


Figure 9. Volcano plots of the differentially expressed genes in bronchial epithelial tissue cultures exposed to 3R4F smoke or THS2.2 aerosol

Each dot in a plot represents a gene. Gene expression changes (on the *x*-axis) are expressed as $\log_2(fold\ change)$. The number of changes increased relative to the air control culture when the value was > 0 and decreased when the value was < 0. The statistical significance (on the *y*-axis) is represented as −log10 (adjusted *p*-value FDR) (N = 9 to 16 in six experimental repetitions with one exposure run per repetition in experiments 1 and 2 and three exposure runs per repetition in experiments 3–6). The horizontal line in each plot represents the level of statistical significance ($-\log_{10}(0.05)$).

9.6.2 Biological Impact Factor

A network-based systems biology approach using the transcriptomic data was used to quantify the effects of the exposure of the cultures to smoke or aerosol on the biological processes in the cultures (Martin 2014). Biological networks, each made up of a series of cause-and-effect relationships, were used in the approach to represent biological processes involved in cell proliferation, cell fate, cell stress, and inflammatory responses (inflammatory process networks) (Boue 2015). The edges of the networks carry a causal directionality (increase or decrease) between two nodes based on information extracted from scientific literature (Martin 2014). The nodes of the networks correspond to biological entities (e.g., protein abundances, protein activities, chemical compounds, or biological processes described as molecular entities) and are referred to as "backbone nodes", which form the functional layer of the network models (Martin 2014).

Information on the abilities of many of the background nodes to regulate the expression of particular genes was included in the network models. Therefore, in addition to the model backbone, each model could have a measurable layer consisting of mRNA nodes. The abundance of the mRNA transcripts in the transcriptomic data could vary, so the transcriptomic data (relative to the control samples) for the four test groups collected 4 h, 24 h, 48 h, and 72 h after the cultures had been exposed to smoke or aerosol were used quantitatively to predict the activity and functionality of the functional layer of the network using the NPA algorithm (Martin 2014). The algorithm was based on backward reasoning, through which a change in gene expression was considered to be caused by the activity of an upstream backbone node. The predicted activation or inhibition of a backbone node and the causality of the network edges allowed inferences to be made and computations for the network as a whole to be performed (Martin 2014). The overall systems effect (the aggregation of network perturbations) is later called the BIF (Thomson 2013).

The overall biological impacts, in the context of the biology covered by the network models, for the different exposure conditions compared with the corresponding air controls are shown in Figure 10. Four hours after the cultures had been exposed to smoke or aerosol, the strongest impact was found in the tissues exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L. The BIF for 3R4F smoke at a nicotine concentration of 0.13 mg/L was therefore used as the reference (REF), i.e., used as the 100% relative BIF.

The impact of 3R4F (0.13) exposure decreased with increasing duration of the post-exposure.
At comparable nicotine concentration, the impact of THS2.2 (0.14) aerosol on the tissues was always lower than that of 3R4F (0.13) smoke for any of the post-exposure time points tested. The impact of THS2.2 aerosol exposure decreased sharply with increasing duration of the post-exposure time. A dose-dependent effect of THS2.2 exposure is observed on the BIF score 4 h after exposure. Interestingly, the δ values around or above 0.9 indicate that the underlying networks perturbed at the 4 h post-exposure time point in the THS2.2 (0.25) and THS2.2 (0.42) groups are very similar to the ones perturbed 4 h after exposure to 3R4F (0.13) smoke (also marked as REF).

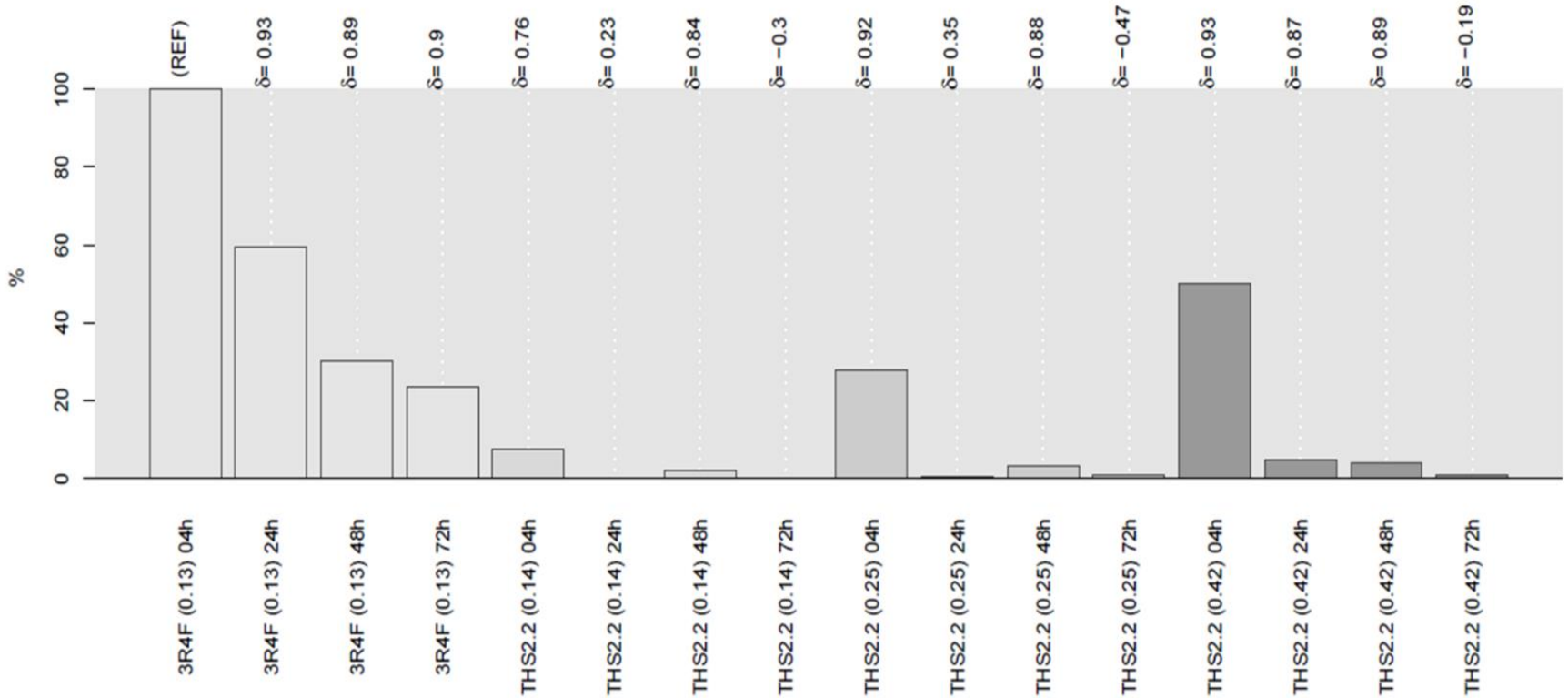


Figure 10. Relative biological impact factors (BIFs) derived from the cumulated network perturbations caused by exposure to 3R4F smoke and THS2.2 aerosol relative to the air control

The relative BIF (in percent) is plotted on the *y*-axis. The highest BIF is labelled REF and was used as the 100% point. The δ values (δ in [-1,1]) given above the plot indicate how similar the underlying networks that were perturbed in a particular test were to the underlying networks in the REF, δ = 0 indicating no similarity, δ = 1 indicating identical underlying networks were perturbed, and δ = −1 indicating completely opposite underlying networks were perturbed.

An overview of the different biological processes, represented as network families that contributed to the overall BIF under the different exposure conditions is shown in Figure 11. In the figure, the contributions of the perturbations in each network family to the BIF are shown in pie charts. Among the network families, the impact of 3R4F (0.13) smoke (for all post-exposure time points) and THS2.2 aerosol (at all tested concentrations but only at 4 h and 24 h post-exposure time point) is the highest on the Cell Stress (CST) network family, followed by the Cell Fate (CFA), Inflammatory Processes (IPN), and cell proliferation (CPR) networks.

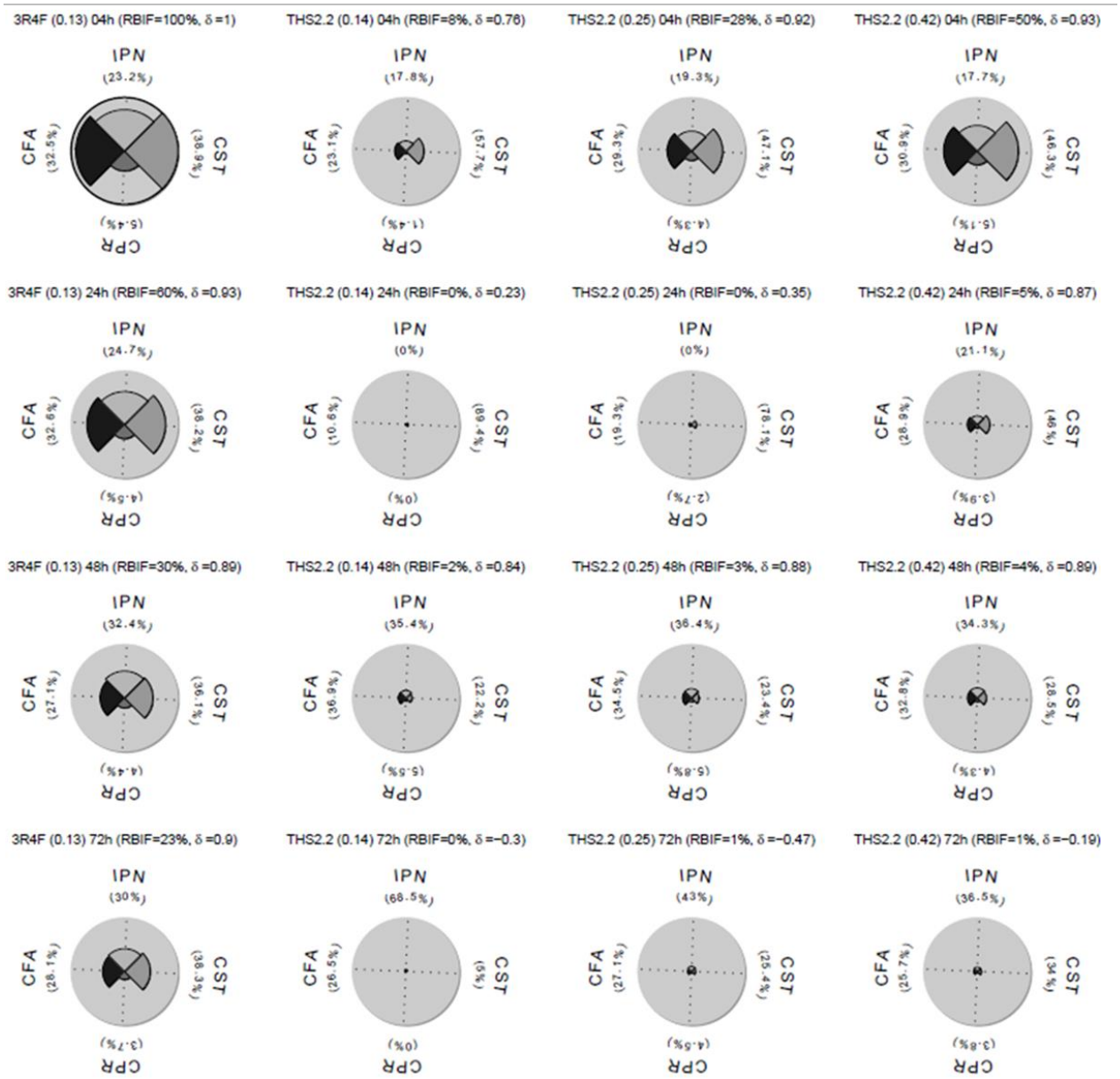


Figure 11. Contributions of the different network perturbations to the biological impact factor (BIF)

Each pie chart represents the corresponding bar in the BIF plot shown in Figure 10. Each chart shows the contributions of the cell fate (CFA), cell stress (CST), cell proliferation (CPR), and inflammatory process networks (IPN) families to the BIF. The area of a slice indicates the relative contribution of the perturbation of that network to the BIF in a given test (shown as the percentage in parentheses underneath the network label). The pie chart for the REF (the test with the highest BIF, taken as the 100% BIF level) has a thick black line around it. The relative BIF (RBIF, in percent) and the δ value are given above each plot. The δ values (δ in [-1,1]) indicate the similarity between the underlying networks that were perturbed in the test and the networks that were perturbed in the REF, δ = 0 indicating no similarity, δ = 1 indicating identical networks were perturbed, and δ = −1 indicating exactly opposite networks were perturbed.

9.6.3 Network Perturbation Amplitude

Each network family consisted of a set of network models, and the BIF could be decomposed to the network level. The perturbations of the different networks in the different tests are shown in Figure 12.

Functional analysis of the transcriptomic changes induced by exposure to 3R4F smoke at a nicotine concentration of 0.13 mg/L indicated that significant perturbations occurred at all post-exposure time points:

- in the cell fate networks (senescence, necroptosis, and apoptosis);
- in the cell stress networks (xenobiotic metabolism response, oxidative stress, osmotic stress, and NFE2L2 signaling);
- in the cell proliferation networks (Wnt signaling, nuclear receptors, MAPK and JAK-STAT signaling, and growth factor pathways); and
- in the inflammatory processes networks (tissue damage, epithelial mucus hypersecretion, and epithelial innate immune activation).

At all concentrations, the exposure to THS2.2 aerosol perturbed almost the same list of subnetworks but typically with a markedly lower NPA score than after 3R4F exposure and often only for a few post-exposure time points. For both, 3R4F and THS2.2 exposures, transcriptomic changes were more or less transient, with an initial peak in the amplitude at the 4h post-exposure time point, gradually decreasing until 72h. There were a few exceptions, such as the delayed Hypoxic Stress response or the sporadic Cell Cycle perturbations. Again, a dose-dependent effect is noticeable for THS2.2 exposure at 4 h post-exposure time point (e.g. Xenobiotic Metabolism, Oxidative Stress, Osmotic stress, NEF2L2 Signalling, Senescence, DNA damage, Necroptosis, Apoptosis, Nuclear Receptors, Mapk, Growth Factors, Cell Cycle, Tissue Damage, Epithelial Mucus Hypersecretion, Epithelial Innate Immune Activation). There were single perturbations in the Calcium, Cell Interaction, and Hedgehog subnetworks of the CPR family that were statistically significant only for a few conditions of THS2.2 aerosol exposure, while these perturbations missed statistical significance for 3R4F (0.13) smoke exposure, though the amplitudes were higher for the latter.

Gene-set analyses were also conducted, and the results are shown as a heatmap of the enrichment scores in Supplementary Figure 3. The heatmap showed that the gene expression data from the tissues exposed to 3R4F (0.13) had higher enrichment scores for the different biological annotations than did the data for tissues exposed to THS2.2 aerosol at all three nicotine concentrations that were tested at all of the post-exposure time points. The findings are also consistent with the results obtained based on the NPA scores, where higher signals were observed with the shorter post-exposure duration for THS2.2.

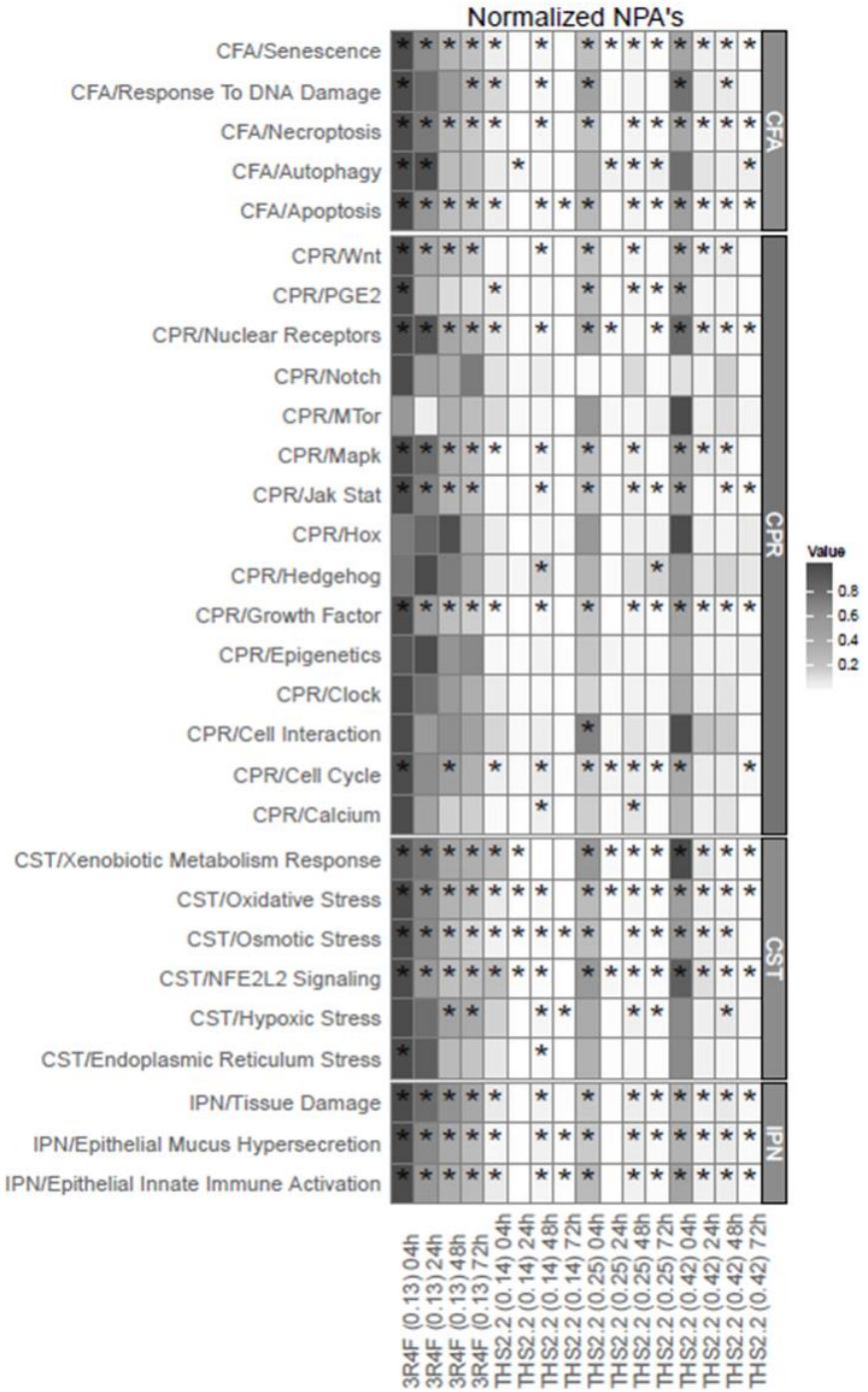


Figure 12. Heatmap of the network perturbation amplitude (NPA) scores for the biological networks affected by exposure to 3R4F smoke or THS2.2 aerosol

The network names are shown on the left of the heatmap, and the network family names are shown on the right. All networks significantly affected in at least one test are shown. The shading gradient for the NPA score was normalized to the maximum NPA score for each network. A network was considered to be significantly perturbed if the NPA score was significant after accounting for experimental variations and if the companion statistics O and K were significant ($p < 0.05$). The O and K statistics indicate the specificity of the NPA score with respect to the biology represented by the network. A * symbol indicates that the NPA score was significant with respect to experimental variations and that the O and K statistics were significant. Abbreviations: CFA, cell fate; CST, cell Stress; CPR, cell proliferation; and IPN, inflammatory process networks.

9.6.4 Alterations in the Expression Levels of the Genes Regulating Cell Stress and Inflammation

In addition to the network analysis, differentially expressed relevant genes known to contribute to cell stress and inflammatory processes are displayed in Figure 13 and Figure 14, respectively.

Many of the genes that are involved in DNA damage response (e.g., DDIT3), endoplasmic reticulum stress (e.g., SLC3A2 and ATF6), heat shock (e.g., DNAJB9), and hypoxic stress (e.g., VEGFA) were differentially expressed (mostly increased compared with the air controls) in cultures exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L between 4 h and 72 h after exposure. However, at comparable nicotine concentrations, exposure to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L only caused increased expression in very few genes (i.e., SLC3A2 for endoplasmic reticulum stress, DNAJB4 for heat shock, and MDM2 for DNA damage), and this only occurred at the 4 h post-exposure time point. Exposure to the higher THS2.2 aerosol concentrations had a larger response in the expression of the genes involved in these stress networks than did exposure to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L, especially 4 h after exposure, but these effects were still less severe than the effects caused by exposure to 3R4F smoke at a nicotine concentration of 0.13 mg/L.

Increased expression of many of the genes that regulate oxidative stress (*SLC7A11, MAFF, HMOX1, NQO1, GSR, FTH1, GCLC, GPX2, TXNRD1, GCLM, MAFG, SQSTM1, SOD2,* and *FTL*) was found in tissues exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L at all of the post-exposure time points, as shown in Figure 13. Most of these genes are regulated by the NRF2 transcription factor (also known as NFE2L2), whereas *MAFG* and *MAFF* play roles in regulating the intracellular location of NRF2, and therefore contribute to its transcriptional activity. The expression of the majority of these genes increased also in THS2.2-exposed tissues. However, while these genes appeared to be permanently activated for 3R4F at all post-exposure time points, for THS2.2, the activation was only transient, decreasing again after 4h to 24h post-exposure.

The expression of many genes involved in the xenobiotic stress network (Figure 13) increased in tissues exposed to 3R4F (0.13). *CYP1A1* and *CYP1B1* were more strongly expressed at all times after tissues had been exposed to 3R4F (0.13). Exposure to THS2.2 aerosol at all concentrations, and principally at the 4 h post-exposure time point, also increased the expression of *CYP1A1* and *CYP1B1* relative to the air controls. Only exposure to THS2.2 aerosol at a nicotine concentration of 0.42 mg/L continued to increase *CYP1A1* expression 24 h after exposure. However, high concentrations, THS2.2 (0.25) and THS2.2 (0.42) exposure induced a sustained increase in the expression of *CYP1B1*. Tissues exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L had increased *AKR1C2*, *TIPARP*, *AHRR*, and *AKR1B10* levels at all post-exposure time points. In contrast, exposure to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L only increased the expression of genes in the xenobiotic stress network (i.e., *AHR*, *AKR1C3*, *AKR1C2*, *AKR1C1*, *AHRR*, and *AKR1B10*) at the 4 h post-exposure time point. Similar to *CYP1B1*, for the higher concentrations of THS2.2 aerosol, the expression of many of these genes was still high at later post-exposure time points.

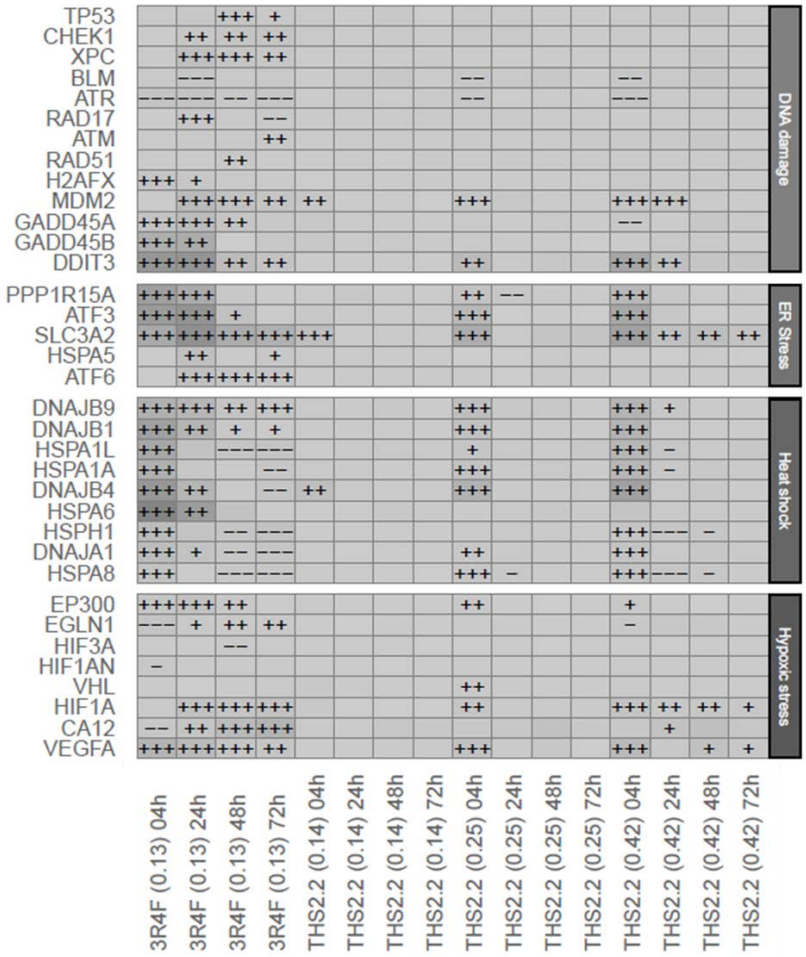


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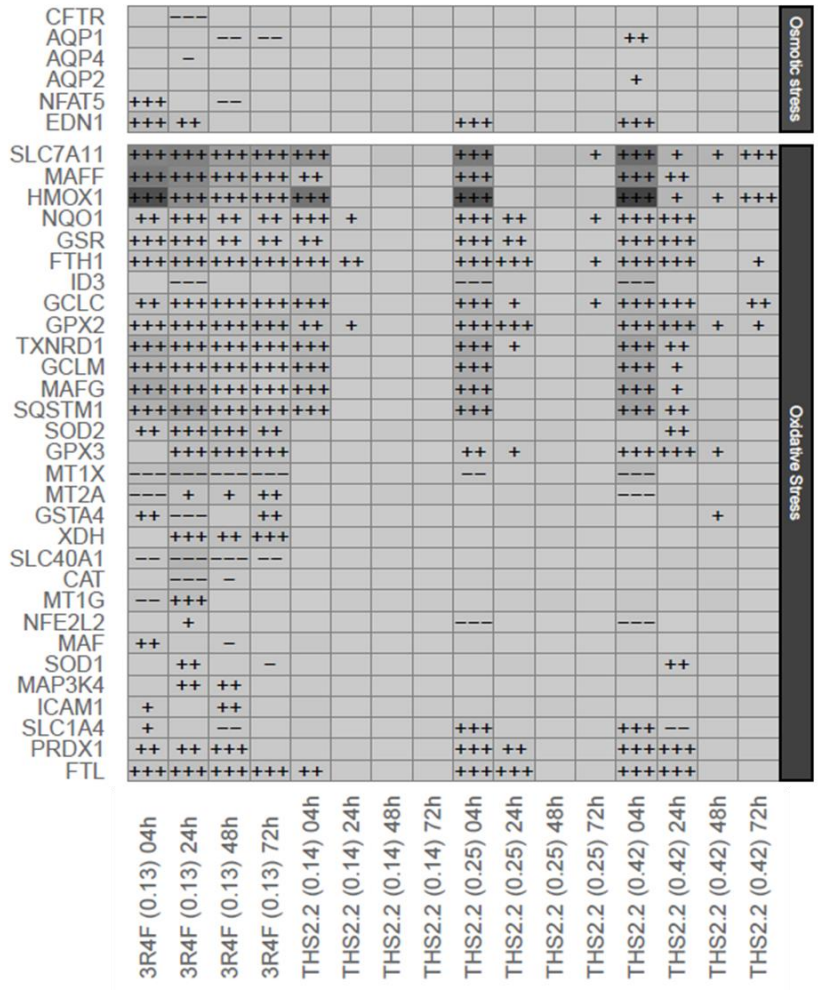


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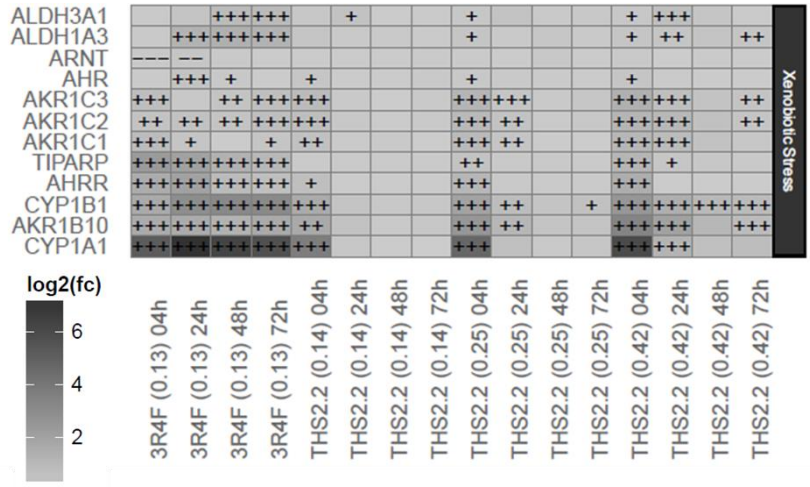


Figure 13. Changes in the expression levels of genes that regulate cellular stress in cultures exposed to 3R4F smoke or THS2.2 aerosol

The heatmap shows the changes in gene expression expressed as $\log_2(fold\ change)$. Upregulated gene expression and downregulated gene expression compared with the appropriate air controls are marked with + and − signs, respectively (+++ and – – – indicate adjusted *p*-values < 0.01, ++ and – – indicate adjusted *p*-values < 0.05, and + and – indicate adjusted *p*-values < 0.1). The gene symbols are given on the left of the heatmap, and stress-related processes are marked on the right. Fold changes is abbreviated to fc.

As shown in Figure 14**,** the expression level of various genes involved in inflammatory processes was changed (mostly increased) after exposure to 3R4F (0.13) smoke and at most exposure time points (e.g. *IL1B, PLAU, CXCL2, EGR1, FOS, IL1A, MMP1, PTGS2*). At the comparable nicotine concentration, THS2.2 did not impact the expression of any of the genes listed in this heatmap and at any post-exposure time points investigated. Only higher concentrations of THS2.2 aerosol showed an effect on the expression levels of genes linked to inflammation and this was mostly observed at the early post-exposure time point (e.g. *IL1A, PTGS2, FOS, JUN, EGR1, CXCL2, CCL20, PLAU, CEBPB*). It is also noticeable that the directionality of the gene expression changes is almost identical to the ones observed for 3R4F, only the amplitude and/or the significance of the changes may vary.

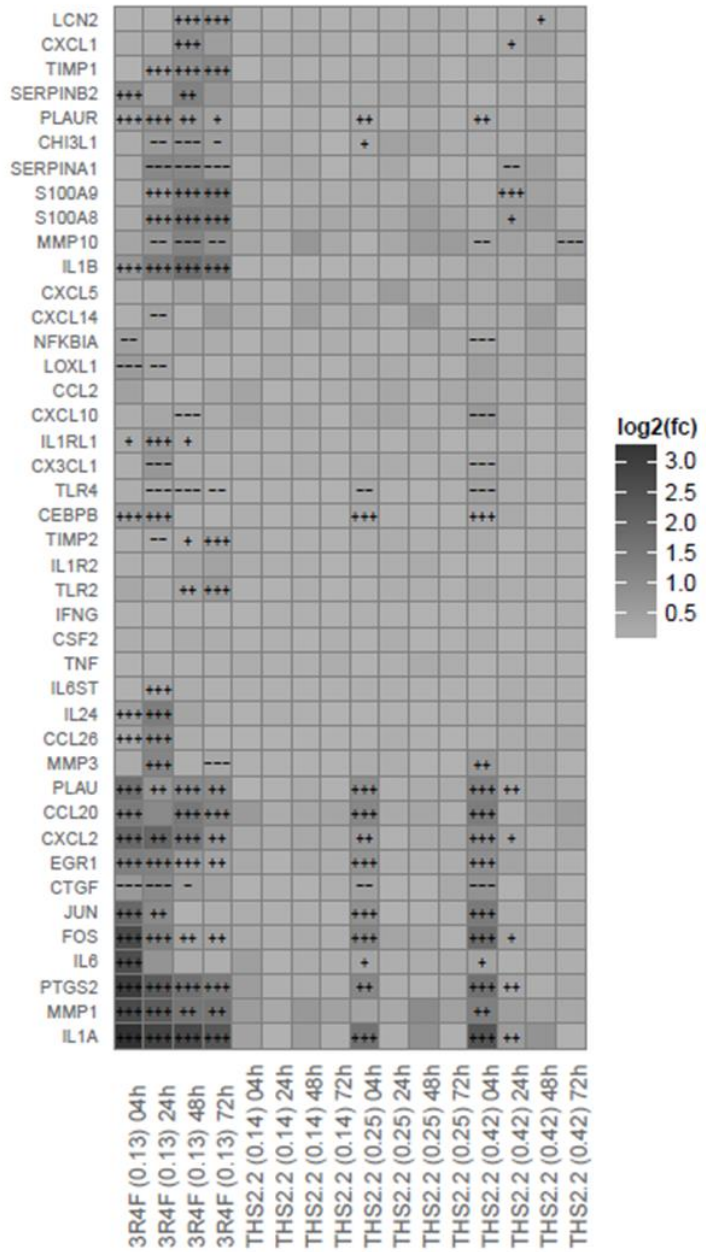


Figure 14. Changes in the levels of expression of genes that regulate inflammation after exposure to 3R4F smoke or THS2.2 aerosol

The heatmap shows the changes in gene expression expressed as $\log_2(fold\ change)$. Upregulated gene expression and downregulated gene expression compared with the appropriate air controls are marked + and −, respectively (+++ and − − − indicate adjusted *p*-values < 0.01, ++ and − − indi cate adjusted *p*-values < 0.05, and + and − indicate adjusted *p*-values < 0.1). The gene symbols are shown on the left. Fold changes is abbreviated to fc.

9.7 MiRNA Transcriptomes of the Bronchial Epithelial Cultures

Gene expression is regulated and many biological processes are influenced by miRNA (Pritchard 2012). Possible mechanisms associated with responses to exposure to 3R4F smoke and THS2.2 aerosol were identified from the miRNA transcriptome data for the bronchial epithelial tissues exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L and to THS2.2 aerosol at nicotine concentrations of 0.14 mg/L, 0.25 mg/L, and 0.42 mg/L relative to the appropriate air controls. The profiles of the differentially expressed miRNAs in all of the exposure groups are shown in Supplementary Figure 4.

The changes found in miRNA expression levels after tissues were exposed to smoke or aerosol are shown in Figure 15. The expressions of more miRNAs were altered in tissue exposed to 3R4F (0.13) than in tissue exposed to THS2.2 aerosol at all of the test concentrations. The expressions of more miRNAs were significantly altered by exposure to 3R4F smoke the longer after exposure the measurements were made. In contrast, almost no miRNAs were significantly differentially expressed in tissues exposed to THS2.2 aerosol at any of the post-exposure time points that were used.

Global miRNA changes found in all of the tests are shown as quantile–quantile (QQ) plots in Supplementary Figure 5. When marked differentially expressed miRNAs were not found (for example, in the volcano plots for the adjusted *p*-values in Figure 15), the QQ plots could provide additional information on miRNA alterations by showing similarities between the miRNA changes found and miRNA changes in randomly permuted miRNA expression matrices. The QQ plots suggested that exposure to 3R4F (0.13) may have been related to significant global effects on miRNA expression that increased with post-exposure time. Exposure to THS2.2 aerosol at all of the test concentrations affected the miRNA expression profiles less at all of the post-exposure time points that were used.

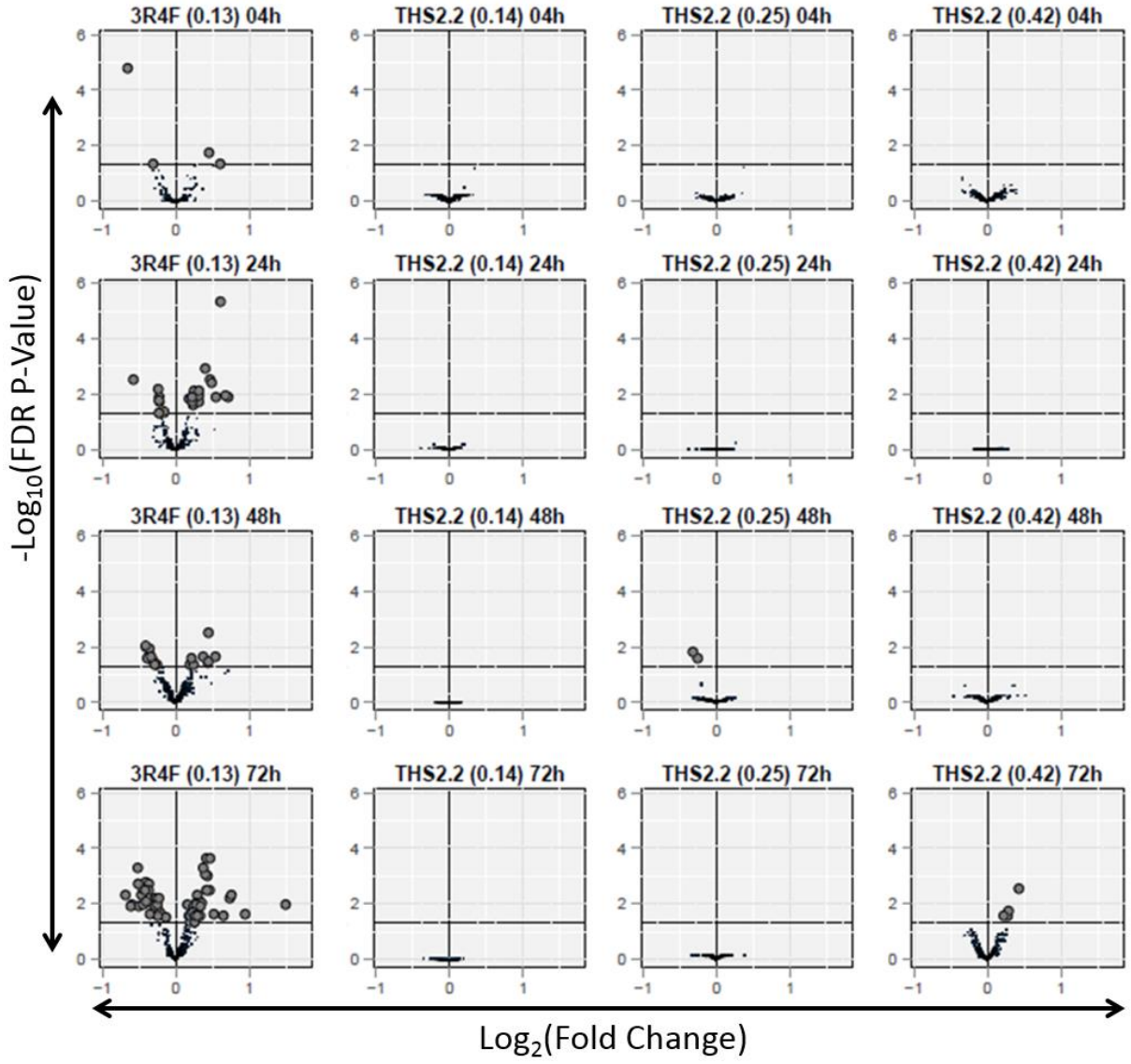


Figure 15. Volcano plots of the differentially expressed miRNAs in bronchial epithelial tissue cultures exposed to 3R4F smoke and THS2.2 aerosol

Each dot in a plot represents a miRNA. A change in miRNA expression (on the *x*-axis) is expressed as $\log_2(fold\ change)$, more changes occurring when the value was > 0 and fewer when the value was < 0. The statistical significance (on the *y*-axis) is expressed as $-\log_{10}(adjusted\ p\ value)$ (N = 8 to 12 in six experimental repetitions with one exposure run per repetition in experiments 1 and 2 and three exposure runs per repetition in experiments 3–6). The threshold line for statistical significance ($-\log_{10}(0.05)$) is shown as a solid line (at 1.3 on the *y*-axis).

The miRNAs that were significantly altered in at least one test are shown in Figure 16. Exposure to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L did not affect the expression of these miRNAs relative to the air controls, whereas exposure to 3R4F smoke at a nicotine concentration of 0.13 mg/L caused various miRNAs to be differentially expressed relative to the air controls at all post-exposure time points that were used. The most differentially expressed miRNAs were found 72 h after exposure to 3R4F smoke at a nicotine concentration of 0.13 mg/L. Only four miRNAs were more expressed after tissue had been exposed to THS2.2 aerosol at a nicotine concentration of 0.42 mg/L at the longest post-exposure time point than in the air controls. Three out of these miRNAs were upregulated 72 h after tissues had been exposed to 3R4F smoke at a nicotine concentration of 0.13 mg/L. Two miRNAs were slightly downregulated relative to the air controls 48 h after tissues had been exposed to THS2.2 aerosol at a nicotine concentration of 0.25 mg/L.

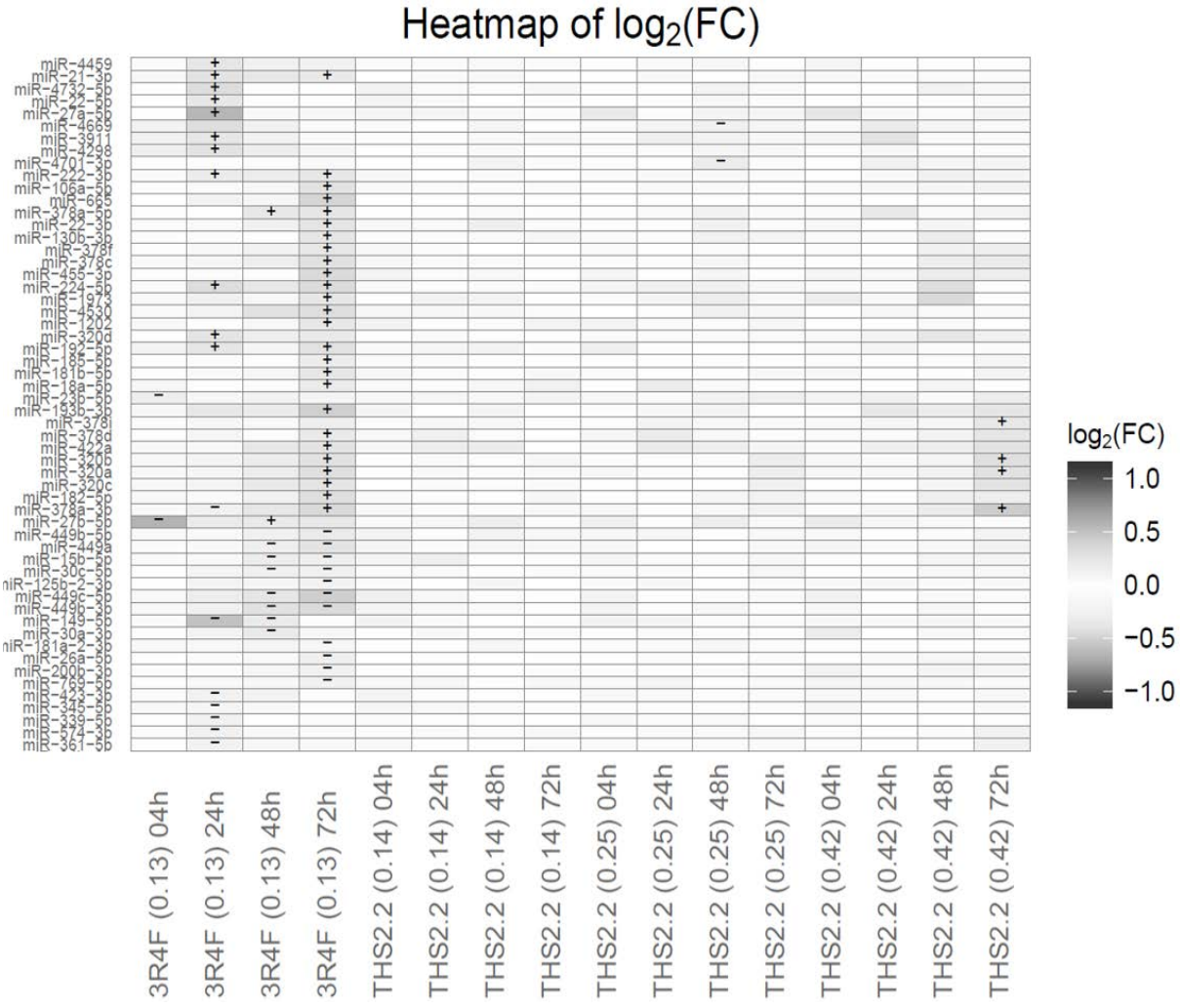


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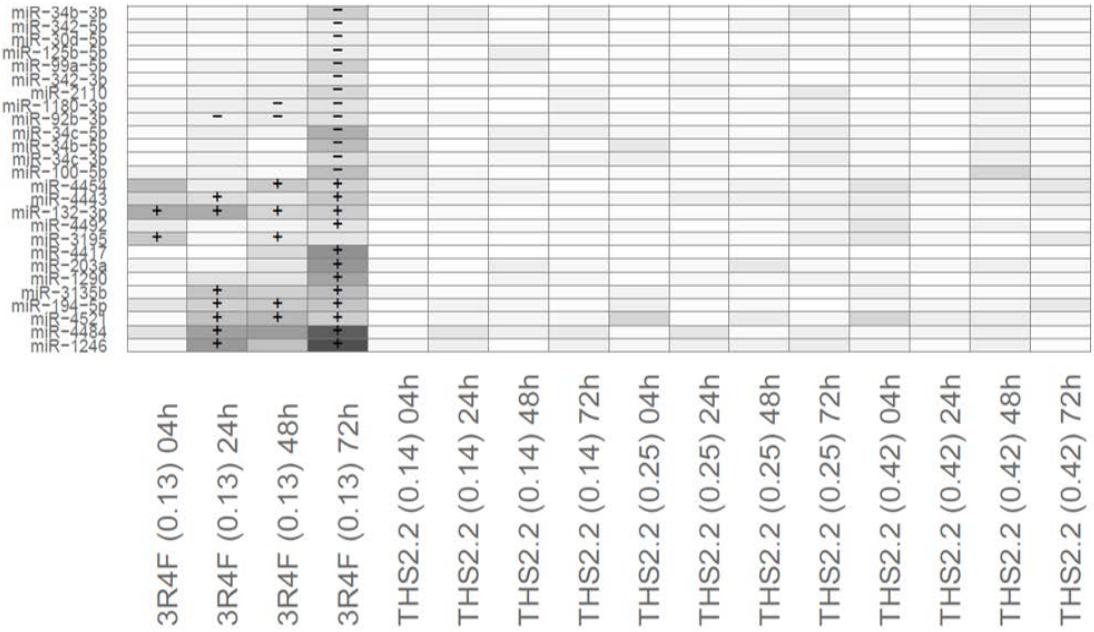


Figure 16. Heatmap of differentially expressed miRNAs after exposure to 3R4F smoke or THS2.2 aerosol

The heatmap shows changes in miRNA expression levels expressed as $\log_2(fold\ change)$, increased expression occurring relative to the appropriate air controls at values > 0 and decreased expression occurring at values < 0. The + and − signs indicate increased and decreased expression, respectively (N = 8 to 12 per group in six experimental repetitions with one exposure run per repetition in experiments 1 and 2 and three exposure runs per repetition in experiments 3–6). The miRNAs that were significantly affected in at least one test are shown on the left. The shading indicates the extent of absolute fold changes relative to the controls.

9.8 Correlation of the Outcomes across the Experimental Repetitions

9.8.1 Correlation of Immunohistochemistry, Cytotoxicity, CYP1A1/1B1 Activity, and Pro-Inflammatory Mediators across the Experimental Repetitions

To assess the variability of the observation across the experimental repetitions, a correlation of the endpoint values from various functional assays (i.e. immunohistochemistry scores [proportion of the positive stained cells]; normalized cytotoxicity level [based on AK assays]; normalized CYP1A1/1B1 activity level; and the concentrations of the secreted pro-inflammatory mediators [the logarithmic base 10 of the concentration]) were computed. To explore the variability across the experimental repetitions, we only considered the experiments 3-6 which had an experimental design with N=3 smoking runs per experimental repetition. For this, for each of these endpoints and post-exposure time points, the difference (Δ) between the exposed and air control was calculated (i.e. the pair sample, see Statistical Comparisons).

Subsequently, we computed the average of these differences; i.e. those obtained in experimental repetitions 3 and 4 (the x-axis, Figure 17) and those obtained in experimental repetitions 5 and 6 (the y-axis, Figure 17). The plots are separated into stimulus exposure type. They illustrate that these averaged values obtained from the experimental repetition 3 and 4, were correlated to those obtained from the experimental repetition 5 and 6. Greater correlations were observed for 3R4F smoke exposure as compared with THS2.2 aerosol exposure. This is consistent with the greater biological impact upon 3R4F exposure (greater cytotoxicity, greater alteration of CYP1A1/1B1 activity, and greater secretion of pro-inflammatory mediators) than upon THS2.2 exposure.

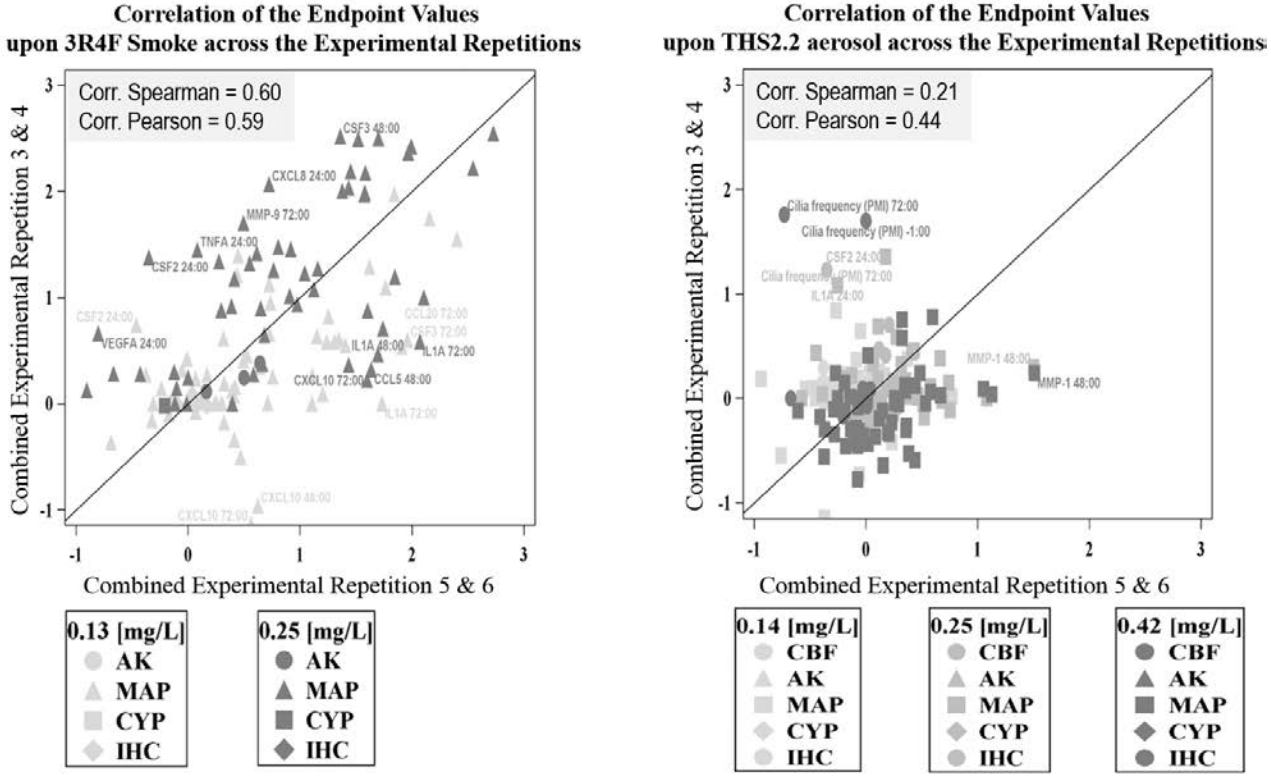


Figure 17. Correlation plots of the immunohistochemistry, cytotoxicity, CYP1A1/1B1 activity, and pro-inflammatory mediators across the experimental repetitions

The average of the differences of the endpoint values (exposed vs. control), which were obtained from the experimental repetition 1 and 2 (x-axis), were correlated to those obtained from the experimental repetition 3, 4 and 5 (y-axis). Different symbols are used to highlight the different endpoints: AK (the normalized cytotoxicity measurement), CYP (the normalized activity of CYP1A1/1B1), and MAP (the logarithm based 10 of the concentration measured using Luminex based analyses of pro-inflammatory mediators), as well as IHC (the proportion of the p63, Ki67, and FoxJ1 positive stained cells). A 45-degree line is drawn to ease the visualization. A Spearman and Pearson correlations were computed. Abbreviations: AK, adenylate kinase; Cor, correlation; CYP, cytochrome P450; Exp; experimental repetition; IHC, immunohistochemistry; MAP, multianalyte profiling.

9.8.2 Reproducibility of the Gene Expression Fold Changes

Correlation plots were produced to allow the reproducibility of the gene expression changes found in the different experimental repetitions to be assessed. Only experiments 3–6, which each had three smoking runs per experimental repetition, were used in this assessment. The gene expression data produced in experiments 3 and 4 were combined, and the average fold change was determined for each gene in the dataset. The gene expression data produced in experiments 5 and 6 were treated in the same way. Correlation plots for the different tests were then produced, each dot in a correlation plot representing an individual gene. The correlation plots are shown in Figure 18.

The plots showed that, at the different post-exposure time points, the gene expression changes in tissues exposed to 3R4F smoke at nicotine concentrations of 0.13 mg/L and 0.25 mg/L obtained in experiments 1 and 2 and in experiments 3–6 strongly correlated (the correlation coefficients were 0.76–0.95). Strong correlations were also found 4 h after tissues had been exposed to THS2.2 aerosol at concentrations of 0.25 mg/L (the correlation coefficient was 0.83) and 0.42 mg/L (the correlation coefficient was 0.86). However, the correlation coefficients were lower (0.01–0.45) for the relationships between the gene expression fold changes 24 h, 48 h, and 72 h after exposure to THS2.2 aerosol at all of the test concentrations and 4 h after exposure to THS2.2 aerosol at a nicotine concentration of 0.14 mg/L. This result was expected because of the few genes for which expression was affected by exposure to THS2.2 aerosol (see Figure 9).

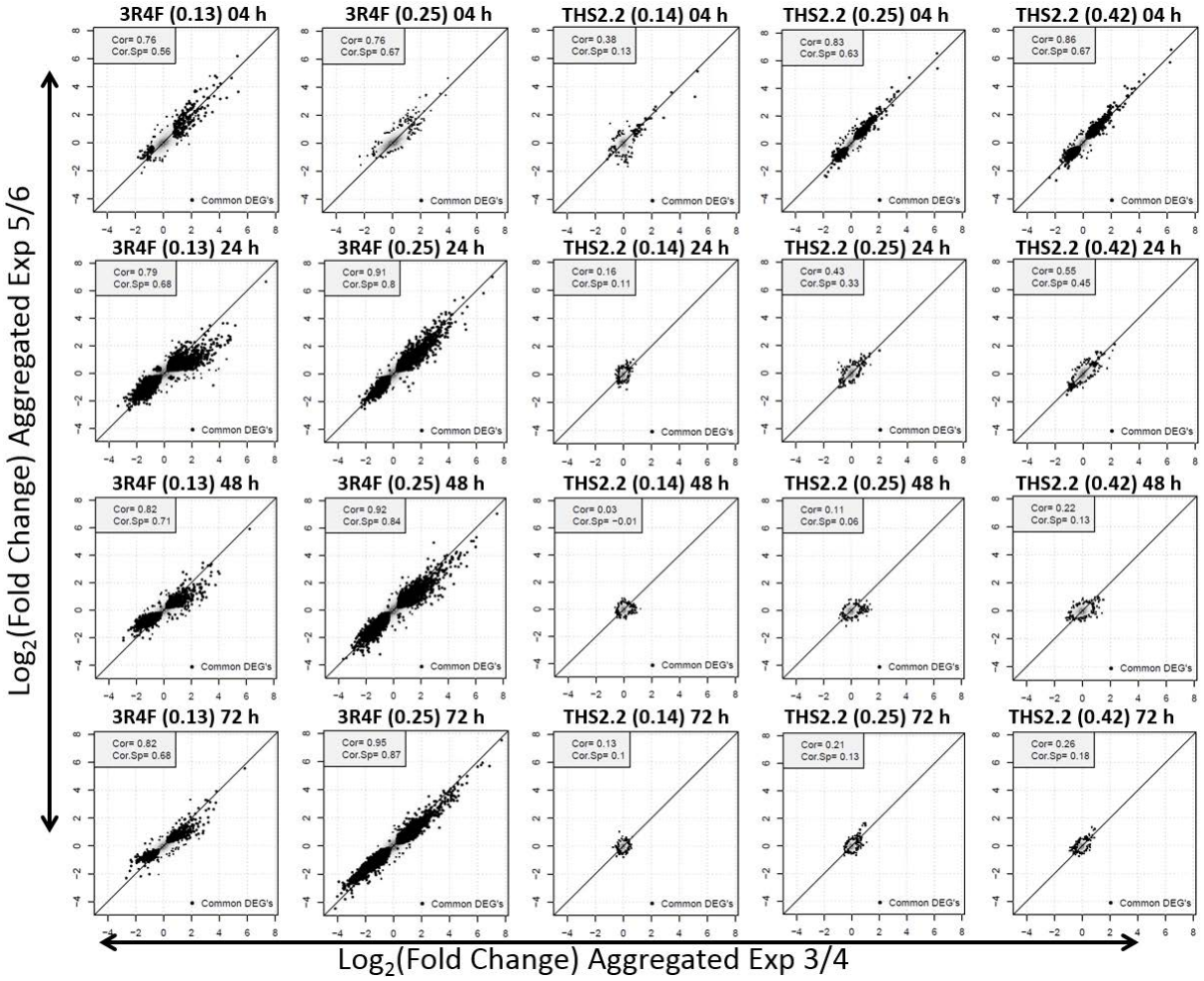


Figure 18. Correlation plots for the gene fold changes found in the experimental repetitions using bronchial MucilAir™ tissue cultures

Each dot represents the results for a gene in four experimental repetitions (N = 2 to 6 per group in experiments 3 and 4 and N = 3 to 6 per group in experiments 5 and 6). The gene fold change results from the aggregated data from experiments 3 and 4 are shown on the *x*-axis, and the gene fold change results from the aggregated data from experiments 5 and 6 are shown on the *y*-axis. The abbreviations used in the plots are: Cor, correlation; Cor. Sp, Spearman correlation; DEG, differentially expressed gene; Exp, experiment; FC, fold change; h, hours.

9.8.3 Reproducibility of the miRNA Expression Fold Change Results

The reproducibility of the miRNA expression change results obtained in experiments 3–6 were assessed using the same approach as was used to assess the reproducibility of the mRNA changes. Only the data produced using Mucilair™ inserts were considered because three smoking runs per experimental repetition were performed. This allowed the miRNA fold change results obtained from a balanced number of replicates to be compared. The correlation plots are shown in Figure 19.

Good correlations were found between the results of the experimental repetitions, and a large number of miRNAs had statistically significant differential expressions after exposure to smoke or aerosol, particularly 24 h, 48 h, and 72 h after exposure to 3R4F smoke at nicotine concentrations of 0.13 mg/L and 0.25 mg/L. The Pearson correlation coefficients for these correlations ranged from 0.6 to 0.89, suggesting that the experimental repetitions gave reproducible results when the exposure conditions clearly affected the level of miRNA expression relative to the air controls. When the number of differentially expressed miRNAs was lower or negligible (see Supplementary Figure 4), the correlation coefficients for the relationships between the results of the experimental repetitions were much lower (< 0.5). This reflected these weak exposure effects mainly being dominated by random noise, which will not correlate in repeat measurements.

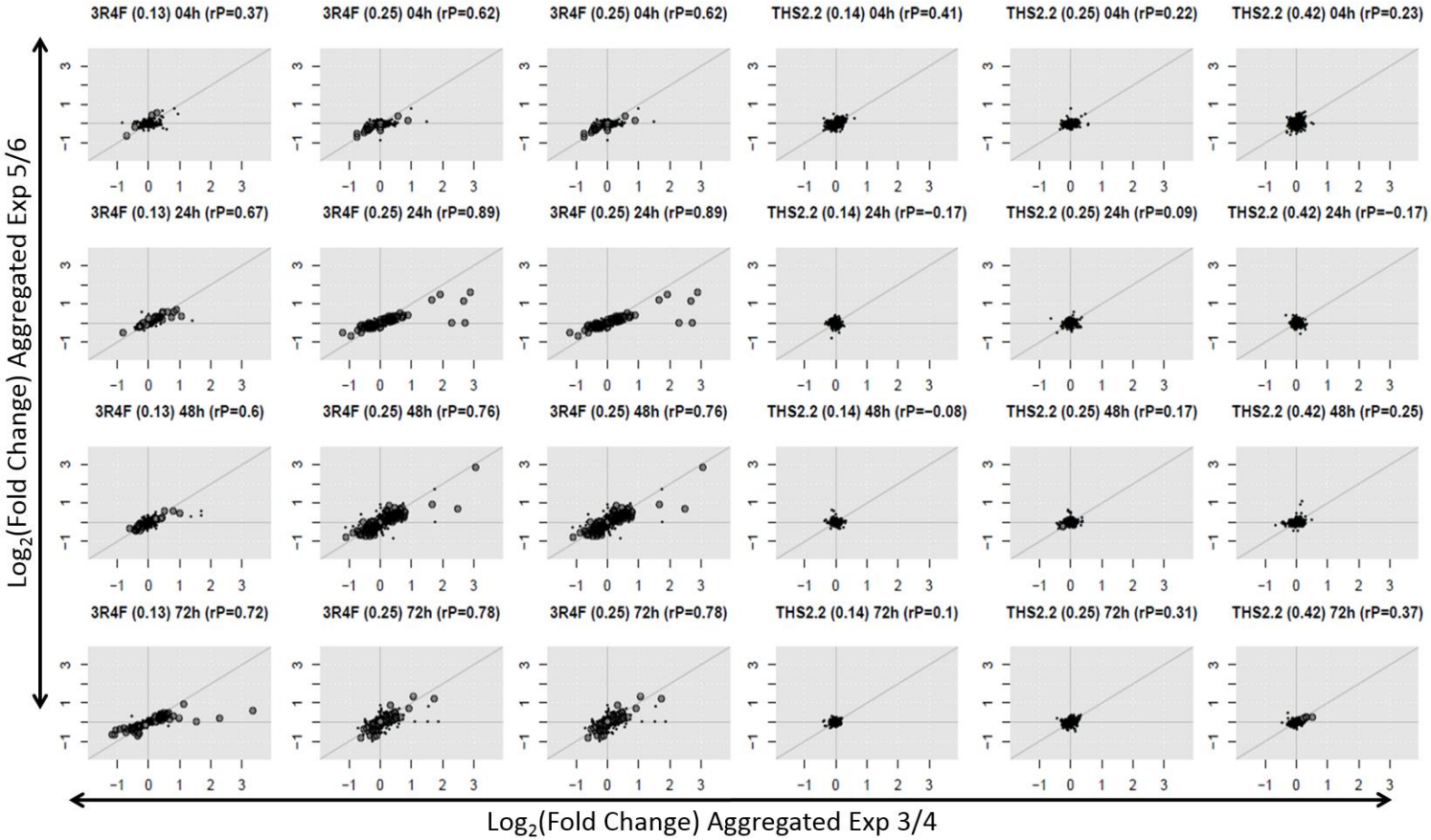


Figure 19. Correlation plots for miRNA expression fold change results in two groups of smoking runs in which only Mucilair™ inserts were used (experiments 3–6) for each post-exposure time point and each exposure condition

The grey dots represent statistically significant differentially expressed miRNA results in the aggregated dataset (experiments 1–6). The abbreviations used in the plots are: Exp, experiment; FC, fold change; h, hours; rP= Pearson correlation coefficient.

9.9 Dose Range Assessment for the Effects of THS2.2 Aerosol on Cytotoxicity and Tissue Histology

In addition to the THS2.2 aerosol concentrations used in the tests for which the results are described above, the effects of a wider range of THS2.2 aerosol concentrations was examined at the histological level and using the AK assay for assessing cytotoxicity. The results are shown in Figure 20. We selected the 72 h post-exposure time point for the analysis, because we hypothesized that the greatest impact on cytotoxicity and overt alterations of the tissue morphology to occur at the later time point.

Various concentrations, ranging from 13-100% of the THS2.2 aerosol were generated within the Vitrocell® 24/48 exposure system to assess the impact of escalating concentrations on the organotypic cultures. Table 12 shows the actual nicotine concentrations measured in the aerosol at the various concentrations that were tested in the dose range assessment. Three independent exposure runs were conducted for this dose range assessment.

Table 12. Actual Nicotine Concentrations in the THS2.2 Aerosol at the Dilutions Used in the Dose Range Assessment

	Dose Range Assessment Performed in the Week Commencing 27 Apr 2015	
Group	**Aerosol Concentrations in the Dilution/Distribution Module of the Vitrocell® 24/48 Exposure System**	**Actual Nicotine Concentration in the Aerosol**
		Mean (mg/L) ± SEM
Air Control (THS2.2)	-	NA
THS2.2 Dilution 1	13%	0.137 ± 0.009 (N = 3)
THS2.2 Dilution 2	23%	0.223 ± 0.007 (N = 3)
THS2.2 Dilution 3	31%	0.369 ± 0.035 (N = 3)
THS2.2 Dilution 4	48%	0.547 ± 0.066 (N = 3)
THS2.2 Dilution 5	60%	0.766 ± 0.024 (N = 3)
THS2.2 Dilution 6	80%	1.024 ± 0.148 (N = 3)
THS2.2 Dilution 7	100%	1.786 ± 0.055 (N = 3)

The actual nicotine concentration in each aerosol dilution was determined from three independent samples, and is shown as the mean ± the standard error of the mean (SEM). THS means the tobacco heating system.

Clear tissue damage (e.g., epithelial degeneration or loss of ciliated cells) was observed 72 h after cultures were exposed to THS2.2 aerosols with nicotine concentrations > 1 mg/L, as is shown in Figure 20A. These morphological observations were also supported by the AK release measurements (shown in Figure 20B) for cultures exposed to THS2.2 aerosol at nicotine concentrations of 1.02 mg/L and 1.79 mg/L. The descriptive statistics for the cytotoxicity results are shown in Supplementary Table 36.

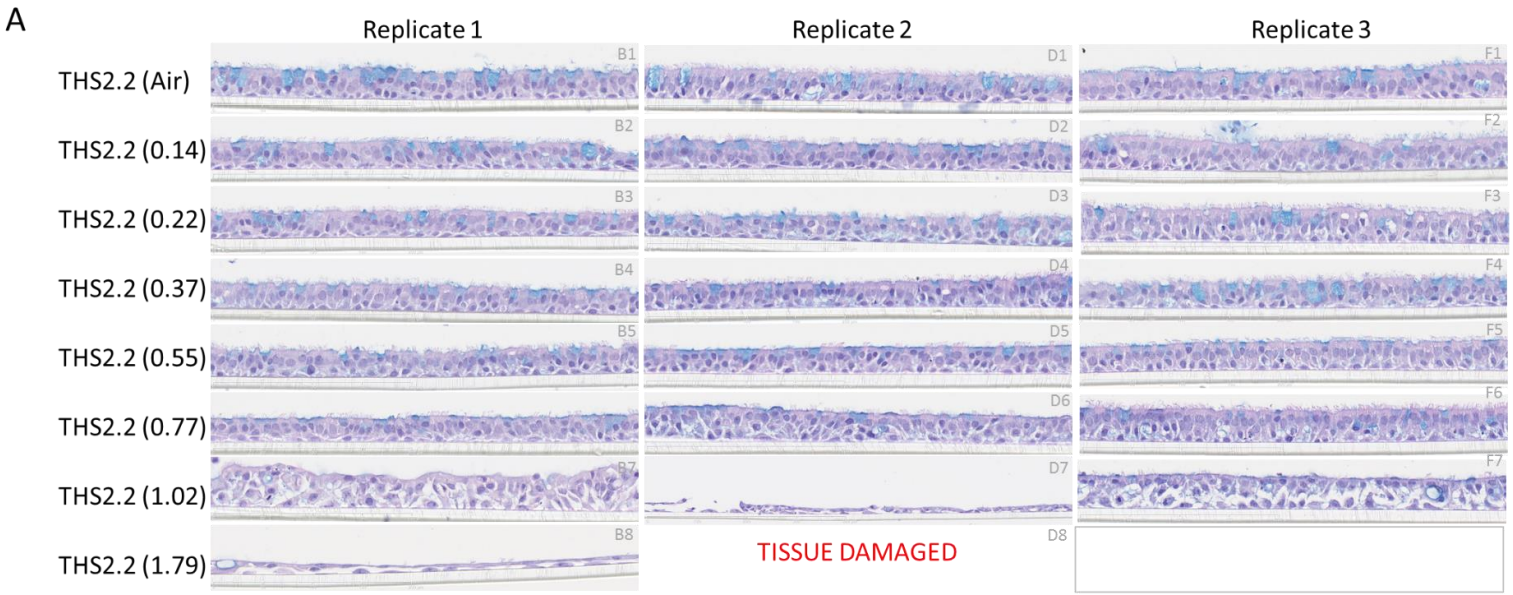


Figure 20. Cytotoxicity and tissue histology results for cultures exposed to THS2.2 aerosol in the dose range assessment (i.e., over an extended dose range)

(A) Tissue sections were stained with haematoxylin and eosin and Alcian Blue. (B) The relative amount of adenylate kinase (AK) released into the basolateral medium. Measurements were made 72 h after the tissue had been exposed to THS2.2 aerosol at the nicotine concentrations (mg/L) indicated in parentheses. The mean cytotoxicity ± the standard error of the mean (N = 3 independent exposure runs, one replicate per exposure run) are shown, with the cytotoxicity normalized to the positive control (a culture treated with Triton X-100 was used to indicate 100% cytotoxicity). A ⋆ symbol indicates the value was significantly different from the corresponding air controls ($p < 0.05$).

10 DISCUSSION AND CONCLUSIONS

The use of *in vitro* models that are more closely resembling human airway tissues as well as systems biology-based approaches to investigate the impact of inhaled environmental pollutants and cigarette smoke is in line with the recent shift of toxicological assessment strategy to find alternatives for animal testing and to tackle systems level questions in an integrative way. In this study, we investigated the biological impact of an acute exposure at the air-liquid interface of human organotypic bronchial tissue cultures MucilAir™ from Epithelix and EpiAirway™ from MatTek to various doses of the aerosol generated by the THS2.2 product. In parallel, exposure with air (control) or with mainstream smoke from 3R4F cigarette (at a concentration where the nicotine level within the smoke are matching those of THS2.2 aerosol) was performed. Various endpoints (cytotoxicity, CYP1A1/1B1 activity, inflammatory markers release, morphological and gene expression changes) were collected at different time-points after exposure (4 h, 24 h, 48 h, 72 h) to identify and compare the concentration- and the time-dependent effect of each exposure condition.

The first two experimental repetitions were performed as a pilot study using organotypic bronchial cultures from MatTek and using an initial experimental design (three inserts per smoking run and one smoking run per experimental repetition). After evaluation of these first two experiments and to further improve the experimental design, four additional experimental repetitions were performed with one insert per smoking run and three smoking runs per experimental repetition. For the experimental repetitions with the improved design, the supplier of the organotypic bronchial cultures, was also changed (from MatTek to Epithelix). The results reported here are the aggregated data obtained from the six experimental repetitions with the exception of the morphology evaluation which was only performed with MucilAir™ cultures. The effect on cytotoxicity and on the epithelial morphology after exposure to a wider range of THS2.2 aerosol concentrations (dose range assessment) was also investigated using MucilAir™ cultures.

An independent smoking machine connected to a Vitrocell® 24/48 exposure system was used to produce 3R4F smoke, and another independent smoking machine connected to a Vitrocell® 24/48 exposure system was used to produce THS2.2 aerosol. The Vitrocell® 24/48 exposure system had a Base Module in which the bronchial organotypic cultures were exposed to smoke or aerosol at the air–liquid interface. The Vitrocell® 24/48 exposure system has been reported before to be suitable for assessing the effects of exposure to smoke (Adamson 2013, Majeed 2014). The experimental system allowed exposures physiologically relevant to actual smoking situations to be used because exposure occurred at the air–liquid interface. The Vitrocell® 24/48 exposure system also had a Dilution/Distribution Module in which different dilutions of 3R4F smoke or THS2.2 aerosol could be administered simultaneously. The biological effects of the test aerosol relative to the effects of the 3R4F reference cigarette smoke were compared by using two THS2.2 aerosol test doses with nicotine concentrations that were comparable to the nicotine concentrations in 3R4F smoke. The reproducibility of the delivery of the target nicotine concentrations after the smoke or aerosol had been diluted in the Vitrocell® 24/48 exposure system was tested by collecting samples of the smoke or aerosol (trapping the smoke/aerosol samples in EXtrelut® 3NT columns) within the same week that each experimental repetition was performed. The data obtained from these tests indicated that diluting the smoke or aerosol effectively gave the target nicotine concentrations.

Cytotoxicity (AK release) was exerted by the high concentration of 3R4F smoke from 24 h post-exposure time point onwards, reaching 40% at 48 h and 72 h; whereas, the low 3R4F concentration elicited a transient marginal response at 24 h only. Exposure to THS2.2 aerosol at comparable nicotine concentrations did not cause cytotoxicity, only concentrations above 1 mg nicotine/L caused substantial AK release in a dose-dependent manner, reaching 80% cytotoxicity for 1.79 mg/L (undiluted aerosol). The histological evaluation of the same tissue cultures confirmed these thresholds for the toxicity of 3R4F smoke and THS2.2 aerosol, respectively. Additional immunohistochemical investigations revealed a small reduction in Fox-J1-positive ciliated cell numbers already for the low 3R4F smoke exposure concentration at 48 h and 72 h post-exposure and a slight increase in proliferating Ki-67-positive cells and in p63-positive basal cells, indicative of a weak basal cell hyperplasia, which is also a typical adaptive response to cigarette smoke exposure in respiratory epithelia *in vivo*. THS2.2 aerosol exposure at the comparable concentrations did not exert these effects, with an apparently opposite tendency to a higher proportion of ciliated cells and lower proportion of proliferating cells.

Xenobiotic metabolizing enzymes such as CYP1A1 and CYP1B1 are known to be involved in the metabolism of tobacco smoke constituents (Anttila 2011, Port 2004), and their inducibility in respiratory tract cells is an indicator of cigarette smoke exposure (Ding 2003, Guengerich 2007, McLemore 1990). CYP-mediated reactions contribute to the generation of toxic metabolites that can bind to DNA and form DNA adducts, which can then interfere with normal DNA replication resulting in gene mutations that can lead to malignant transformation (Anttila 2011, Port 2004). These carcinogen metabolite-to-DNA adducts can be considered useful biomarkers of the biologically effective dose (US Department of Health and Human Services 2010). Although the transcriptomic results indicated a robust and sustained induction of CYP1A1 and CYP1B1 mRNA expression in bronchial epithelial tissue cultures exposed to 3R4F smoke under all conditions tested, the measurable enzyme activity did not significantly increase following cigarette smoke exposure. THS2.2 aerosol exposure at the comparable concentrations was only linked to transient RNA increases at 4 h post-exposure with no increase in enzyme activity. These observations, which resemble a similar effect seen in organotypic oral epithelial cultures (1743_Organotypic_Buccal_SP), may indicate a possible translational inhibitory mechanism of the CYP1A1/1B1 enzyme activity that warrants further investigations.

In previous published studies (Iskandar 2013, Mathis 2013) the impact of 3R4F smoke exposure (28 min and 15% smoke) has been also investigated using both types of bronchial organotypic cultures (EpiAirway™ and MucilAir™), and following 3R4F smoke (0.13 mg nicotine/L) exposure, in which increased release of various pro-inflammatory mediators including MMP-1, VEGFA, TIMP-1, CXCL8 was reported. In the present study, these analytes were also significantly higher in 3R4F smoke-exposed cultures than in the air-exposed controls, at all post-exposure time-points. The effect of THS2.2 aerosol exposure on the release of inflammatory mediators was significantly less pronounced than after 3R4F exposure at comparable nicotine concentration or even when comparing the higher concentration of THS2.2 with the high concentration of 3R4F at all post-exposure time points.

Systems toxicology-based risk assessment approaches, combining computable biological network models and gene expression changes, were applied to investigate the molecular perturbations triggered by different exposures, using only tissues with minimal damage: 3R4F (0.13), and THS2.2 (0.14), (0.25), and (0.42). This approach has been previously used to deduce successfully the impact

of CS exposure on various organotypic epithelial culture models (Hoeng 2014, Iskandar 2015, Talikka 2014). In the present study, the molecular perturbation linked to 3R4F (0.13) smoke exposure was in concordance with the overall gene expression response profiles—a stronger biological impact (BIF) than exposure to THS2.2 aerosol at the comparable and higher concentrations. The analysis of the perturbations of relevant biological networks (i.e. Cellular Stress, Cellular Proliferation, Cellular Fate, and Inflammatory Response) indicated that 3R4F (0.13) smoke exposure induced significant effect on Senescence, Necroptosis, Apoptosis Xenobiotic Metabolism Response, Oxidative stress, Osmotic stress, NFE2L2 signaling as well as Wnt signaling, nuclear receptors, Mapk and Jak Stat signaling, growth factors modeled in the context of cell proliferation at all post-exposure time points. The inflammatory processes that were impacted included Tissue damage, Epithelial Mucus Hypersecretion, and Epithelial Innate Immune Activation. Similar network perturbations were observed following THS2.2 exposure however at a much lower level than after 3R4F exposure and only at the early post-exposure time point. Gene expression analysis with an independent method, GSA, indicated a number of differentially regulated gene-sets that may point to the same or related mechanisms of toxicity as predicted by the network-based modeling, and also the expression of single differentially expressed genes was in support of these interpretations. Unlike the RNA response profiles, the miRNA response profiles for 3R4F (0.13) smoke exposure showed an increasing number of differentially expressed miRNA species with increasing post-exposure duration. For THS2.2 aerosol exposure at the comparable concentration, no differential regulation was observed, and for higher exposure concentrations, a few significantly down- or upregulated miRNAs were seen at 48 h and 72 h post-exposure, respectively.

Correlations in the results obtained across the six different experimental repetitions were estimated to explore the consistency of the cytotoxicity, CYP1A1/CYP1B1 activity, secreted inflammatory mediators, and mRNA/miRNA expression fold changes observations. The correlation analysis indicated that most of the observations were consistent across the different experimental repetitions, suggesting that the study design was robust. Donor-specific factors that could have been associated with the response of the cultures to exposure to smoke or aerosol could not be excluded. For example, undetected genetic variations may have influenced the outcome of the experiment. However, the overall findings clearly demonstrate that exposure to THS2.2 aerosol had less effect on the bronchial tissue cultures than did exposure to 3R4F smoke at a comparable nicotine concentration. A similar evaluation of exposure to THS2.2 aerosol using tissue cultures from a larger number of donors may be necessary in the future to confirm the observations described here.

11 ARCHIVING

After the study is completed, the study plan (with any amendments), the retained test and reference item samples, all raw data, the report (with any amendments), and all further study-related records needed to reconstruct the study will be archived. The archived material will be retained for at least 10 years, in accordance with applicable archiving procedures. Paper records will be kept in the archive at Philip Morris Products S.A, Research & Development, PMI Product Testing, Neuchâtel, Switzerland. Electronic records will be managed by the PMI Product Testing e-archivist on the central archiving server at Philip Morris S.A, Lausanne, Switzerland.

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Wolz L, Krause G, Scherer G, Aufderheide M, Mohr U. In vitro genotoxicity assay of sidestream smoke using a human bronchial epithelial cell line. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2002;40(6):845-50. PubMed PMID: 11983279.

12.2 List of Standard Operating Procedures (SOP) and Work Instruction (WKI)

The standard operating procedures and WKIs that were followed when performing the study are listed below.

PMI_RD_WKI_001066. Title: EXtrelut® trapping for measuring nicotine
PMI_RD_WKI_000409. Title: Performing analysis: determination of nicotine in diluted aerosol
PMI_RD_WKI_001094. Title: Sample preparation for carbonyl measurements
PMI_RD_WKI_001065. Title: Placing Epithelix tissues in culture and maintenance
PMI_RD_WKI_001064. Title: Placing MatTek tissues in culture and maintenance
PMI_RD_WKI_001087. Title: Apical wash of 3D cultures
PMI_RD_WKI_001048.Title: Adenylate kinase assay for 3D cultures
PMI_RD_SOP_000361. Title: Luminex
PMI_RD_WKI_001032. Title: Calibration and maintenance of LMX200
PMI_RD_WKI_001274. Title: Procedure for Luminex assay (FM3D)
PMI_RD_FOR_000803. Title: Luminex assay working sheet
PMI_RD_FOR_000849. Title: Treatment with controls for MAP analysis
PMI_RD_WKI_001242. Title: Treatment with controls for MAP analysis
PMI_RD_WKI_001243. Title: Tissue processing using Leica ASP300S
PMI_RD_WKI_001260. Title: Embedding tissue in paraffin
PMI_RD_WKI_001262. Title: Sectioning paraffin blocks using a microtome
PMI_RD_WKI_001266. Title: Haematoxylin and eosin, with or without Alcian blue, staining procedure for formalin-fixed paraffin-embedded and frozen tissue sections
PMI_RD_WKI_001314. Title: Scanning slides with a nanozoomer
PMI_RD_WKI_001309. Title: Immunohistochemistry procedure using a Leica Bond-Max autostainer
PMI_RD_WKI_001049. Title: CYP assay of 3D cultures
PMI_RD_FOR_000846. Title: Treatment and substrate preparation for CYP1A1/CYP1B1 activity assay
PMI_RD_WKI_001117. Title: Isolation of total RNA, including microRNAs, from tissues and organotypic tissue inserts using the miRNeasy minikit protocol
PMI_RD_WKI_000978. Title: Quality controls after RNA extractions
PMI_RD_WKI_001109. Title: Affymetrix IVT PLUS protocol
PMI_RD_WKI_001125. Title: Quality control assessment of the Affymetrix IVT and Nugen amplification products using a fragment analyser
PMI_RD_WKI_001126. Title: Affymetrix-3' array cartridge hybridization (IVT_PLUS, NugenWB, and PicoV2)
PMI_RD_WKI_001123. Title: miRNA labelling using FlashTag biotin HSR kits
PMI_RD_WKI_001228. Title: Computational practices for omics (mRNA and miRNA) analyses
PMI_RD_SOP_000346. Title: Computational processing of mRNA and miRNA Affymetrix data

12.3 Philip Morris International (PMI) International Documents

1709_Organotypic_Bronch_SP. PMI Internal Document. Unpublished data. Study Plan Translational Organotypic Acute Human Bronchial Tissues Study (THS2.2). Study Plan Number S170900.

1743_Organotypic_Buccal_SP. PMI Internal Document. TRANSLATIONAL ORGANOTYPIC ACUTE HUMAN BUCCAL TISSUES STUDY (THS2.2). Study Plan Number S174300.

Certificate of THS2.2 Stick Batch B-05771. PMI Internal Document. Unpublished data. Certificate of THS2.2 Batch B-05771. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Certificate_No_030_StickBatchNo_B-05771_Manufactured_Jun_2013.pdf

Certificate of THS2.2 Stick Batch B-08163. PMI Internal Document. Unpublished data. Certificate of THS2.2 Batch B-08163. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Certificate_No_045_StickBatchNo_B-08163_Manufactured_Oct_2013.pdf

Certificate of THS2.2 Stick Batch B-13055. PMI Internal Document. Unpublished data. Certificate of THS2.2 Batch B-13055. Stored at: \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Certificate_No_072_StickBatchNo_B-13055_Manufactured_Jun_2014.pdf

Certificate of THS2.2 Device Batch B-06050. PMI Internal Document. Unpublished data. Certificate of Device THS2.2 B-06050. Stored at: \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Device_Information_B-06050_Release_Date_Sep_2013.pdf

Certificate of THS2.2 Device Batch B-18731. PMI Internal Document. Unpublished data. Certificate of Device THS2.2 B-18731. Stored at: \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Device_Information_B-18731_Release_Date_Feb_2015.pdf

Certificate of Analysis of the Test System Seeded on 05-Feb-2015. PMI Internal Document. Unpublished data. Certificate of Bronchial MucilAir™ CoA_seeded_05-Feb-2015. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Acute human bronchial - ZRH - part2-S179000\MP4_CW12_2015\Epithelix_TissueQC\S170900_MP4_CW12_2015_CoA.pdf

Certificate of Analysis of the Test System Seeded on 21-May-2015. PMI Internal Document. Unpublished data. Certificate of Bronchial MucilAir™ CoA_seeded_21-May-2015. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Acute human bronchial - ZRH - part2-S179000\MP6_CW27_2015\Epithelix_Tissue_QC\S170900_MP6_CW27_2015_CoA.pdf

Certificate of Analysis of the Test System Seeded on 26-May-2015. PMI Internal Document. Unpublished data. Certificate of Bronchial MucilAir™ CoA_seeded_26-May-2015. Stored at: \\rd-

bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Acute human bronchial - ZRH - part2-S179000\MP7_CW28_2015\Epithelix_Tissue_QC\S170900_MP7_CW28_2015_CoA.pdf

Certificate of Analysis of the Test System Seeded on 24-Jul-2015. PMI Internal Document. Unpublished data. Certificate of Bronchial MucilAir™ CoA_seeded_24-Jul-2015. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Acute human bronchial - ZRH - part2-S179000\MP8_CW36_2015\Epithelix_tissue_QC\S170900_MP8_CW36_2015_CoA.pdf

Certificate of Analysis of the Test System Seeded on 16-Mar-2016. PMI Internal Document. Unpublished data. Certificate of Bronchial MucilAir™ CoA_seeded_16-Mar-2016. Stored at: \\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology\Acute human bronchial - ZRH - part2-S179000\DRF_CW18_2015\Epithelix_Tissue QC\S170900_DRA_CW18_2015_CoA.pdf

13 ABBREVIATIONS

Abbreviation/Term	
AK	Adenylate kinase
ALI	Air–liquid interface
BIF	Biological impact factor
CCL	Chemokine (C–C motif) ligand
cRNA	Complementary RNA
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
FDR	False discovery rate
LCLM	Lower confidence limit of the mean
MAP	Multi-analyte profiling
miRNA	MicroRNA
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NPA	Network perturbation amplitude
PBS	Phosphate buffered saline
QQ	Quantile–quantile
REF	Reference
RNA	Ribonucleic acid
sICAM-1	Soluble inter-cellular adhesion molecule-1
THS	Tobacco heating system (also referred to as ZRH)
TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumour necrosis factor
UCLM	Upper confidence limit of the mean
VEGFA	Vascular endothelial growth factor A
WKI	Work instruction

ZRH	See THS

14 TABLES AND SUPPLEMENTARY FIGURES

14.1 Supplementary Tables

14.1.1 **Supplementary Table 1**. Descriptive Statistics of Acetaldehyde Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	1.06	0.09	1.93	0.17	0.00	0.00	0.38	0.02	0.75	0.13	NA.	NA.	1.13	0.05
2014.19	0.00	0.00	1.08	0.03	2.04	0.04	0.00	0.00	0.00	0.00	0.27	0.01	NA.	NA.	0.95	0.05
2014.25	0.00	0.00	0.86	0.04	1.95	0.13	0.00	0.00	0.00	0.00	0.18	0.02	0.45	0.01	NA.	NA.
2015.11	0.00	0.00	1.34	0.03	2.32	0.03	0.00	0.00	0.22	0.01	0.53	0.01	0.61	0.02	NA.	NA.
2015.12	0.00	0.00	1.86	0.14	3.47	0.47	0.00	0.00	0.48	0.11	1.03	0.09	1.17	0.23	NA.	NA.
2015.15	0.00	0.00	NA.	NA.	2.95	0.12	0.00	0.00	NA.	NA.	NA.	NA.	1.08	0.05	NA.	NA.
2015.16	0.00	0.00	0.99	0.06	1.91	0.03	0.00	0.00	0.10	0.01	0.31	0.04	0.44	0.02	NA.	NA.
2015.23	0.00	0.00	0.69	0.05	1.61	0.17	0.00	0.00	0.00	0.00	0.25	0.04	0.23	0.07	NA.	NA.
2015.25	0.00	0.00	1.88	0.04	3.55	0.14	0.00	0.00	0.35	0.03	0.94	0.05	NA.	NA.	1.87	0.08
2015.27	0.00	0.00	1.39	0.02	2.62	0.10	0.00	0.00	0.24	0.00	0.59	0.03	NA.	NA.	1.52	0.02

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.2 **Supplementary Table 2.** Descriptive Statistics of Acetone Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.03	0.00	0.60	0.07	1.07	0.15	0.05	0.00	0.12	0.01	0.19	0.03	NA.	NA.	0.27	0.00
2014.19	0.04	0.00	0.79	0.02	1.36	0.02	0.05	0.00	0.12	0.00	0.17	0.01	NA.	NA.	0.29	0.01
2014.25	0.03	0.00	0.67	0.02	1.21	0.09	0.02	0.01	0.08	0.00	0.13	0.00	0.17	0.00	NA.	NA.
2015.11	0.09	0.04	0.80	0.03	1.33	0.03	0.01	0.01	0.07	0.00	0.11	0.00	0.13	0.01	NA.	NA.
2015.12	0.00	0.00	0.84	0.07	1.36	0.12	0.00	0.00	0.09	0.01	0.17	0.02	0.19	0.02	NA.	NA.
2015.15	0.03	0.00	NA.	NA.	1.38	0.03	0.02	0.01	NA.	NA.	NA.	NA.	0.20	0.00	NA.	NA.
2015.16	0.00	0.00	0.70	0.03	1.26	0.01	0.01	0.01	0.07	0.00	0.11	0.00	0.13	0.01	NA.	NA.
2015.23	0.00	0.00	0.62	0.03	1.12	0.10	0.00	0.00	0.06	0.00	0.12	0.00	0.12	0.01	NA.	NA.
2015.25	0.19	0.12	1.03	0.07	1.79	0.01	0.08	0.00	0.16	0.01	0.23	0.01	NA.	NA.	0.38	0.02
2015.27	0.03	0.00	0.78	0.01	1.35	0.00	0.02	0.00	0.08	0.00	0.13	0.01	NA.	NA.	0.28	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.3 **Supplementary Table 3.** Descriptive Statistics of Acrolein Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.06	0.00	0.10	0.00	0.00	0.00	0.01	0.00	0.03	0.01	NA.	NA.	0.03	0.00
2014.19	0.00	0.00	0.07	0.00	0.12	0.00	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.04	0.00
2014.25	0.00	0.00	0.08	0.01	0.15	0.01	0.00	0.00	0.01	0.00	0.02	0.00	0.03	0.00	NA.	NA.
2015.11	0.01	0.01	0.10	0.00	0.15	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.12	0.00	0.00	0.11	0.01	0.18	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.02	NA.	NA.
2015.15	0.00	0.00	NA.	NA.	0.17	0.00	0.00	0.00	NA.	NA.	NA.	NA.	0.03	0.00	NA.	NA.
2015.16	0.00	0.00	0.08	0.00	0.14	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.23	0.00	0.00	0.09	0.00	0.16	0.02	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.25	0.00	0.00	0.12	0.00	0.22	0.01	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.05	0.00
2015.27	0.00	0.00	0.07	0.00	0.11	0.00	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.03	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.4 **Supplementary Table 4**. Descriptive Statistics of Propionaldehyde Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.01	0.00	0.02	0.01	NA.	NA.	0.02	0.00
2014.19	0.00	0.00	0.06	0.00	0.11	0.00	0.00	0.00	0.02	0.00	0.03	0.00	NA.	NA.	0.06	0.00
2014.25	0.00	0.00	0.06	0.00	0.10	0.01	0.00	0.00	0.02	0.00	0.03	0.00	0.04	0.00	NA.	NA.
2015.11	0.01	0.01	0.08	0.00	0.13	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.04	0.00	NA.	NA.
2015.12	0.00	0.00	0.10	0.01	0.21	0.01	0.00	0.00	0.03	0.01	0.05	0.00	0.07	0.02	NA.	NA.
2015.15	0.00	0.00	NA.	NA.	0.18	0.01	0.00	0.00	NA.	NA.	NA.	NA.	0.07	0.00	NA.	NA.
2015.16	0.00	0.00	0.06	0.00	0.10	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.03	0.00	NA.	NA.
2015.23	0.00	0.00	0.05	0.00	0.10	0.01	0.00	0.00	0.01	0.00	0.03	0.00	0.03	0.00	NA.	NA.
2015.25	0.00	0.00	0.14	0.00	0.24	0.01	0.00	0.00	0.03	0.00	0.07	0.00	NA.	NA.	0.13	0.01
2015.27	0.00	0.00	0.08	0.00	0.14	0.00	0.00	0.00	0.02	0.00	0.04	0.00	NA.	NA.	0.08	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.5 **Supplementary Table 5.** Descriptive Statistics of Crotonaldehyde Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.08	0.00	0.14	0.00	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.02	0.00
2014.19	0.00	0.00	0.07	0.00	0.12	0.00	0.00	0.00	0.01	0.00	0.01	0.00	NA.	NA.	0.02	0.00
2014.25	0.00	0.00	0.07	0.00	0.13	0.01	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	NA.	NA.
2015.11	0.01	0.00	0.07	0.00	0.12	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	NA.	NA.
2015.12	0.00	0.00	0.07	0.00	0.12	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.02	0.00	NA.	NA.
2015.15	0.00	0.00	NA.	NA.	0.13	0.00	0.00	0.00	NA.	NA.	NA.	NA.	0.02	0.00	NA.	NA.
2015.16	0.00	0.00	0.07	0.01	0.13	0.01	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	NA.	NA.
2015.23	0.00	0.00	0.05	0.00	0.10	0.01	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	NA.	NA.
2015.25	0.00	0.00	0.09	0.01	0.16	0.02	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.03	0.00
2015.27	0.00	0.00	0.07	0.01	0.11	0.01	0.00	0.00	0.01	0.00	0.01	0.00	NA.	NA.	0.02	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.6 **Supplementary Table 6.** Descriptive Statistics of Methyl Ethyl Ketone Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.03	0.00	0.17	0.01	0.30	0.02	0.03	0.00	0.05	0.00	0.06	0.00	NA.	NA.	0.07	0.00
2014.19	0.03	0.00	0.21	0.00	0.35	0.00	0.04	0.00	0.05	0.00	0.06	0.00	NA.	NA.	0.07	0.00
2014.25	0.03	0.00	0.18	0.00	0.31	0.02	0.03	0.00	0.04	0.00	0.05	0.00	0.06	0.00	NA.	NA.
2015.11	0.05	0.02	0.19	0.01	0.32	0.00	0.01	0.01	0.02	0.00	0.03	0.00	0.03	0.00	NA.	NA.
2015.12	0.01	0.00	0.20	0.01	0.32	0.02	0.00	0.00	0.02	0.00	0.04	0.00	0.04	0.00	NA.	NA.
2015.15	0.02	0.00	NA.	NA.	0.35	0.00	0.02	0.00	NA.	NA.	NA.	NA.	0.05	0.00	NA.	NA.
2015.16	0.02	0.01	0.19	0.01	0.33	0.01	0.01	0.00	0.04	0.01	0.03	0.00	0.05	0.01	NA.	NA.
2015.23	0.01	0.00	0.16	0.01	0.29	0.03	0.01	0.00	0.02	0.00	0.03	0.00	0.03	0.00	NA.	NA.
2015.25	0.07	0.00	0.25	0.01	0.42	0.03	0.08	0.00	0.09	0.00	0.11	0.00	NA.	NA.	0.14	0.00
2015.27	0.02	0.00	0.22	0.01	0.35	0.00	0.02	0.00	0.03	0.00	0.04	0.00	NA.	NA.	0.07	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.7 **Supplementary Table 7.** Descriptive Statistics of Butyraldehyde Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.03	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.03	0.01	NA.	NA.	0.05	0.00
2014.19	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA.	NA.	0.02	0.00
2014.25	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	NA.	NA.
2015.11	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.12	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.01	0.00	0.03	0.00	0.03	0.00	NA.	NA.
2015.15	0.00	0.00	NA.	NA.	0.03	0.00	0.00	0.00	NA.	NA.	NA.	NA.	0.03	0.00	NA.	NA.
2015.16	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.23	0.00	0.00	0.01	0.00	0.03	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.00	NA.	NA.
2015.25	0.00	0.00	0.03	0.00	0.05	0.00	0.00	0.00	0.02	0.00	0.06	0.03	NA.	NA.	0.06	0.00
2015.27	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.01	0.00	0.02	0.00	NA.	NA.	0.05	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. Due to a lack of dose-dependency observed in the measured values of butyraldehyde at the different smoke/aerosol concentrations, these data were not used for smoke/aerosol characterization. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.8 **Supplementary Table 8.** Descriptive Statistics of Formaldehyde Concentrations (in µg/mL) in Phosphate Buffered Saline Exposed to Air, 3R4F Smoke, or THS2.2 Aerosol

Year. Calendar	3R4F (Air)		3R4F (0.13)		3R4F (0.25)		THS2.2 (Air)		THS2.2 (0.14)		THS2.2 (0.25)		THS2.2 (0.42)		THS2.2 (0.56)	
Week	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.03	0.01	0.07	0.03	0.22	0.09	0.18	0.06	0.16	0.04	0.28	0.07	NA.	NA.	0.13	0.08
2014.19	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA.	NA.	0.00	0.00
2014.25	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA.	NA.
2015.11	0.02	0.00	0.07	0.00	0.10	0.01	0.01	0.00	0.02	0.00	0.03	0.01	0.03	0.01	NA.	NA.
2015.12	0.07	0.02	0.15	0.01	0.24	0.03	0.04	0.01	0.09	0.03	0.11	0.02	0.10	0.04	NA.	NA.
2015.15	0.05	0.01	NA.	NA.	0.19	0.01	0.04	0.02	NA.	NA.	NA.	NA.	0.07	0.00	NA.	NA.
2015.16	0.00	0.00	0.02	0.00	0.05	0.00	0.03	0.03	0.02	0.02	0.02	0.02	0.01	0.01	NA.	NA.
2015.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA.	NA.
2015.25	0.10	0.02	0.16	0.01	0.22	0.00	0.11	0.01	0.10	0.01	0.13	0.01	NA.	NA.	0.12	0.01
2015.27	0.05	0.01	0.08	0.01	0.12	0.01	0.04	0.00	0.03	0.00	0.03	0.00	NA.	NA.	0.05	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. Due to a lack of dose-dependency observed in the measured values of formaldehyde at the different smoke/aerosol concentrations, these data were not used for smoke/aerosol characterization. The concentrations of carbonyl compounds deposited in 18.5 mL of phosphate buffered saline exposed to THS2.2 aerosol, 3R4F smoke, or air for 5 min were measured and are shown in the table above. Abbreviations: M, mean; NA, not applicable; SEM, standard error of the mean; THS, tobacco heating system. The nicotine concentration in each test is given in parentheses.

14.1.9 **Supplementary Table 9.** Descriptive Statistics Immunohistochemistry: P63 Positive Staining (%)

		Descriptive Statistics: p63-positive Cells							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
48	3R4F (Air)	11	20.99	1.52	15.98	20.55	26.39	17.61	24.37
	3R4F (0.13)	9	28.79	2.19	28.26	28.97	33.10	23.73	33.85
	THS2.2 (Air)	6	21.47	2.72	14.16	22.83	23.90	14.48	28.46
	THS2.2 (0.14)	6	19.99	2.13	16.47	17.52	24.03	14.53	25.46
	THS2.2 (0.25)	6	19.91	2.30	15.65	18.08	24.40	14.00	25.82
	THS2.2 (0.42)	6	18.14	1.21	14.78	19.24	20.37	15.04	21.25
72	3R4F (Air)	9	24.11	1.50	20.26	24.78	26.15	20.66	27.57
	3R4F (0.13)	8	29.98	1.99	25.11	28.44	35.64	25.27	34.70
	THS2.2 (Air)	6	19.64	2.95	12.95	19.63	21.24	12.06	27.22
	THS2.2 (0.14)	6	20.94	1.55	19.05	21.89	23.06	16.96	24.93
	THS2.2 (0.25)	6	18.68	0.53	18.17	18.53	18.95	17.32	20.03
	THS2.2 (0.42)	6	22.86	4.17	17.05	19.42	21.01	12.14	33.58

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.10 **Supplementary Table 10.** Descriptive Statistics Immunohistochemistry: Ki67 Positive Staining (%)

		Descriptive Statistics: Ki67-positive Cells							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
48	3R4F (Air)	11	4.58	0.59	2.51	4.70	6.43	3.27	5.90
	3R4F (0.13)	9	6.73	1.24	3.76	5.01	10.17	3.87	9.59
	THS2.2 (Air)	6	3.96	0.94	2.84	3.18	3.55	1.53	6.39
	THS2.2 (0.14)	6	5.20	0.91	4.03	4.68	5.34	2.87	7.52
	THS2.2 (0.25)	6	2.14	0.37	1.58	1.93	2.85	1.20	3.08
	THS2.2 (0.42)	6	2.90	0.44	1.97	2.70	3.77	1.77	4.03
72	3R4F (Air)	9	5.81	0.44	5.31	5.81	7.08	4.78	6.83
	3R4F (0.13)	8	11.14	3.63	3.70	4.38	22.61	2.55	19.72
	THS2.2 (Air)	6	4.71	0.72	4.34	5.11	5.85	2.86	6.56
	THS2.2 (0.14)	6	3.24	0.57	2.56	3.23	4.22	1.77	4.70
	THS2.2 (0.25)	6	1.96	0.45	1.04	1.81	2.33	0.80	3.13
	THS2.2 (0.42)	6	2.28	0.93	1.23	1.34	1.63	−0.11	4.68

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.11 **Supplementary Table 11.** Descriptive Statistics Immunohistochemistry: FoxJ1 Positive Staining (%)

		Descriptive Statistics: FoxJ1-positive Cells							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
48	3R4F (Air)	11	19.90	4.17	8.97	18.66	25.99	10.62	29.18
	3R4F (0.13)	9	10.64	4.13	5.10	6.31	8.94	1.12	20.16
	THS2.2 (Air)	6	23.09	2.21	19.68	20.38	29.18	17.40	28.78
	THS2.2 (0.14)	6	31.61	5.08	20.61	29.21	45.20	18.55	44.67
	THS2.2 (0.25)	6	25.72	4.90	18.39	22.04	27.53	13.12	38.33
	THS2.2 (0.42)	6	29.32	3.70	22.90	24.51	37.55	19.81	38.83
72	3R4F (Air)	9	17.34	5.69	5.62	10.26	19.62	4.20	30.47
	3R4F (0.13)	8	8.67	1.82	5.33	7.36	9.68	4.36	12.97
	THS2.2 (Air)	6	25.56	5.64	13.33	25.85	36.05	11.06	40.06
	THS2.2 (0.14)	6	26.63	6.98	9.64	25.19	41.51	8.68	44.58
	THS2.2 (0.25)	6	28.56	7.45	8.81	27.74	47.72	9.42	47.70
	THS2.2 (0.42)	6	28.25	6.14	15.08	26.41	43.91	12.46	44.04

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.12 **Supplementary Table 12.** Descriptive Statistics: Cytotoxicity (RLU)

		Descriptive Statistics								
PE[h]	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**	**Skew**
N/A	Untreated	5	1005	337	898	938	976	69	1942	0.66
	PBS	2	2419	367	2052	2419	2786	-2248	7086	.
	Triton X-100	8	56249	9384	45372	67116	73260	34059	78439	-1.63
4:00	3R4F (Air)	9	1128	263	646	672	1363	522	1735	1.48
	3R4F (0.13)	8	1062	226	669	735	1354	527	1597	1.57
	3R4F (0.25)	9	1080	250	658	824	851	504	1655	2.16
	THS2.2 (Air)	9	1103	256	724	759	801	513	1692	1.82
	THS2.2 (0.14)	9	1041	216	700	706	864	544	1538	1.90
	THS2.2 (0.25)	9	1162	231	687	740	1497	628	1695	1.28
	THS2.2 (0.42)	6	718	28	657	720	738	645	790	0.74
24:00	3R4F (Air)	12	1574	413	791	902	1705	666	2482	1.92
	3R4F (0.13)	11	2337	466	1219	1313	3461	1300	3375	1.12
	3R4F (0.25)	12	11306	2578	6723	9008	13616	5631	16980	2.26
	THS2.2 (Air)	9	1623	403	900	1012	1645	694	2552	1.56
	THS2.2 (0.14)	9	1759	498	850	895	2032	612	2907	1.45
	THS2.2 (0.25)	9	1717	473	889	1012	1600	626	2808	1.51
	THS2.2 (0.42)	6	831	15	813	832	861	793	868	-0.61
48:00	3R4F (Air)	12	2823	1047	968	1480	2228	519	5128	2.42
	3R4F (0.13)	11	3574	923	1382	1793	6536	1516	5632	1.40
	3R4F (0.25)	12	29581	5933	16640	19582	42806	16523	42640	0.83
	THS2.2 (Air)	9	3083	1180	1320	1443	2048	361	5805	2.15
	THS2.2 (0.14)	9	3067	1290	1186	1476	1693	92	6042	2.38
	THS2.2 (0.25)	9	2899	1224	1185	1302	1850	77	5722	2.45
	THS2.2 (0.42)	6	1269	78	1119	1367	1398	1068	1469	-1.25
72:00	3R4F (Air)	14	3202	1119	876	1679	2371	785	5618	2.59
	3R4F (0.13)	13	5090	1292	1860	2515	5765	2277	7904	1.30
	3R4F (0.25)	14	30248	4572	21238	27829	32566	20370	40126	0.64
	THS2.2 (Air)	9	4412	1494	1927	2015	5805	968	7856	2.13
	THS2.2 (0.14)	9	4526	1576	1985	2076	4670	892	8159	2.04
	THS2.2 (0.25)	9	3852	1382	1476	1855	4224	666	7039	1.98
	THS2.2 (0.42)	6	1695	157	1366	1731	1986	1292	2097	-0.18

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.13 **Supplementary Table 13.** Descriptive Statistics: CYP1A1/CYP1B1 Activity (RLU)

		Descriptive Statistics (RLU): Experimental 1/2/3/4/5 (All Post-Exposure Time Points)							
PE[h]	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
NA	Untreated	2	1676	663	1013	1676	2339	-6749	10101
	PBS	1	3132	.	3132	3132	3132	.	.
	Luciferin	3	1134	158	909	1054	1440	453	1816
	TCDD	6	20905	10116	6326	12798	20194	-5099	46909
4:00	3R4F (Air)	1	3244	.	3244	3244	3244	.	.
	3R4F (0.13)	1	2979	.	2979	2979	2979	.	.
	3R4F (0.25)	1	2779	.	2779	2779	2779	.	.
	THS2.2 (Air)	1	3332	.	3332	3332	3332	.	.
	THS2.2 (0.14)	1	3486	.	3486	3486	3486	.	.
	THS2.2 (0.25)	1	3328	.	3328	3328	3328	.	.
24:00	3R4F (Air)	2	2890	300	2590	2890	3190	-920	6700
	3R4F (0.13)	2	7514	993	6521	7514	8508	-5107	20136
	3R4F (0.25)	2	1945	637	1308	1945	2582	-6153	10043
	THS2.2 (Air)	2	3956	1144	2812	3956	5100	-10578	18490
	THS2.2 (0.14)	2	3142	254	2889	3142	3396	-81	6365
	THS2.2 (0.25)	2	3581	200	3381	3581	3782	1036	6127
48:00	3R4F (Air)	11	1707	235	1208	1558	1781	1182	2231
	3R4F (0.13)	10	3163	1236	1113	1643	2617	367	5958
	3R4F (0.25)	11	2820	1720	182	231	521	-1013	6654
	THS2.2 (Air)	11	1542	318	946	1149	1354	833	2252
	THS2.2 (0.14)	11	1474	254	1049	1161	1342	907	2041
	THS2.2 (0.25)	11	1393	273	807	1080	1443	783	2002
	THS2.2 (0.42)	9	852	32	810	865	899	778	926
72:00	3R4F (Air)	2	2655	12	2643	2655	2667	2507	2803
	3R4F (0.13)	2	6626	1376	5250	6626	8002	-10860	24112
	3R4F (0.25)	2	13712	2774	10938	13712	16485	-21531	48954
	THS2.2 (Air)	2	2429	62	2367	2429	2491	1637	3221
	THS2.2 (0.14)	2	2786	29	2757	2786	2815	2422	3150
	THS2.2 (0.25)	2	2678	23	2655	2678	2702	2382	2975

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

		Descriptive Statistics (RLU): Experimental 1/2 (Measured at 48 h Post-Exposure)							
PE[h]	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
48:00	3R4F (Air)	2	3182	69	3113	3182	3251	2306	4059
	3R4F (0.13)	2	10319	1615	8704	10319	11935	-10205	30844
	3R4F (0.25)	2	14309	1184	13125	14309	15494	-739	29358
	THS2.2 (Air)	2	3592	509	3083	3592	4101	-2880	10064
	THS2.2 (0.14)	2	3143	110	3032	3143	3253	1743	4542
	THS2.2 (0.25)	2	3173	11	3163	3173	3184	3038	3309

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

		Descriptive Statistics (RLU): Experimental 3/4/5 (Measured at 48 h Post-Exposure)							
PE[h]	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
48:00	3R4F (Air)	9	1379	103	1208	1382	1566	1142	1615
	3R4F (0.13)	8	1373	272	733	1552	1742	731	2016
	3R4F (0.25)	9	267	45	182	225	351	164	371
	THS2.2 (Air)	9	1087	71	946	1142	1257	923	1250
	THS2.2 (0.14)	9	1103	64	1049	1132	1260	956	1250
	THS2.2 (0.25)	9	997	81	807	1021	1107	810	1184
	THS2.2 (0.42)	9	852	32	810	865	899	778	926

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.14 **Supplementary Table 14.** Descriptive Statistics for EGF Protein in the Basolateral Media

		Descriptive Statistics: EGF (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	2	5.58	4.18	1.40	5.58	9.77	−47.56	58.73
	TNFα+IL1β	2	5.58	4.18	1.40	5.58	9.77	−47.56	58.73
4	3R4F (Air)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
	3R4F (0.13)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
	3R4F (0.25)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
	THS2.2 (Air)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
	THS2.2 (0.14)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
	THS2.2 (0.25)	2	1.40	0.00	1.40	1.40	1.40	NA.	NA.
24	3R4F (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.13)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.14)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
48	3R4F (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.13)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.14)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
72	3R4F (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.13)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	3R4F (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (Air)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.14)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40
	THS2.2 (0.25)	4	1.40	0.00	1.40	1.40	1.40	1.40	1.40

Note: EGF was only measured in experiments 1 and 2 (see Table 9). Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.15 **Supplementary Table 15.** Descriptive Statistics for CCL11/Eotaxin Protein in the Basolateral Media

		Descriptive Statistics: CCL11/Eotaxin (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	2	3.15	1.15	2.00	3.15	4.30	−11.48	17.78
	TNFα+IL1β	2	3.94	0.26	3.68	3.94	4.20	0.66	7.22
4	3R4F (Air)	2	5.79	2.14	3.65	5.79	7.93	−21.44	33.02
	3R4F (0.13)	2	18.65	11.77	6.88	18.65	30.42	−130.94	168.24
	3R4F (0.25)	2	13.89	1.94	11.95	13.89	15.83	−10.78	38.56
	THS2.2 (Air)	2	5.56	0.90	4.66	5.56	6.47	−5.91	17.04
	THS2.2 (0.14)	2	8.14	0.39	7.75	8.14	8.53	3.21	13.07
	THS2.2 (0.25)	2	9.24	1.54	7.70	9.24	10.78	−10.34	28.82
24	3R4F (Air)	4	2.43	0.26	2.00	2.35	2.86	1.62	3.24
	3R4F (0.13)	4	3.96	1.21	2.35	3.19	5.56	0.11	7.80
	3R4F (0.25)	4	2.42	0.42	2.00	2.00	2.84	1.08	3.76
	THS2.2 (Air)	4	3.13	0.44	2.39	3.06	3.87	1.75	4.52
	THS2.2 (0.14)	4	3.49	0.86	2.00	3.43	4.99	0.74	6.25
	THS2.2 (0.25)	4	3.43	0.82	2.00	3.43	4.86	0.80	6.05
48	3R4F (Air)	4	2.00	0.00	2.00	2.00	2.00	NA	NA
	3R4F (0.13)	4	2.91	0.91	2.00	2.00	3.81	0.02	5.79
	3R4F (0.25)	4	2.00	0.00	2.00	2.00	2.00	NA	NA
	THS2.2 (Air)	4	2.74	0.43	2.00	2.65	3.48	1.36	4.12
	THS2.2 (0.14)	4	2.25	0.25	2.00	2.00	2.50	1.46	3.04
	THS2.2 (0.25)	4	3.06	0.32	2.56	3.02	3.57	2.04	4.08
72	3R4F (Air)	4	6.05	2.22	2.21	6.15	9.90	−1.01	13.12
	3R4F (0.13)	4	4.59	1.53	2.00	4.23	7.19	−0.26	9.45
	3R4F (0.25)	4	5.64	2.00	2.21	5.44	9.07	−0.72	11.99
	THS2.2 (Air)	4	6.66	0.96	5.06	6.44	8.27	3.59	9.73
	THS2.2 (0.14)	4	6.92	3.76	2.75	3.77	11.09	−5.06	18.90
	THS2.2 (0.25)	4	6.10	1.05	4.40	5.76	7.80	2.74	9.45

Note: CCL11/Eotaxin was only measured in experiments 1 and 2 (see Table 9). Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.16 **Supplementary Table 16.** Descriptive Statistics for CSF3 Protein in the Basolateral Media

		Descriptive Statistics: CSF3 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	124.43	54.47	45.25	80.88	140.51	−26.80	275.66
	TNFα+IL1β	5	33039.24	7367.43	20128.78	41358.61	42209.31	12583.98	53494.51
4	3R4F (Air)	2	78.78	13.41	65.37	78.78	92.19	−91.57	249.13
	3R4F (0.13)	2	73.93	19.92	54.02	73.93	93.85	−179.13	326.99
	3R4F (0.25)	2	38.79	0.29	38.50	38.79	39.09	35.06	42.53
	THS2.2 (Air)	2	95.73	31.58	64.15	95.73	127.32	−305.57	497.04
	THS2.2 (0.14)	2	93.53	0.10	93.43	93.53	93.63	92.27	94.79
	THS2.2 (0.25)	2	30.31	8.30	22.02	30.31	38.61	−75.13	135.76
24	3R4F (Air)	13	1320.61	1042.15	90.85	141.24	389.52	−950.04	3591.27
	3R4F (0.13)	12	363.57	112.60	102.04	203.04	563.62	115.73	611.41
	3R4F (0.25)	13	637.22	239.81	225.01	326.52	551.50	114.72	1159.72
	THS2.2 (Air)	13	1250.88	973.52	93.23	141.05	319.16	−870.25	3372.00
	THS2.2 (0.14)	13	372.64	168.04	65.98	106.19	198.57	6.51	738.78
	THS2.2 (0.25)	13	404.43	158.06	89.90	150.84	260.87	60.04	748.83
	THS2.2 (0.42)	9	272.86	152.49	60.49	88.80	231.99	−78.77	624.49
48	3R4F (Air)	13	1137.69	525.78	99.40	387.21	1295.00	−7.89	2283.27
	3R4F (0.13)	12	2936.51	1841.67	260.91	955.57	1624.14	−1116.97	6990.00
	3R4F (0.25)	13	2530.20	1338.83	810.47	967.60	1521.45	−386.86	5447.26
	THS2.2 (Air)	13	809.21	259.97	181.05	264.56	1223.36	242.77	1375.65
	THS2.2 (0.14)	13	1246.70	641.65	129.88	306.88	1035.90	−151.34	2644.74
	THS2.2 (0.25)	13	1325.26	680.56	156.54	300.18	720.25	−157.55	2808.07
	THS2.2 (0.42)	9	258.49	96.17	82.69	144.10	308.38	36.72	480.25
72	3R4F (Air)	13	2579.62	862.25	93.80	1391.75	5188.51	700.94	4458.30
	3R4F (0.13)	12	3146.73	932.36	433.46	2192.98	5199.69	1094.63	5198.83
	3R4F (0.25)	13	2179.26	370.74	990.58	2325.23	3105.73	1371.48	2987.04
	THS2.2 (Air)	13	1304.30	498.83	162.56	256.87	1914.41	217.45	2391.14
	THS2.2 (0.14)	13	1238.41	458.50	206.76	274.46	1913.40	239.41	2237.40
	THS2.2 (0.25)	13	1824.47	843.90	209.39	251.67	1540.30	−14.24	3663.18
	THS2.2 (0.42)	9	237.09	53.88	97.01	186.68	393.61	112.85	361.33

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.17 **Supplementary Table 17.** Descriptive Statistics for CSF2 Protein in the Basolateral Media

		Descriptive Statistics: CSF2 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	6.28	1.55	3.75	3.75	9.60	1.96	10.59
	TNFα+ILß	5	87.88	30.25	22.65	102.72	150.05	3.88	171.89
4	3R4F (Air)	2	4.28	0.53	3.75	4.28	4.81	−2.46	11.02
	3R4F (0.13)	2	5.39	1.64	3.75	5.39	7.02	−15.40	26.17
	3R4F (0.25)	2	4.53	0.78	3.75	4.53	5.30	−5.35	14.40
	THS2.2 (Air)	2	3.75	0.00	3.75	3.75	3.75	NA	NA
	THS2.2 (0.14)	2	3.75	0.00	3.75	3.75	3.75	NA	NA
	THS2.2 (0.25)	2	3.75	0.00	3.75	3.75	3.75	NA	NA
24	3R4F (Air)	13	26.47	12.22	3.75	3.75	24.03	−0.15	53.08
	3R4F (0.13)	12	15.90	4.90	3.75	9.18	21.29	5.12	26.68
	3R4F (0.25)	13	20.94	3.91	12.75	18.81	24.07	12.42	29.46
	THS2.2 (Air)	13	15.43	7.20	3.75	5.81	12.15	−0.26	31.13
	THS2.2 (0.14)	13	13.01	4.38	3.75	5.73	11.21	3.47	22.54
	THS2.2 (0.25)	13	22.14	7.79	3.75	10.42	23.20	5.18	39.11
	THS2.2 (0.42)	9	20.47	7.64	8.27	10.17	20.83	2.85	38.09
48	3R4F (Air)	13	34.40	8.52	14.73	19.68	34.28	15.83	52.97
	3R4F (0.13)	12	75.73	36.09	13.17	19.02	87.37	−3.70	155.16
	3R4F (0.25)	13	82.51	20.85	41.49	57.97	87.17	37.08	127.94
	THS2.2 (Air)	13	23.15	6.26	12.63	14.54	26.99	9.51	36.78
	THS2.2 (0.14)	13	27.57	7.39	12.26	19.82	26.31	11.47	43.67
	THS2.2 (0.25)	13	36.18	15.10	14.49	17.88	23.19	3.28	69.08
	THS2.2 (0.42)	9	43.04	17.42	11.06	13.78	65.94	2.87	83.21
72	3R4F (Air)	13	57.04	22.60	17.82	29.58	40.26	7.81	106.27
	3R4F (0.13)	12	62.44	21.74	25.04	30.44	45.29	14.58	110.29
	3R4F (0.25)	13	110.44	28.03	59.03	82.10	102.13	49.37	171.52
	THS2.2 (Air)	13	32.05	5.71	16.33	24.79	45.27	19.60	44.49
	THS2.2 (0.14)	13	33.68	5.46	20.85	23.20	39.81	21.77	45.58
	THS2.2 (0.25)	13	36.02	7.79	24.29	25.67	37.25	19.04	52.99
	THS2.2 (0.42)	9	39.46	11.37	16.75	19.29	66.15	13.23	65.68

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.18 **Supplementary Table 18.** Descriptive Statistics for CXCL1 Protein in the Basolateral Media

		Descriptive Statistics: CXCL1 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	4267.92	959.80	3173.30	3631.20	4249.80	1603.09	6932.75
	TNFα+ILß	5	131428.8	35637.04	66687.65	157851.8	175659.4	32484.47	230373.0
4	3R4F (Air)	2	527.64	106.26	421.38	527.64	633.89	−822.46	1877.74
	3R4F (0.13)	2	330.95	24.73	306.22	330.95	355.68	16.74	645.17
	3R4F (0.25)	2	194.88	10.99	183.89	194.88	205.87	55.25	334.52
	THS2.2 (Air)	2	673.15	110.69	562.47	673.15	783.84	−733.26	2079.57
	THS2.2 (0.14)	2	585.83	56.40	529.43	585.83	642.23	−130.80	1302.46
	THS2.2 (0.25)	2	351.10	14.94	336.16	351.10	366.04	161.26	540.93
24	3R4F (Air)	13	12812.11	6235.06	2949.38	6592.15	7899.81	−772.92	26397.13
	3R4F (0.13)	12	7801.49	2262.03	2312.42	4887.78	11708.28	2822.80	12780.17
	3R4F (0.25)	13	14762.46	2973.81	3032.24	16067.96	18984.02	8283.10	21241.83
	THS2.2 (Air)	13	11291.63	5883.69	3734.96	5870.30	8219.04	−1527.82	24111.08
	THS2.2 (0.14)	13	6067.64	1487.11	3357.56	5323.24	6408.12	2827.50	9307.78
	THS2.2 (0.25)	13	6611.77	1163.90	3756.71	6593.75	8243.18	4075.85	9147.69
	THS2.2 (0.42)	9	9133.50	2846.09	5500.89	6682.36	7894.36	2570.40	15696.61
48	3R4F (Air)	13	20170.47	7733.92	7495.96	10551.40	19020.99	3319.70	37021.25
	3R4F (0.13)	12	30616.32	11171.82	10467.11	14534.96	38211.01	6027.31	55205.33
	3R4F (0.25)	13	37986.61	7935.14	21545.36	28533.61	45205.94	20697.43	55275.78
	THS2.2 (Air)	13	13447.98	1950.16	8196.40	12607.40	17051.69	9198.95	17697.01
	THS2.2 (0.14)	13	18732.53	6863.60	9474.98	12508.93	15919.96	3778.04	33687.03
	THS2.2 (0.25)	13	17520.02	6091.14	9307.26	13751.56	14538.49	4248.57	30791.47
	THS2.2 (0.42)	9	18166.87	3982.03	9709.73	13883.17	24279.00	8984.29	27349.46
72	3R4F (Air)	13	32910.26	7282.01	12138.34	20275.88	52632.07	17044.12	48776.39
	3R4F (0.13)	12	49385.87	15385.19	17166.30	33067.41	48582.31	15523.30	83248.44
	3R4F (0.25)	13	48216.82	11332.40	20443.33	41848.57	48628.18	23525.64	72908.00
	THS2.2 (Air)	13	21805.18	3727.30	13145.74	16355.81	20512.73	13684.09	29926.27
	THS2.2 (0.14)	13	22895.90	4053.09	15786.37	17939.24	20118.39	14064.99	31726.81
	THS2.2 (0.25)	13	31016.35	10040.81	14566.58	17763.44	21283.34	9139.30	52893.41
	THS2.2 (0.42)	9	19138.75	3519.45	12198.89	14099.98	24351.70	11022.88	27254.63

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.19 **Supplementary Table 19.** Descriptive Statistics for IL-1α Protein in the Basolateral Media

		Descriptive Statistics: IL-1α (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	4.70	0.00	4.70	4.70	4.70	NA	NA
	TNFα+ILß	5	4.70	0.00	4.70	4.70	4.70	NA.	NA
4	3R4F (Air)	2	5.62	0.92	4.70	5.62	6.54	−6.06	17.30
	3R4F (0.13)	2	11.78	7.08	4.70	11.78	18.86	−78.17	101.72
	3R4F (0.25)	2	6.20	0.26	5.94	6.20	6.47	2.85	9.55
	THS2.2 (Air)	2	4.70	0.00	4.70	4.70	4.70	NA	NA
	THS2.2 (0.14)	2	5.19	0.49	4.70	5.19	5.68	−1.03	11.41
	THS2.2 (0.25)	2	5.99	0.20	5.79	5.99	6.20	3.40	8.59
24	3R4F (Air)	13	6.39	1.44	4.70	4.70	4.70	3.26	9.52
	3R4F (0.13)	12	7.41	1.80	4.70	4.70	6.27	3.46	11.37
	3R4F (0.25)	13	15.95	7.80	4.70	5.73	10.85	−1.05	32.95
	THS2.2 (Air)	13	5.99	1.29	4.70	4.70	4.70	3.18	8.81
	THS2.2 (0.14)	13	4.70	0.00	4.70	4.70	4.70	NA	NA
	THS2.2 (0.25)	13	13.56	8.86	4.70	4.70	4.70	−5.74	32.86
	THS2.2 (0.42)	9	4.70	0.00	4.70	4.70	4.70	NA	NA
48	3R4F (Air)	13	12.73	5.79	4.70	4.70	10.68	0.10	25.35
	3R4F (0.13)	12	40.87	24.42	4.70	9.87	30.61	−12.88	94.62
	3R4F (0.25)	13	68.04	25.46	13.91	25.31	67.47	12.56	123.52
	THS2.2 (Air)	13	8.05	3.14	4.70	4.70	4.70	1.20	14.90
	THS2.2 (0.14)	13	5.80	0.74	4.70	4.70	4.70	4.18	7.42
	THS2.2 (0.25)	13	7.98	2.81	4.70	4.70	4.70	1.86	14.10
	THS2.2 (0.42)	9	9.03	4.33	4.70	4.70	4.70	−0.96	19.02
72	3R4F (Air)	13	8.13	1.44	4.70	4.70	10.36	4.98	11.27
	3R4F (0.13)	12	28.26	7.30	5.98	22.31	45.43	12.18	44.33
	3R4F (0.25)	13	38.88	8.20	18.73	32.72	46.56	21.00	56.75
	THS2.2 (Air)	13	6.72	1.23	4.70	4.70	4.70	4.04	9.40
	THS2.2 (0.14)	13	7.12	1.50	4.70	4.70	4.70	3.85	10.39
	THS2.2 (0.25)	13	6.06	0.72	4.70	4.70	5.59	4.48	7.64
	THS2.2 (0.42)	9	4.70	0.00	4.70	4.70	4.70	NA	NA

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.20 **Supplementary Table 20.** Descriptive Statistics for IL-1β Protein in the Basolateral Media

		Descriptive Statistics: IL-1β (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	1.00	0.31	0.47	0.66	1.50	0.13	1.87
	TNFα+ILß	5	7599.02	687.89	6564.55	7293.24	7544.10	5689.14	9508.90
4	3R4F (Air)	2	1.17	0.77	0.40	1.17	1.95	−8.64	10.99
	3R4F (0.13)	2	4.58	3.15	1.43	4.58	7.73	−35.40	44.56
	3R4F (0.25)	2	1.84	0.20	1.64	1.84	2.04	−0.67	4.35
	THS2.2 (Air)	2	1.48	0.67	0.81	1.48	2.16	−7.07	10.04
	THS2.2 (0.14)	2	1.25	0.00	1.25	1.25	1.25	1.24	1.26
	THS2.2 (0.25)	2	1.16	0.33	0.83	1.16	1.49	−3.05	5.37
24	3R4F (Air)	13	0.58	0.11	0.40	0.40	0.40	0.35	0.82
	3R4F (0.13)	12	0.50	0.05	0.40	0.40	0.55	0.38	0.62
	3R4F (0.25)	13	0.60	0.09	0.40	0.40	0.69	0.39	0.81
	THS2.2 (Air)	13	0.70	0.13	0.40	0.40	0.81	0.43	0.97
	THS2.2 (0.14)	13	1.37	0.74	0.40	0.40	1.05	−0.25	2.99
	THS2.2 (0.25)	13	0.66	0.10	0.40	0.40	0.81	0.44	0.89
	THS2.2 (0.42)	9	0.76	0.17	0.40	0.40	1.13	0.37	1.15
48	3R4F (Air)	13	1.02	0.50	0.40	0.40	0.64	−0.06	2.10
	3R4F (0.13)	12	0.79	0.13	0.40	0.66	1.13	0.51	1.07
	3R4F (0.25)	13	0.69	0.09	0.40	0.62	0.93	0.48	0.89
	THS2.2 (Air)	13	0.75	0.12	0.40	0.40	1.06	0.48	1.01
	THS2.2 (0.14)	13	0.80	0.12	0.40	0.69	1.16	0.53	1.06
	THS2.2 (0.25)	13	0.62	0.07	0.40	0.62	0.77	0.47	0.76
	THS2.2 (0.42)	9	0.77	0.10	0.65	0.69	0.88	0.54	1.00
72	3R4F (Air)	13	0.53	0.07	0.40	0.40	0.40	0.37	0.68
	3R4F (0.13)	12	0.57	0.07	0.40	0.44	0.70	0.42	0.72
	3R4F (0.25)	13	0.76	0.19	0.40	0.40	0.84	0.36	1.17
	THS2.2 (Air)	13	0.80	0.10	0.40	0.77	1.09	0.58	1.03
	THS2.2 (0.14)	13	0.78	0.16	0.40	0.63	0.73	0.44	1.12
	THS2.2 (0.25)	13	0.71	0.12	0.40	0.40	1.12	0.46	0.97
	THS2.2 (0.42)	9	0.77	0.16	0.40	0.40	1.12	0.40	1.14

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.21 **Supplementary Table 21.** Descriptive Statistics for IL-6 Protein in the Basolateral Media

		Descriptive Statistics: IL-6 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	5.52	2.05	2.21	3.06	9.05	−0.17	11.22
	TNFα+ILß	5	491.44	275.44	25.48	179.44	816.12	−273.30	1256.17
4	3R4F (Air)	2	1.16	0.71	0.45	1.16	1.87	−7.86	10.18
	3R4F (0.13)	2	5.03	2.14	2.90	5.03	7.17	−22.10	32.17
	3R4F (0.25)	2	2.00	0.93	1.07	2.00	2.92	−9.78	13.77
	THS2.2 (Air)	2	4.85	2.17	2.68	4.85	7.01	−22.68	32.37
	THS2.2 (0.14)	2	1.81	0.34	1.47	1.81	2.15	−2.53	6.14
	THS2.2 (0.25)	2	1.45	0.25	1.19	1.45	1.70	−1.79	4.68
24	3R4F (Air)	13	94.71	64.16	6.39	16.47	40.56	−45.08	234.50
	3R4F (0.13)	12	132.28	58.05	8.80	58.57	131.22	4.52	260.04
	3R4F (0.25)	13	222.51	85.33	47.88	180.60	225.74	36.60	408.42
	THS2.2 (Air)	13	68.84	37.40	5.81	12.97	30.71	−12.65	150.34
	THS2.2 (0.14)	13	35.72	27.11	1.95	9.43	18.54	−23.36	94.79
	THS2.2 (0.25)	13	67.02	37.63	2.75	21.63	34.70	−14.98	149.02
	THS2.2 (0.42)	9	39.04	13.70	13.99	20.24	51.96	7.45	70.63
48	3R4F (Air)	13	112.95	76.31	11.73	18.74	41.54	−53.32	279.22
	3R4F (0.13)	12	297.01	163.12	8.60	70.75	154.59	−62.01	656.04
	3R4F (0.25)	13	327.41	112.01	44.16	214.85	266.72	83.35	571.47
	THS2.2 (Air)	13	55.15	25.90	9.43	25.46	59.71	−1.29	111.58
	THS2.2 (0.14)	13	58.02	31.49	9.03	15.18	32.45	−10.59	126.62
	THS2.2 (0.25)	13	65.16	36.88	11.15	18.30	46.99	−15.19	145.52
	THS2.2 (0.42)	9	72.02	39.91	18.32	29.80	38.31	−20.02	164.06
72	3R4F (Air)	13	125.85	69.04	8.53	19.43	60.24	−24.58	276.29
	3R4F (0.13)	12	113.10	38.07	9.29	72.17	178.01	29.31	196.89
	3R4F (0.25)	13	234.66	54.69	38.98	224.37	386.56	115.49	353.82
	THS2.2 (Air)	13	27.35	5.16	15.92	22.87	47.88	16.12	38.59
	THS2.2 (0.14)	13	33.90	8.71	13.49	14.93	59.96	14.91	52.88
	THS2.2 (0.25)	13	57.28	28.82	14.66	23.78	39.02	−5.51	120.06
	THS2.2 (0.42)	9	28.78	8.28	14.64	20.99	32.54	9.68	47.87

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.22 **Supplementary Table 22.** Descriptive Statistics for CXCL8/IL-8 Protein in the Basolateral Media

		Descriptive Statistics: CXCL8/IL-8 (pg/mL)							
PE (h)	Exposure	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	1334.85	451.79	733.48	769.39	1394.32	80.50	2589.21
	TNFα+ILß	5	71622.52	15399.95	40176.48	85976.23	92106.41	28865.40	114379.6
4	3R4F (Air)	2	442.55	33.05	409.50	442.55	475.60	22.58	862.52
	3R4F (0.13)	2	1058.99	50.31	1008.68	1058.99	1109.31	419.68	1698.30
	3R4F (0.25)	2	374.45	131.29	243.16	374.45	505.74	−1293.77	2042.67
	THS2.2 (Air)	2	532.48	35.71	496.77	532.48	568.20	78.72	986.25
	THS2.2 (0.14)	2	520.27	63.83	456.44	520.27	584.10	−290.75	1331.29
	THS2.2 (0.25)	2	363.77	62.20	301.57	363.77	425.97	−426.54	1154.08
24	3R4F (Air)	13	6695.29	3696.75	1122.23	2517.84	3523.10	−1359.24	14749.81
	3R4F (0.13)	12	8185.89	1760.59	4001.01	5477.67	12132.94	4310.86	12060.93
	3R4F (0.25)	13	10451.06	1713.10	4596.90	9795.22	14623.76	6718.53	14183.60
	THS2.2 (Air)	13	5280.71	2728.54	1033.32	1909.89	3604.39	−664.26	11225.68
	THS2.2 (0.14)	13	3071.52	969.86	1111.92	2015.87	2959.63	958.38	5184.66
	THS2.2 (0.25)	13	4718.73	1184.94	1478.26	3424.82	6695.15	2136.96	7300.49
	THS2.2 (0.42)	9	6172.45	1963.13	2771.78	3484.50	6959.68	1645.46	10699.44
48	3R4F (Air)	13	9258.18	4383.32	2489.78	5510.06	6678.14	−292.26	18808.63
	3R4F (0.13)	12	28343.39	10161.72	7946.53	9201.89	34100.99	5977.59	50709.20
	3R4F (0.25)	13	24544.69	4827.90	13922.36	20718.72	26903.16	14025.60	35063.78
	THS2.2 (Air)	13	6053.28	1062.92	4247.71	6478.63	7238.07	3737.38	8369.17
	THS2.2 (0.14)	13	8524.83	3517.29	3097.73	4525.21	7419.33	861.31	16188.35
	THS2.2 (0.25)	13	9917.43	4042.14	3487.61	6532.24	9869.95	1110.36	18724.49
	THS2.2 (0.42)	9	10292.19	3260.97	2977.08	7250.64	13198.42	2772.38	17811.99
72	3R4F (Air)	13	12043.37	3102.54	3017.64	8345.33	14284.27	5283.51	18803.23
	3R4F (0.13)	12	34034.39	10720.28	9915.09	14994.43	56258.16	10439.23	57629.56
	3R4F (0.25)	13	37492.24	9126.57	14985.61	33594.13	43877.38	17607.14	57377.33
	THS2.2 (Air)	13	7647.97	1213.37	4308.58	7774.06	9701.50	5004.27	10291.67
	THS2.2 (0.14)	13	7879.46	1017.18	4675.25	8282.63	10028.85	5663.22	10095.69
	THS2.2 (0.25)	13	1. 1238 1.72	2. 4442 .55	3. 6945 .83	4. 7681 .85	5. 1014 0.79	6. 2702 .24	7. 2206 1.20
	THS2.2 (0.42)	9	9218.77	2393.91	3997.53	5327.89	13643.66	3698.40	14739.15

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.23 **Supplementary Table 23.** Descriptive Statistics for IL-10 Protein in the Basolateral Media

		Descriptive Statistics: IL-10 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	2	3.70	3.15	0.55	3.70	6.84	−36.28	43.68
	TNFα+ILß	2	4.32	3.66	0.66	4.32	7.97	−42.14	50.78
4	3R4F (Air)	2	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	3R4F (0.13)	2	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	3R4F (0.25)	2	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	THS2.2 (Air)	2	0.88	0.33	0.55	0.88	1.21	−3.29	5.05
	THS2.2 (0.14)	2	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	THS2.2 (0.25)	2	0.55	0.00	0.55	0.55	0.55	NA.	NA.
24	3R4F (Air)	4	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	3R4F (0.13)	4	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	3R4F (0.25)	4	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	THS2.2 (Air)	4	0.63	0.08	0.55	0.55	0.70	0.39	0.86
	THS2.2 (0.14)	4	0.63	0.08	0.55	0.55	0.70	0.39	0.86
	THS2.2 (0.25)	4	0.63	0.08	0.55	0.55	0.70	0.39	0.86
48	3R4F (Air)	4	2.52	1.15	0.55	2.33	4.49	−1.13	6.17
	3R4F (0.13)	4	7.29	6.34	0.55	1.16	14.03	−12.88	27.45
	3R4F (0.25)	4	0.65	0.10	0.55	0.55	0.75	0.33	0.96
	THS2.2 (Air)	4	2.24	1.17	0.55	1.44	3.92	−1.50	5.97
	THS2.2 (0.14)	4	1.31	0.45	0.55	1.19	2.06	−0.12	2.73
	THS2.2 (0.25)	4	2.02	0.88	0.55	1.76	3.49	−0.77	4.81
72	3R4F (Air)	4	0.69	0.08	0.55	0.66	0.82	0.43	0.94
	3R4F (0.13)	4	0.63	0.05	0.55	0.60	0.71	0.47	0.80
	3R4F (0.25)	4	0.68	0.09	0.55	0.60	0.80	0.38	0.98
	THS2.2 (Air)	4	0.66	0.11	0.55	0.55	0.77	0.31	1.01
	THS2.2 (0.14)	4	0.55	0.00	0.55	0.55	0.55	NA.	NA.
	THS2.2 (0.25)	4	0.55	0.00	0.55	0.55	0.55	NA.	NA.

Note: IL-10 was only measured in experiments 1 and 2 (see Table 9). Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.24 **Supplementary Table 24.** Descriptive Statistics for IL-13 Protein in the Basolateral Media

		Descriptive Statistics: IL-13 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	2	0.65	0.00	0.65	0.65	0.65	NA.	NA
	TNFα+ILß	2	2.39	1.74	0.65	2.39	4.14	−19.78	24.57
4	3R4F (Air)	2	0.73	0.08	0.65	0.73	0.82	−0.33	1.80
	3R4F (0.13)	2	0.65	0.00	0.65	0.65	0.65	NA	NA
	3R4F (0.25)	2	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (Air)	2	0.72	0.07	0.65	0.72	0.80	−0.20	1.65
	THS2.2 (0.14)	2	0.72	0.07	0.65	0.72	0.80	−0.20	1.65
	THS2.2 (0.25)	2	0.71	0.06	0.65	0.71	0.76	−0.02	1.44
24	3R4F (Air)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	3R4F (0.13)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	3R4F (0.25)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (Air)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (0.14)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (0.25)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
48	3R4F (Air)	4	1.78	0.65	0.65	1.78	2.92	−0.30	3.87
	3R4F (0.13)	4	0.95	0.30	0.65	0.65	1.25	0.00	1.90
	3R4F (0.25)	4	1.07	0.24	0.65	1.02	1.48	0.29	1.84
	THS2.2 (Air)	4	0.86	0.21	0.65	0.65	1.06	0.20	1.51
	THS2.2 (0.14)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (0.25)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
72	3R4F (Air)	4	0.69	0.04	0.65	0.65	0.73	0.56	0.81
	3R4F (0.13)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	3R4F (0.25)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (Air)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (0.14)	4	0.65	0.00	0.65	0.65	0.65	NA	NA
	THS2.2 (0.25)	4	0.65	0.00	0.65	0.65	0.65	NA	NA

Note: IL-13 was only measured in experiments 1 and 2 (see Table 9). Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.25 **Supplementary Table 25.** Descriptive Statistics for CXCL10 Protein in the Basolateral Media

		Descriptive Statistics: CXCL10 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	83.23	45.57	9.18	22.54	149.39	−43.30	209.76
	TNFα+ILß	5	764.98	121.51	690.78	698.33	812.92	427.61	1102.35
4	3R4F (Air)	2	8.25	1.49	6.75	8.25	9.74	−10.73	27.22
	3R4F (0.13)	2	16.08	0.83	15.25	16.08	16.92	5.48	26.69
	3R4F (0.25)	2	7.34	3.04	4.30	7.34	10.38	−31.28	45.96
	THS2.2 (Air)	2	6.26	0.96	5.30	6.26	7.22	−5.95	18.46
	THS2.2 (0.14)	2	4.30	0.00	4.30	4.30	4.30	NA.	NA.
	THS2.2 (0.25)	2	4.30	0.00	4.30	4.30	4.30	NA.	NA.
24	3R4F (Air)	13	432.11	146.13	19.67	52.86	945.50	113.72	750.51
	3R4F (0.13)	12	701.26	278.00	5.33	179.88	1246.69	89.37	1313.14
	3R4F (0.25)	13	2560.20	931.79	70.72	435.08	4134.17	530.01	4590.38
	THS2.2 (Air)	13	523.32	222.38	47.47	70.36	714.73	38.78	1007.85
	THS2.2 (0.14)	13	305.78	111.80	9.68	112.12	427.83	62.20	549.36
	THS2.2 (0.25)	13	648.70	270.30	8.14	127.77	673.92	59.78	1237.63
	THS2.2 (0.42)	9	542.75	184.66	89.63	469.49	678.26	116.93	968.57
48	3R4F (Air)	13	590.54	206.68	82.46	287.15	825.15	140.21	1040.87
	3R4F (0.13)	12	1121.64	390.54	10.40	258.87	2331.12	262.06	1981.22
	3R4F (0.25)	13	2636.17	821.15	284.98	868.85	3607.11	847.04	4425.30
	THS2.2 (Air)	13	787.90	8. 46 4. 47	9. 71 .0 7	10. 171. 62	11. 747 .88	−224.10	1799.90
	THS2.2 (0.14)	13	837.66	333.68	59.57	215.49	858.17	110.63	1564.69
	THS2.2 (0.25)	13	944.31	371.10	55.07	185.23	967.94	135.75	1752.88
	THS2.2 (0.42)	9	1079.25	326.06	105.16	980.78	1856.14	327.35	1831.14
72	3R4F (Air)	13	586.81	138.46	105.58	341.27	971.08	285.14	888.48
	3R4F (0.13)	12	1182.92	476.06	37.74	411.58	1875.57	135.11	2230.72
	3R4F (0.25)	13	2540.63	758.07	320.03	455.71	4802.13	888.94	4192.32
	THS2.2 (Air)	13	783.04	260.99	144.96	221.04	981.05	214.40	1351.68
	THS2.2 (0.14)	13	922.83	365.99	129.72	384.05	1438.65	125.41	1720.25
	THS2.2 (0.25)	13	943.78	449.01	112.09	219.37	956.86	−34.52	1922.08
	THS2.2 (0.42)	9	934.82	285.01	138.34	876.26	1381.81	277.58	1592.07

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.26 **Supplementary Table 26.** Descriptive Statistics for CCL2 Protein in the Basolateral Media

		Descriptive Statistics: CCL2 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	203.47	90.15	4.66	219.27	331.99	−46.84	453.77
	TNFα+ILß	5	48128.82	19255.88	1286.68	76394.76	80454.48	−5334.06	101591.7
4	3R4F (Air)	2	1.55	0.42	1.13	1.55	1.97	−3.75	6.86
	3R4F (0.13)	2	2.57	0.52	2.05	2.57	3.09	−4.01	9.16
	3R4F (0.25)	2	2.15	0.64	1.50	2.15	2.79	−6.03	10.33
	THS2.2 (Air)	2	4.32	0.60	3.73	4.32	4.92	−3.28	11.93
	THS2.2 (0.14)	2	2.12	0.50	1.62	2.12	2.63	−4.28	8.52
	THS2.2 (0.25)	2	1.86	0.24	1.62	1.86	2.10	−1.17	4.89
24	3R4F (Air)	13	1096.53	662.16	11.85	440.94	653.47	−346.19	2539.26
	3R4F (0.13)	12	651.30	236.87	1.40	389.78	1011.99	129.96	1172.64
	3R4F (0.25)	13	1935.56	640.78	14.11	1078.82	2915.43	539.41	3331.71
	THS2.2 (Air)	13	833.40	465.26	25.64	364.08	782.23	−180.31	1847.12
	THS2.2 (0.14)	13	358.13	131.78	9.66	327.92	485.40	71.01	645.25
	THS2.2 (0.25)	13	556.47	197.21	4.87	352.02	665.03	126.79	986.15
	THS2.2 (0.42)	9	591.64	142.16	427.70	468.56	604.28	263.82	919.45
48	3R4F (Air)	13	1448.49	723.03	94.79	1071.00	1150.92	−126.87	3023.85
	3R4F (0.13)	12	2417.00	1394.77	4.94	854.73	1563.46	−652.85	5486.86
	3R4F (0.25)	13	3898.85	1544.96	16.27	2235.47	4183.51	532.67	7265.02
	THS2.2 (Air)	13	1015.39	313.48	66.49	860.86	1323.53	332.37	1698.42
	THS2.2 (0.14)	13	1233.39	573.49	68.79	655.19	1148.23	−16.15	2482.93
	THS2.2 (0.25)	13	1049.40	363.22	103.68	706.94	1189.60	258.00	1840.79
	THS2.2 (0.42)	9	842.29	165.54	480.74	599.64	1018.70	460.57	1224.02
72	3R4F (Air)	13	2042.99	921.72	112.51	718.74	1524.16	34.73	4051.25
	3R4F (0.13)	12	1090.82	319.53	7.17	952.36	1694.86	387.53	1794.11
	3R4F (0.25)	13	3411.21	1870.97	5.78	1514.37	1988.37	−665.28	7487.70
	THS2.2 (Air)	13	914.85	213.15	106.94	731.02	1544.35	450.43	1379.27
	THS2.2 (0.14)	13	891.10	232.89	83.26	749.06	1265.64	383.68	1398.52
	THS2.2 (0.25)	13	1661.08	733.47	102.55	888.95	1663.43	63.00	3259.17
	THS2.2 (0.42)	9	974.13	97.00	755.18	817.39	1325.50	750.45	1197.81

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.27 **Supplementary Table 27.** Descriptive Statistics for CCL5 Protein in the Basolateral Media

		Descriptive Statistics: CCL5 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	4.56	2.44	0.60	2.90	4.92	−2.22	11.34
	TNFα+ILß	5	98.79	14.68	78.91	110.92	115.14	58.03	139.55
4	3R4F (Air)	2	1.16	0.56	0.60	1.16	1.72	−5.94	8.26
	3R4F (0.13)	2	1.73	0.33	1.40	1.73	2.07	−2.50	5.97
	3R4F (0.25)	2	1.06	0.46	0.60	1.06	1.52	−4.76	6.88
	THS2.2 (Air)	2	1.71	0.27	1.45	1.71	1.98	−1.66	5.09
	THS2.2 (0.14)	2	1.66	0.01	1.65	1.66	1.67	1.54	1.79
	THS2.2 (0.25)	2	1.51	0.08	1.43	1.51	1.59	0.45	2.57
24	3R4F (Air)	13	9.25	3.43	0.74	4.31	10.23	1.79	16.71
	3R4F (0.13)	12	13.31	4.70	0.67	3.90	23.67	2.98	23.65
	3R4F (0.25)	13	45.80	16.98	0.60	11.29	90.95	8.80	82.80
	THS2.2 (Air)	13	7.12	2.49	0.60	3.26	6.97	1.70	12.55
	THS2.2 (0.14)	13	3.82	1.47	0.60	1.94	2.89	0.63	7.01
	THS2.2 (0.25)	13	7.51	2.36	0.60	6.67	7.15	2.36	12.66
	THS2.2 (0.42)	9	6.15	1.59	2.57	4.99	7.98	2.48	9.82
48	3R4F (Air)	13	12.24	3.30	0.60	9.05	22.23	5.05	19.42
	3R4F (0.13)	12	22.98	10.10	0.60	8.42	32.99	0.75	45.21
	3R4F (0.25)	13	59.30	19.98	6.83	11.50	133.84	15.77	102.82
	THS2.2 (Air)	13	16.10	4.71	0.60	12.04	19.18	5.84	26.35
	THS2.2 (0.14)	13	16.00	4.13	0.60	15.61	22.19	7.01	25.00
	THS2.2 (0.25)	13	17.41	5.19	1.09	13.37	23.48	6.10	28.72
	THS2.2 (0.42)	9	23.81	4.10	12.21	26.34	28.92	14.37	33.26
72	3R4F (Air)	13	18.41	7.09	1.76	11.37	19.10	2.96	33.86
	3R4F (0.13)	12	21.27	6.19	1.36	13.54	38.08	7.66	34.88
	3R4F (0.25)	13	53.28	15.58	5.32	18.17	104.51	19.35	87.22
	THS2.2 (Air)	13	18.03	4.59	2.07	11.60	32.80	8.03	28.02
	THS2.2 (0.14)	13	16.55	4.20	2.10	11.84	24.11	7.40	25.70
	THS2.2 (0.25)	13	17.56	5.16	2.16	9.86	23.76	6.32	28.80
	THS2.2 (0.42)	9	21.79	3.90	12.27	21.29	31.75	12.79	30.79

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.28 **Supplementary Table 28.** Descriptive Statistics for CCL20/MIP-3A Protein in the Basolateral Media

		Descriptive Statistics: CCL20/MIP-3α (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	19.12	3.57	15.60	21.10	23.01	9.20	29.04
	TNFα+ILß	5	8115.06	2874.51	2701.25	8475.78	11165.10	134.15	16095.98
4	3R4F (Air)	2	6.75	2.47	4.28	6.75	9.22	−24.62	38.12
	3R4F (0.13)	2	2.27	0.19	2.08	2.27	2.46	−0.17	4.71
	3R4F (0.25)	2	2.08	0.00	2.08	2.08	2.08	NA	NA
	THS2.2 (Air)	2	11.60	4.45	7.14	11.60	16.05	−45.00	68.19
	THS2.2 (0.14)	2	9.21	0.02	9.19	9.21	9.23	9.00	9.42
	THS2.2 (0.25)	2	5.13	0.16	4.96	5.13	5.29	3.04	7.22
24	3R4F (Air)	13	85.14	47.43	18.51	44.63	47.15	−18.21	188.49
	3R4F (0.13)	12	57.27	19.06	7.47	37.76	79.05	15.32	99.22
	3R4F (0.25)	13	85.19	23.65	39.70	83.91	97.00	33.66	136.73
	THS2.2 (Air)	13	96.31	66.44	16.35	28.05	40.30	−48.44	241.06
	THS2.2 (0.14)	13	36.90	10.28	14.47	31.60	39.39	14.51	59.30
	THS2.2 (0.25)	13	33.95	8.36	22.23	31.13	40.89	15.74	52.17
	THS2.2 (0.42)	9	49.53	12.85	24.33	33.41	68.92	19.90	79.17
48	3R4F (Air)	13	160.38	68.69	42.61	79.47	175.14	10.72	310.04
	3R4F (0.13)	12	343.49	150.19	44.56	109.90	391.01	12.92	674.06
	3R4F (0.25)	13	385.36	112.95	193.02	284.99	388.04	139.27	631.44
	THS2.2 (Air)	13	96.79	21.22	52.26	77.18	100.59	50.55	143.03
	THS2.2 (0.14)	13	147.23	59.18	50.34	73.10	108.94	18.29	276.17
	THS2.2 (0.25)	13	139.75	49.13	47.80	69.12	111.05	32.71	246.78
	THS2.2 (0.42)	9	104.41	33.27	40.87	63.78	97.21	27.68	181.14
72	3R4F (Air)	13	242.80	53.14	58.02	285.92	361.50	127.02	358.58
	3R4F (0.13)	12	747.82	304.24	119.57	177.20	1173.79	78.19	1417.45
	3R4F (0.25)	13	599.29	128.90	245.70	628.46	885.51	318.45	880.13
	THS2.2 (Air)	13	166.13	36.52	70.83	109.60	275.61	86.55	245.71
	THS2.2 (0.14)	13	174.28	45.77	69.62	80.25	377.21	74.55	274.00
	THS2.2 (0.25)	13	223.77	72.13	71.68	76.64	312.92	66.60	380.93
	THS2.2 (0.42)	9	96.15	20.82	47.94	70.55	122.09	48.15	144.16

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.29 **Supplementary Table 29.** Descriptive Statistics for MMP-1 Protein in the Basolateral Media

		Descriptive Statistics: MMP-1 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	677.19	301.43	141.37	614.13	890.83	−159.72	1514.10
	TNFα+ILß	5	3667.79	2185.00	836.24	2455.47	2701.43	−2398.76	9734.33
4	3R4F (Air)	2	37.37	29.77	7.61	37.37	67.14	−340.83	415.58
	3R4F (0.13)	2	150.47	8.87	141.60	150.47	159.35	37.71	263.24
	3R4F (0.25)	2	112.63	83.13	29.50	112.63	195.76	−943.63	1168.89
	THS2.2 (Air)	2	78.97	60.38	18.59	78.97	139.35	−688.23	846.17
	THS2.2 (0.14)	2	26.53	3.69	22.84	26.53	30.22	−20.39	73.45
	THS2.2 (0.25)	2	17.15	0.45	16.70	17.15	17.60	11.42	22.88
24	3R4F (Air)	13	1968.62	865.01	238.10	866.88	1853.48	83.92	3853.31
	3R4F (0.13)	12	10149.59	2375.22	4184.59	7328.41	15514.48	4921.76	15377.41
	3R4F (0.25)	13	4785.88	755.49	3588.72	3936.26	5500.85	3139.82	6431.94
	THS2.2 (Air)	13	1909.82	892.77	393.22	853.54	1210.11	−35.35	3855.00
	THS2.2 (0.14)	13	1606.36	467.29	759.07	999.17	1254.30	588.23	2624.50
	THS2.2 (0.25)	13	4477.32	1895.52	820.05	1294.59	4955.24	347.34	8607.30
	THS2.2 (0.42)	9	7471.43	3803.86	1067.79	2029.38	3739.29	−1300.29	16243.15
48	3R4F (Air)	13	3130.09	930.63	1607.69	2260.48	2987.80	1102.42	5157.77
	3R4F (0.13)	12	24912.28	8326.27	3777.69	15019.28	30137.12	6586.29	43238.27
	3R4F (0.25)	13	15404.95	1504.35	12518.21	15988.74	16993.28	12127.26	18682.64
	THS2.2 (Air)	13	2075.53	415.62	835.18	2020.68	2443.17	1169.97	2981.08
	THS2.2 (0.14)	13	5317.71	2550.83	412.39	1984.15	2714.64	−240.06	10875.48
	THS2.2 (0.25)	13	10192.47	4661.58	391.51	2667.24	5209.01	35.75	20349.19
	THS2.2 (0.42)	9	14040.34	5621.36	2580.12	4029.07	27644.79	1077.47	27003.21
72	3R4F (Air)	13	6136.08	2572.43	2346.70	2894.29	6415.15	531.23	11740.93
	3R4F (0.13)	12	59012.50	25771.21	10987.78	17009.65	57788.90	2290.46	115734.5
	3R4F (0.25)	13	48637.46	13458.13	9000.17	43184.34	65222.89	19314.73	77960.20
	THS2.2 (Air)	13	4280.55	1276.21	1060.23	2868.70	3588.44	1499.92	7061.18
	THS2.2 (0.14)	13	3904.73	988.57	1454.05	2324.61	4536.47	1750.82	6058.63
	THS2.2 (0.25)	13	9132.63	3800.88	2368.38	3579.06	4306.92	851.21	17414.04
	THS2.2 (0.42)	9	14179.62	4991.74	3892.39	4738.04	20637.97	2668.65	25690.59

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.30 **Supplementary Table 30.** Descriptive Statistics for MMP-9 Protein in the Basolateral Media

		Descriptive Statistics: MMP-9 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	444.88	174.25	44.04	666.49	667.63	−38.91	928.66
	TNFα+ILß	5	740.79	189.97	693.91	842.22	1050.90	213.35	1268.24
4	3R4F (Air)	2	35.32	29.35	5.97	35.32	64.67	−337.63	408.27
	3R4F (0.13)	2	4.94	1.31	3.64	4.94	6.25	−11.65	21.54
	3R4F (0.25)	2	7.38	0.14	7.24	7.38	7.51	5.63	9.12
	THS2.2 (Air)	2	123.06	112.59	10.47	123.06	235.65	−1307.55	1553.68
	THS2.2 (0.14)	2	5.80	0.01	5.79	5.80	5.81	5.63	5.97
	THS2.2 (0.25)	2	1.87	0.10	1.77	1.87	1.98	0.54	3.20
24	3R4F (Air)	13	998.38	462.13	148.94	465.48	1096.52	−8.51	2005.28
	3R4F (0.13)	12	1081.05	380.96	133.50	651.66	1333.70	242.56	1919.55
	3R4F (0.25)	13	1841.73	555.44	897.85	1229.50	1636.73	631.52	3051.94
	THS2.2 (Air)	13	497.16	85.77	333.47	436.89	748.87	310.27	684.05
	THS2.2 (0.14)	13	587.47	151.07	376.08	471.10	756.66	258.32	916.62
	THS2.2 (0.25)	13	452.88	104.20	87.17	450.98	720.71	225.85	679.90
	THS2.2 (0.42)	9	562.86	80.04	364.47	618.79	721.94	378.29	747.42
48	3R4F (Air)	13	986.93	222.16	389.75	815.17	1587.41	502.88	1470.97
	3R4F (0.13)	12	1174.57	372.26	52.37	915.55	1765.10	355.24	1993.90
	3R4F (0.25)	13	7734.95	3769.46	2033.30	2715.96	3932.16	−478.01	15947.91
	THS2.2 (Air)	13	891.27	294.59	99.66	657.56	834.51	249.41	1533.13
	THS2.2 (0.14)	13	738.18	161.84	112.81	650.27	1298.76	385.56	1090.79
	THS2.2 (0.25)	13	920.98	165.15	506.13	825.81	1531.00	561.14	1280.81
	THS2.2 (0.42)	9	1173.75	259.54	676.57	806.75	1296.68	575.24	1772.26
72	3R4F (Air)	13	3071.65	2167.58	186.05	911.93	1439.35	−1651.09	7794.39
	3R4F (0.13)	12	1720.69	529.18	138.97	1280.55	2704.46	555.98	2885.39
	3R4F (0.25)	13	4962.81	2214.47	1622.88	2727.83	4693.61	137.89	9787.73
	THS2.2 (Air)	13	1201.59	252.14	474.58	1074.40	1705.34	652.22	1750.96
	THS2.2 (0.14)	13	1024.95	245.21	338.93	727.42	1529.94	490.68	1559.21
	THS2.2 (0.25)	13	1138.57	254.10	658.81	1004.66	1368.70	584.94	1692.20
	THS2.2 (0.42)	9	1118.51	180.73	768.89	1092.16	1240.52	701.76	1535.27

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.31 **Supplementary Table 31.** Descriptive Statistics for TSLP Protein in the Basolateral Media

		Descriptive Statistics: TSLP (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	2.66	0.58	1.55	2.11	3.77	1.04	4.28
	TNFα+ILß	5	13.52	6.39	3.17	4.73	27.29	−4.23	31.26
4	3R4F (Air)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
	3R4F (0.13)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
	3R4F (0.25)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
	THS2.2 (Air)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
	THS2.2 (0.14)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
	THS2.2 (0.25)	2	1.55	0.00	1.55	1.55	1.55	NA	NA
24	3R4F (Air)	13	3.09	0.48	1.55	1.55	4.88	2.04	4.13
	3R4F (0.13)	12	3.22	0.50	1.55	3.22	4.88	2.11	4.32
	3R4F (0.25)	13	3.22	0.46	1.55	3.22	4.88	2.21	4.22
	THS2.2 (Air)	13	2.70	0.44	1.55	1.55	4.88	1.75	3.66
	THS2.2 (0.14)	13	2.83	0.47	1.55	1.55	4.88	1.81	3.85
	THS2.2 (0.25)	13	3.09	0.48	1.55	1.55	4.88	2.04	4.13
	THS2.2 (0.42)	9	3.77	0.56	1.55	4.88	4.88	2.49	5.05
48	3R4F (Air)	13	5.67	3.03	1.55	1.55	4.88	−0.94	12.27
	3R4F (0.13)	12	1.97	0.22	1.55	1.55	2.38	1.49	2.45
	3R4F (0.25)	13	2.96	0.46	1.55	1.55	4.88	1.97	3.95
	THS2.2 (Air)	13	3.86	0.44	1.55	4.88	4.88	2.89	4.82
	THS2.2 (0.14)	13	3.22	0.46	1.55	3.22	4.88	2.21	4.22
	THS2.2 (0.25)	13	3.09	0.48	1.55	1.55	4.88	2.04	4.13
	THS2.2 (0.42)	9	4.70	0.19	4.88	4.88	4.88	4.27	5.12
72	3R4F (Air)	13	3.14	0.40	1.55	3.51	4.23	2.28	4.01
	3R4F (0.13)	12	2.92	0.45	1.55	2.07	4.55	1.94	3.91
	3R4F (0.25)	13	2.88	0.42	1.55	1.55	4.12	1.95	3.80
	THS2.2 (Air)	13	3.35	0.44	1.55	3.28	4.88	2.39	4.32
	THS2.2 (0.14)	13	3.15	0.47	1.55	2.35	4.88	2.13	4.17
	THS2.2 (0.25)	13	3.35	0.45	1.55	3.99	4.88	2.37	4.33
	THS2.2 (0.42)	9	4.44	0.62	4.88	4.88	4.88	3.01	5.86

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.32 **Supplementary Table 32.** Descriptive Statistics for VEGFA Protein in the Basolateral Media

		Descriptive Statistics: VEGFA (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	191.29	30.97	129.12	172.46	245.54	105.29	277.28
	TNFα+ILß	5	881.34	168.08	560.94	849.45	1107.02	414.69	1348.00
4	3R4F (Air)	2	111.31	1.86	109.45	111.31	113.18	87.62	135.01
	3R4F (0.13)	2	139.04	2.28	136.77	139.04	141.32	110.13	167.95
	3R4F (0.25)	2	56.29	2.81	53.47	56.29	59.10	20.55	92.02
	THS2.2 (Air)	2	128.80	1.47	127.33	128.80	130.27	110.15	147.45
	THS2.2 (0.14)	2	124.45	13.68	110.77	124.45	138.13	−49.39	298.28
	THS2.2 (0.25)	2	122.05	3.16	118.89	122.05	125.22	81.84	162.26
24	3R4F (Air)	13	267.04	33.11	201.59	229.64	357.20	194.89	339.18
	3R4F (0.13)	12	439.71	28.71	362.52	449.47	496.38	376.51	502.90
	3R4F (0.25)	13	281.49	56.23	182.73	220.45	355.01	158.97	404.01
	THS2.2 (Air)	13	222.98	23.05	173.21	201.16	224.23	172.76	273.20
	THS2.2 (0.14)	13	206.54	26.22	172.87	201.61	212.65	149.41	263.66
	THS2.2 (0.25)	13	263.17	23.34	207.72	259.70	317.65	212.31	314.02
	THS2.2 (0.42)	9	374.84	34.77	314.32	337.97	449.43	294.66	455.02
48	3R4F (Air)	13	561.05	63.65	419.97	478.31	576.30	422.38	699.73
	3R4F (0.13)	12	1023.15	133.53	689.99	789.43	1319.20	729.25	1317.05
	3R4F (0.25)	13	561.82	122.12	316.26	378.49	616.07	295.74	827.90
	THS2.2 (Air)	13	547.40	73.31	430.83	450.02	502.38	387.68	707.13
	THS2.2 (0.14)	13	532.04	66.49	383.14	413.48	539.27	387.17	676.91
	THS2.2 (0.25)	13	616.55	96.67	413.77	494.33	665.13	405.92	827.18
	THS2.2 (0.42)	9	761.78	159.86	465.49	622.14	860.32	393.14	1130.43
72	3R4F (Air)	13	907.00	126.22	602.19	800.61	978.03	631.98	1182.02
	3R4F (0.13)	12	1521.03	144.78	1105.59	1540.06	1782.03	1202.38	1839.69
	3R4F (0.25)	13	1018.28	293.22	388.40	638.44	905.77	379.41	1657.15
	THS2.2 (Air)	13	906.74	98.21	703.80	730.48	950.32	692.77	1120.72
	THS2.2 (0.14)	13	858.67	96.01	680.30	746.82	846.29	649.47	1067.86
	THS2.2 (0.25)	13	989.43	94.63	740.50	933.99	995.23	783.25	1195.60
	THS2.2 (0.42)	9	989.72	71.74	865.54	962.15	1178.43	824.30	1155.15

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.33 **Supplementary Table 33.** Descriptive Statistics for TNFA Protein in the Basolateral Media

		Descriptive Statistics: TNFA (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	1.98	0.67	0.98	1.90	2.43	0.11	3.86
	TNFα+ILß	5	8767.09	2768.98	4517.32	7502.42	14290.66	1079.17	16455.02
4	3R4F (Air)	2	0.35	0.00	0.35	0.35	0.35	NA.	NA.
	3R4F (0.13)	2	0.35	0.00	0.35	0.35	0.35	NA.	NA.
	3R4F (0.25)	2	0.35	0.00	0.35	0.35	0.35	NA.	NA.
	THS2.2 (Air)	2	0.49	0.14	0.35	0.49	0.63	−1.26	2.24
	THS2.2 (0.14)	2	0.35	0.00	0.35	0.35	0.35	NA.	NA.
	THS2.2 (0.25)	2	0.35	0.00	0.35	0.35	0.35	NA.	NA.
24	3R4F (Air)	13	4.76	2.74	0.42	1.24	2.11	−1.20	10.73
	3R4F (0.13)	12	2.93	1.11	0.35	1.19	4.40	0.48	5.38
	3R4F (0.25)	13	4.69	0.73	2.71	5.48	5.75	3.10	6.27
	THS2.2 (Air)	13	1.58	0.50	0.35	0.70	1.74	0.49	2.68
	THS2.2 (0.14)	13	1.75	0.75	0.35	1.32	1.59	0.12	3.38
	THS2.2 (0.25)	13	1.61	0.53	0.35	1.29	1.42	0.45	2.77
	THS2.2 (0.42)	9	1.91	0.26	1.60	1.94	2.53	1.31	2.51
48	3R4F (Air)	13	3.78	0.86	1.85	2.90	5.71	1.91	5.65
	3R4F (0.13)	12	5.83	2.18	0.35	3.52	6.06	1.03	10.64
	3R4F (0.25)	13	16.34	3.47	9.55	11.32	25.48	8.79	23.90
	THS2.2 (Air)	13	3.03	1.33	0.78	1.73	2.78	0.12	5.93
	THS2.2 (0.14)	13	2.44	0.53	1.46	2.49	2.87	1.27	3.60
	THS2.2 (0.25)	13	3.46	1.40	0.50	2.03	2.99	0.42	6.50
	THS2.2 (0.42)	9	5.28	2.98	1.92	2.37	2.64	−1.59	12.15
72	3R4F (Air)	13	6.76	3.85	1.63	2.89	4.43	−1.63	15.16
	3R4F (0.13)	12	5.22	1.42	0.87	3.20	9.78	2.08	8.35
	3R4F (0.25)	13	11.58	1.80	7.15	13.54	17.32	7.66	15.50
	THS2.2 (Air)	13	3.17	0.54	1.56	2.80	4.80	2.00	4.34
	THS2.2 (0.14)	13	2.70	0.49	1.27	2.50	4.46	1.64	3.77
	THS2.2 (0.25)	13	3.79	0.82	1.99	3.27	4.59	2.00	5.58
	THS2.2 (0.42)	9	3.93	0.66	2.35	3.90	5.22	2.42	5.45

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; NA, not applicable; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.34 **Supplementary Table 34.** Descriptive Statistics for sICAM1 Protein in the Basolateral Media

		Descriptive Statistics: sICAM1 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	127.69	71.35	52.76	64.28	73.18	−70.42	325.79
	TNFα+ILß	5	672.74	165.87	496.75	528.54	540.28	212.20	1133.29
4	3R4F (Air)	2	31.07	1.73	29.33	31.07	32.80	9.04	53.10
	3R4F (0.13)	2	25.26	1.48	23.78	25.26	26.74	6.44	44.08
	3R4F (0.25)	2	31.43	1.71	29.72	31.43	33.15	9.68	53.19
	THS2.2 (Air)	2	35.10	3.85	31.25	35.10	38.94	−13.81	84.00
	THS2.2 (0.14)	2	34.88	2.52	32.36	34.88	37.40	2.88	66.88
	THS2.2 (0.25)	2	24.06	4.83	19.24	24.06	28.89	−37.25	85.38
24	3R4F (Air)	13	199.44	63.52	50.53	107.59	200.77	61.04	337.84
	3R4F (0.13)	12	1004.04	478.69	63.79	190.43	1163.31	−49.55	2057.63
	3R4F (0.25)	13	2722.93	765.26	403.79	1890.89	3519.21	1055.58	4390.28
	THS2.2 (Air)	13	222.88	90.42	58.09	105.65	238.98	25.88	419.88
	THS2.2 (0.14)	13	159.71	52.40	61.32	100.02	153.41	45.54	273.88
	THS2.2 (0.25)	13	161.55	37.44	66.70	110.95	199.70	79.97	243.13
	THS2.2 (0.42)	9	166.92	41.70	79.55	122.74	216.39	70.76	263.09
48	3R4F (Air)	13	353.79	109.85	143.41	227.96	302.86	114.46	593.12
	3R4F (0.13)	12	1590.18	922.88	105.59	242.37	1696.03	−441.08	3621.44
	3R4F (0.25)	13	4634.38	1154.77	2315.41	3512.30	5216.29	2118.35	7150.42
	THS2.2 (Air)	13	332.86	66.98	140.35	263.04	511.13	186.92	478.80
	THS2.2 (0.14)	13	390.44	149.15	135.00	246.20	343.51	65.46	715.41
	THS2.2 (0.25)	13	360.68	92.15	154.92	313.54	333.44	159.90	561.47
	THS2.2 (0.42)	9	620.14	222.92	184.56	308.86	800.91	106.09	1134.19
72	3R4F (Air)	13	639.93	231.71	236.59	274.30	803.03	135.09	1144.78
	3R4F (0.13)	12	1091.31	494.72	218.18	279.89	1040.19	2.44	2180.19
	3R4F (0.25)	13	3880.99	788.12	2275.02	3448.57	6028.83	2163.82	5598.15
	THS2.2 (Air)	13	459.03	125.04	208.00	319.34	414.89	186.58	731.48
	THS2.2 (0.14)	13	456.62	109.23	193.82	323.00	621.14	218.61	694.62
	THS2.2 (0.25)	13	553.39	242.03	237.64	317.16	361.35	26.04	1080.74
	THS2.2 (0.42)	9	539.32	189.84	150.50	237.14	908.74	101.54	977.10

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.35 **Supplementary Table 35.** Descriptive Statistics for TIMP-1 Protein in the Basolateral Media

		Descriptive Statistics: TIMP-1 (pg/mL)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
0	PBS	5	13427.40	6214.40	5478.46	7386.74	12873.59	−3826.55	30681.35
	TNFα+ILß	5	12084.22	3617.13	6038.62	10489.62	13126.00	2041.45	22126.98
4	3R4F (Air)	2	2355.61	77.13	2278.49	2355.61	2432.74	1375.62	3335.61
	3R4F (0.13)	2	1609.62	194.86	1414.76	1609.62	1804.48	−866.30	4085.53
	3R4F (0.25)	2	875.01	329.32	545.69	875.01	1204.33	−3309.42	5059.44
	THS2.2 (Air)	2	2630.62	535.81	2094.81	2630.62	3166.42	−4177.45	9438.68
	THS2.2 (0.14)	2	2304.40	191.85	2112.56	2304.40	2496.25	−133.22	4742.03
	THS2.2 (0.25)	2	1870.74	225.54	1645.21	1870.74	2096.28	−994.95	4736.44
24	3R4F (Air)	13	9074.31	1610.97	5854.24	6862.17	8450.00	5564.30	12584.32
	3R4F (0.13)	12	13019.05	1803.72	8543.97	11591.38	15858.21	9049.08	16989.01
	3R4F (0.25)	13	14362.94	1307.80	12642.62	14512.75	17038.99	11513.50	17212.38
	THS2.2 (Air)	13	9754.58	2080.07	4208.79	8067.57	16198.95	5222.50	14286.67
	THS2.2 (0.14)	13	8825.23	1897.61	4649.64	5955.94	11000.21	4690.71	12959.76
	THS2.2 (0.25)	13	11604.18	1555.91	6832.95	12412.32	15354.61	8214.15	14994.21
	THS2.2 (0.42)	9	9832.65	1320.83	7166.93	8632.54	11055.96	6786.80	12878.50
48	3R4F (Air)	13	19831.62	3564.37	8419.82	14799.27	31277.35	12065.52	27597.71
	3R4F (0.13)	12	29078.22	1994.44	23210.04	28590.24	34744.90	24688.48	33467.96
	3R4F (0.25)	13	25836.21	1963.42	23400.51	24323.59	28559.10	21558.29	30114.12
	THS2.2 (Air)	13	17988.78	3101.37	9485.23	12738.84	20295.17	11231.48	24746.08
	THS2.2 (0.14)	13	17264.32	2992.15	10530.20	13208.33	21420.29	10744.99	23783.65
	THS2.2 (0.25)	13	21781.02	4414.20	12981.05	15998.61	23300.02	12163.30	31398.73
	THS2.2 (0.42)	9	18215.17	2582.49	14623.37	15180.98	19614.54	12259.95	24170.39
72	3R4F (Air)	13	26925.15	4421.24	13702.40	20979.58	35095.50	17292.10	36558.20
	3R4F (0.13)	12	56503.98	6723.85	35690.60	52001.77	74512.98	41704.90	71303.07
	3R4F (0.25)	13	48650.35	6400.68	29113.01	46127.31	70740.70	34704.48	62596.22
	THS2.2 (Air)	13	24056.59	3773.45	14560.11	18948.16	29405.82	15834.94	32278.24
	THS2.2 (0.14)	13	24730.34	4014.17	15519.11	19890.02	23974.36	15984.21	33476.47
	THS2.2 (0.25)	13	31221.44	4853.18	18814.77	26505.77	40104.75	20647.27	41795.62
	THS2.2 (0.42)	9	24631.02	4004.20	17801.04	20993.28	28211.71	15397.31	33864.73

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PBS, phosphate-buffered saline; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.1.36 **Supplementary Table 36.** Descriptive Statistics for Cytotoxicity (RLU) in the THS2.2 Aerosol Dose Range Assessment

		Descriptive Statistics: Cytotoxicity (RLU)							
PE (h)	Exposure Test	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
NA	Untreated	1	7961	.	7961	7961	7961	.	.
NA	TX-100	1	222018	.	222018	222018	222018	.	.
72	THS2.2 (Air)	3	6483	1132	5128	5591	8731	1614	11353
	THS2.2 (0.14)	3	8227	1254	6270	7847	10564	2831	13623
	THS2.2 (0.22)	3	10910	952	9014	11702	12015	6812	15008
	THS2.2 (0.37)	3	8343	1100	6626	8012	10392	3612	13075
	THS2.2 (0.55)	3	9397	1696	7288	8150	12753	2098	16696
	THS2.2 (0.77)	3	17156	3105	11948	16831	22689	3797	30515
	THS2.2 (1.02)	3	72460	19647	50900	54790	111691	-12076	156996
	THS2.2 (1.79)	3	184493	14525	168112	171906	213461	121995	246991

Abbreviations: LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; PE, post-exposure time point; Q1, first quartile; Q3, third quartile; SEM, standard error of the mean; THS, tobacco heating system; UCLM, upper confidence limit of the mean. The nicotine concentration in each test is given in parentheses.

14.2 Supplementary Figures

14.2.1 **Supplementary Figure 1.** Concentrations of the inflammatory mediators found in the basolateral media

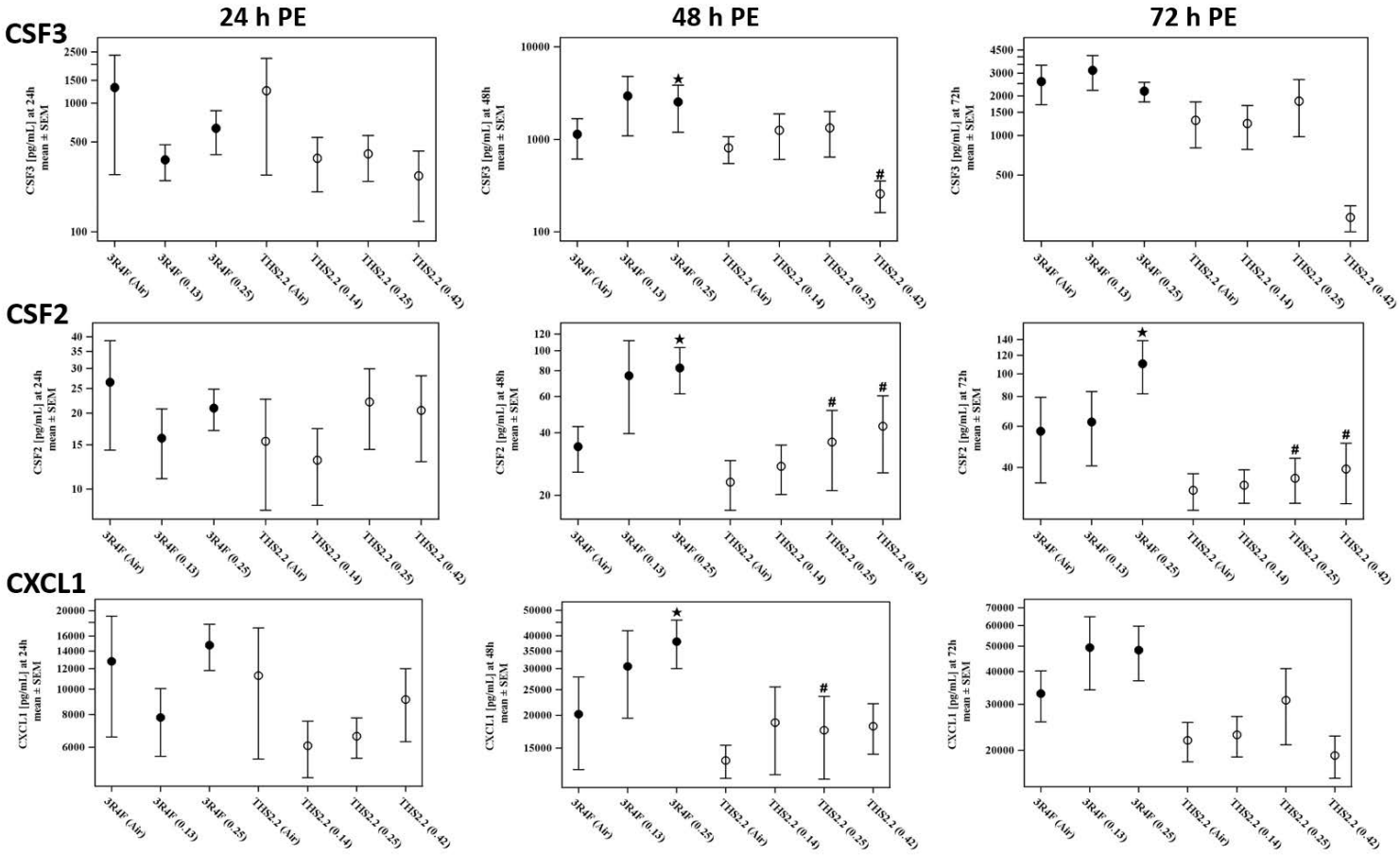


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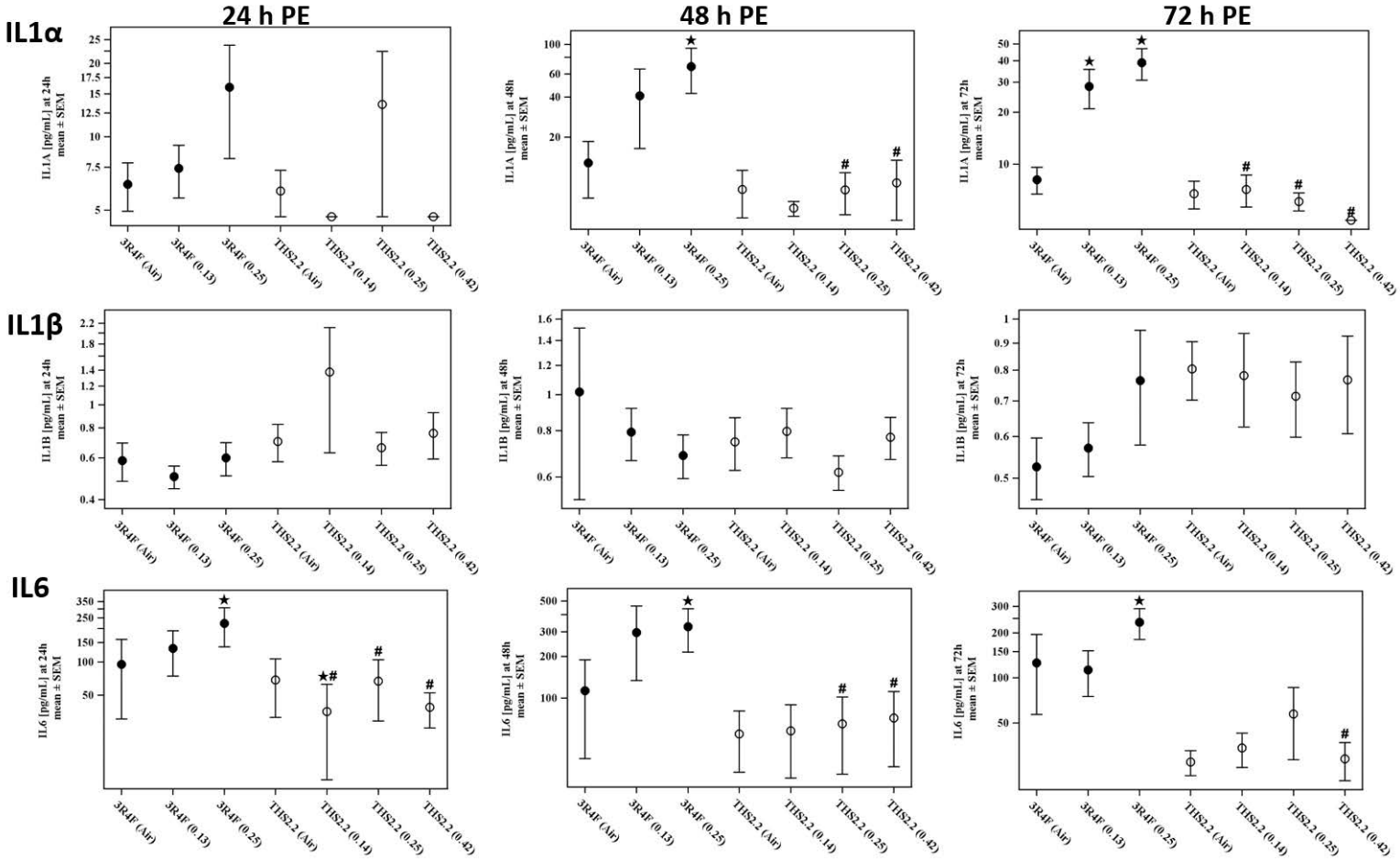


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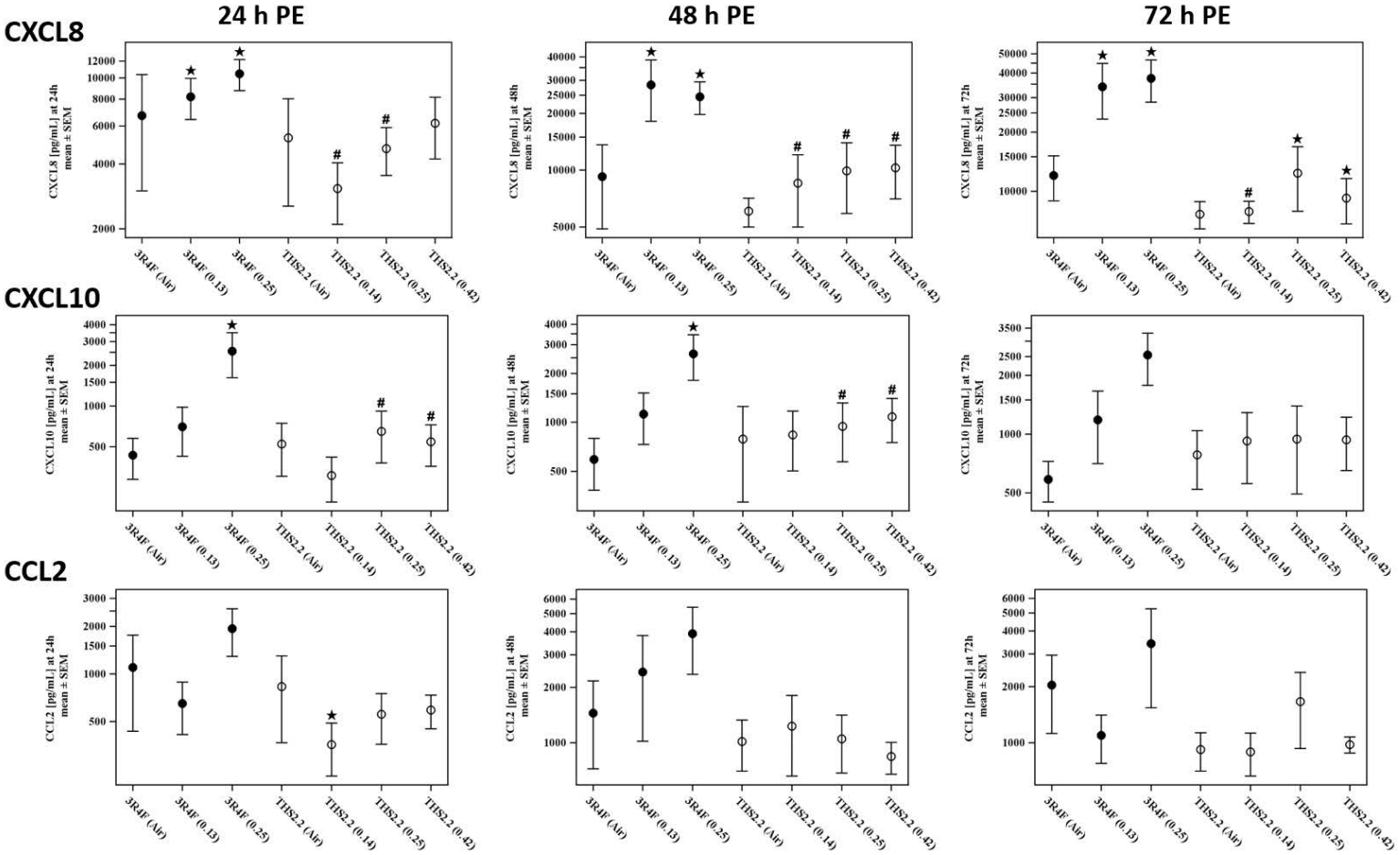


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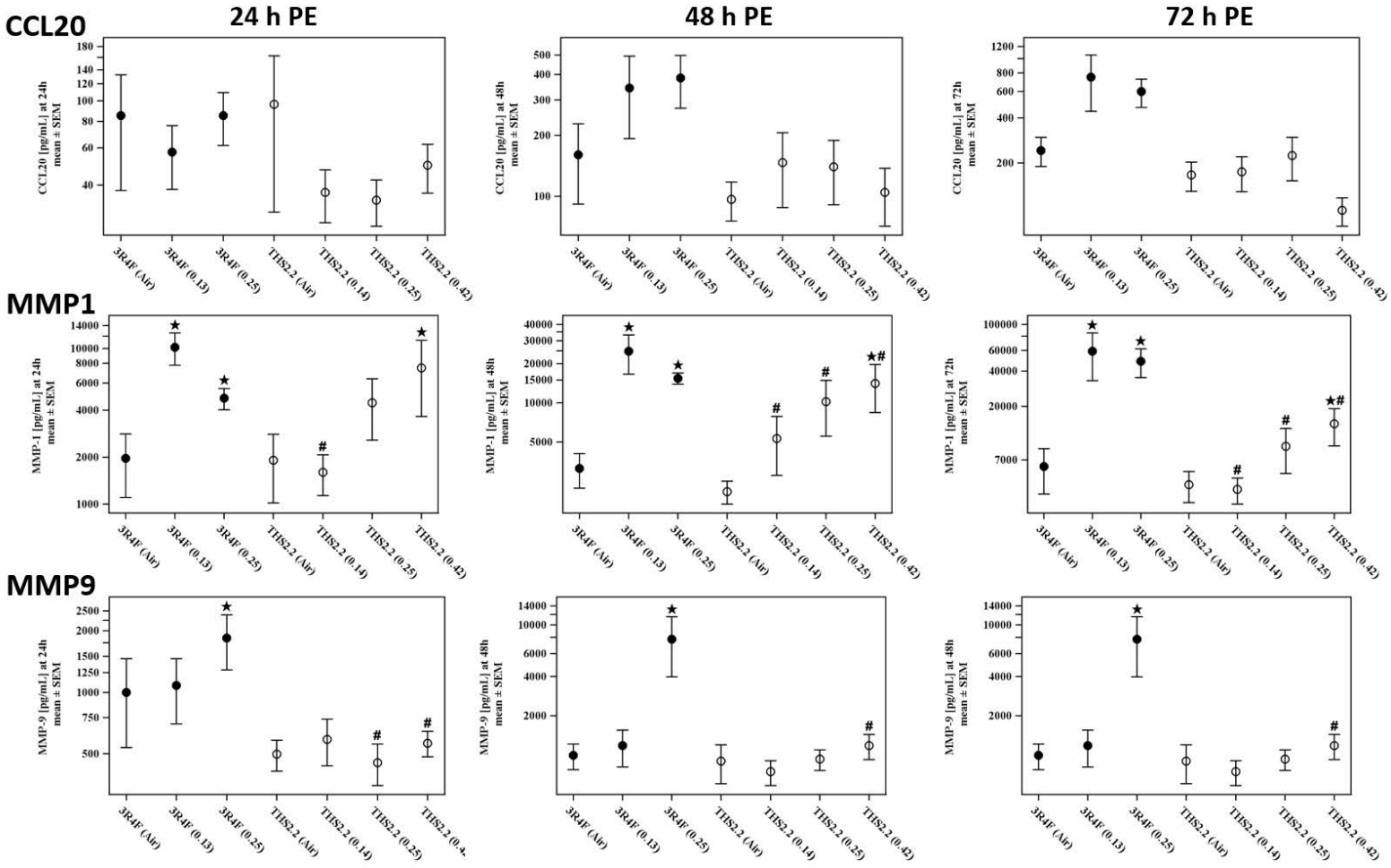


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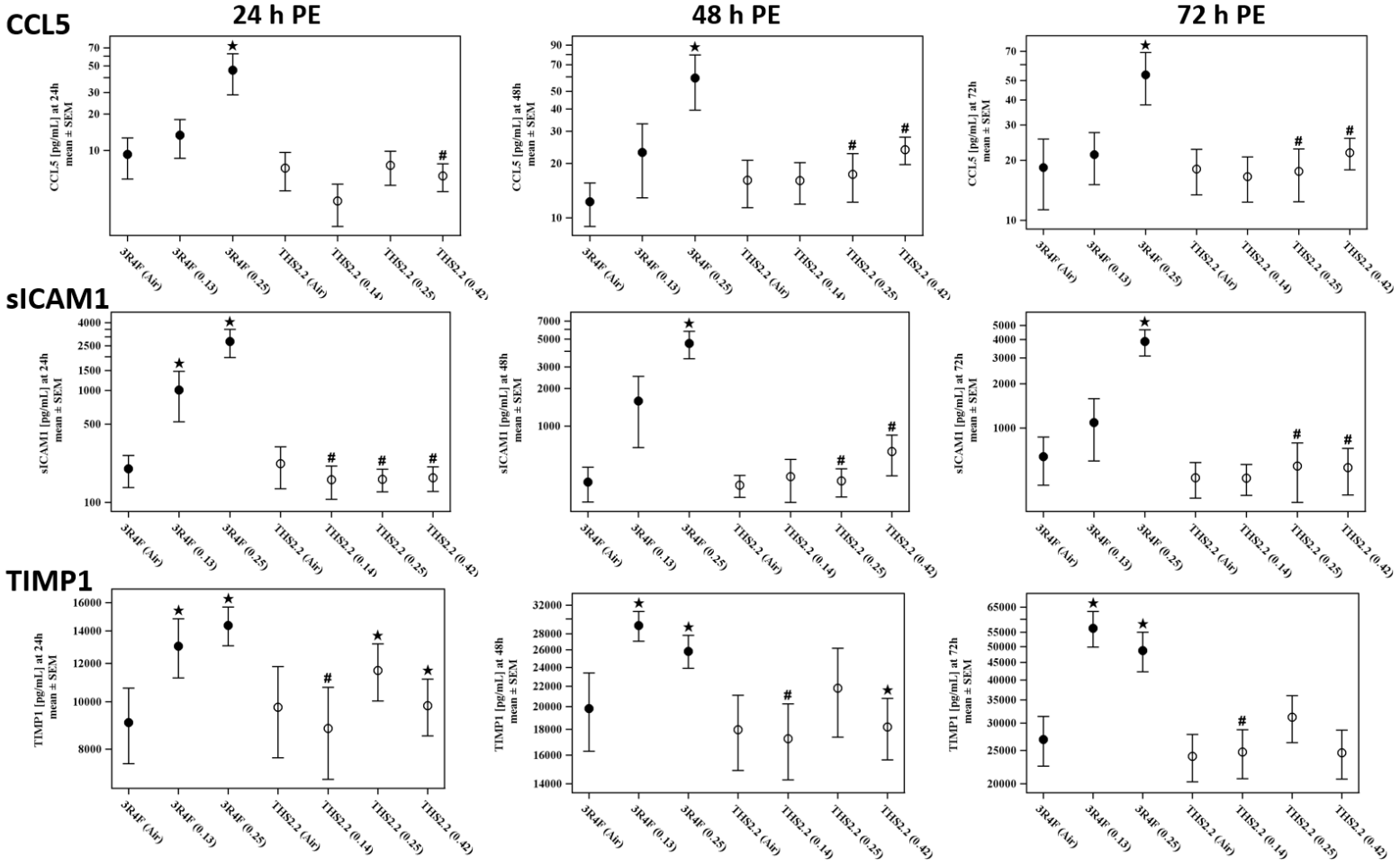


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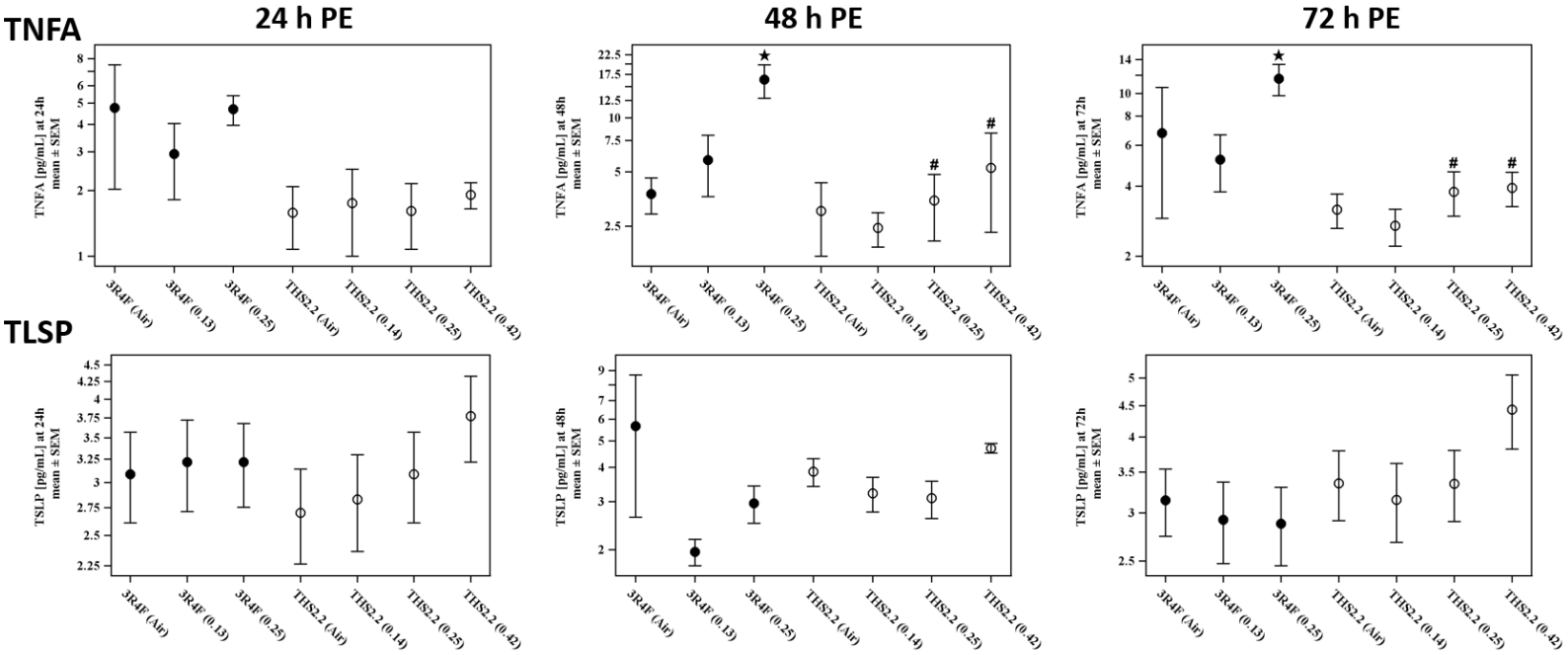
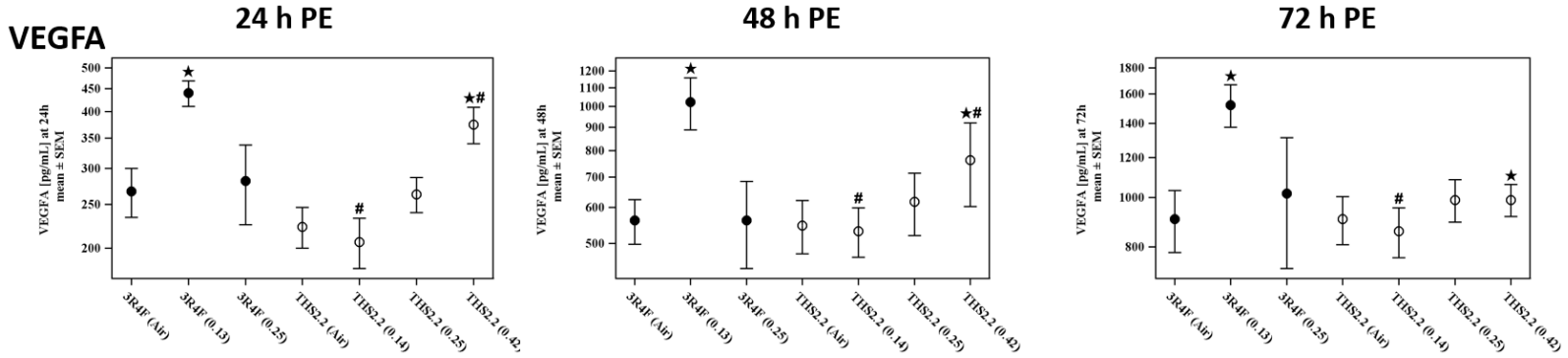


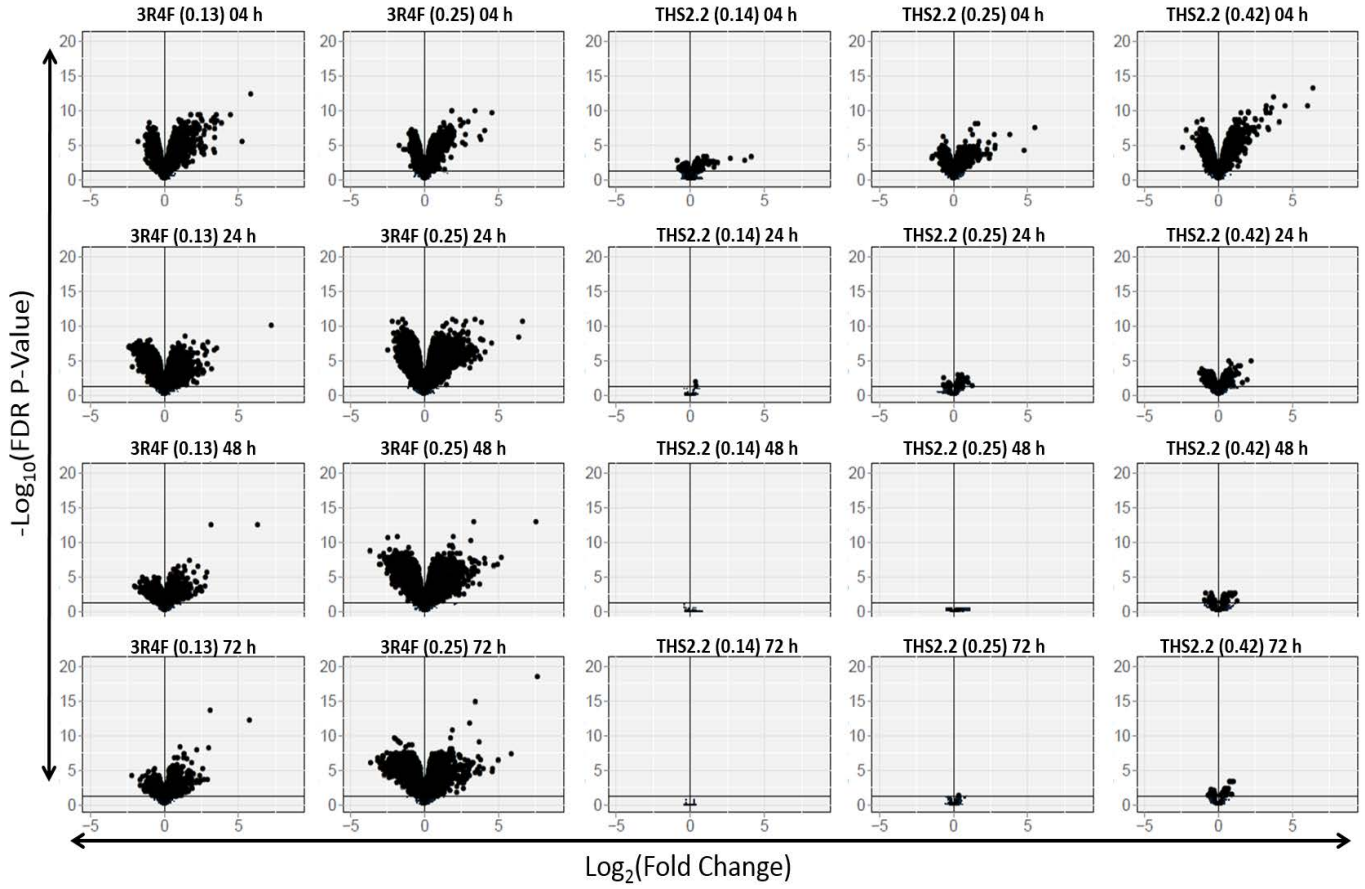
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Supplementary Figure 1. Concentrations of the inflammatory mediators found in the basolateral media

The mean concentration of each indicated mediator and the standard error of the mean (N = 9 to 13, in six experimental repetitions) are shown. A ⋆ symbol indicates that the value was significantly different from the corresponding air controls ($p < 0.05$). A # value indicates that the value was significantly different from the value for cultures exposed to 3R4F smoke at a comparable nicotine concentration ($p < 0.05$). Abbreviations: CCL, chemokine (C–C motif) ligand; CSF, colony stimulating factor; CXCL, chemokine (C–X–C motif) ligand; IL, interleukin; MMP, matrix metalloproteinase; SEM, standard error of the mean; sICAM, soluble inter-cellular adhesion molecule; THS, tobacco heating system; TIMP, tissue inhibitor of metalloproteinase; TNFA, tumour necrosis factor alpha, TSLP, thymic stromal lymphopoietin; VEGFA, vascular endothelial growth factor alpha.

14.2.2 **Supplementary Figure 2.** Volcano Plots of the Differentially Expressed Genes for All Experimental Groups Relative to the Air Controls



Supplementary Figure 2. Volcano plots of the differentially expressed genes

Each dot represents a gene in six experimental repetitions (N = 9 to 15). The change in gene expression (on the x-axis) is expressed as $\log_2(fold\ change)$, which increased when the value was > 0 and decreased when the value was < 0. The statistical significance (on the y-axis) is represented as the negative log10(adjusted p-value for the false discovery rate). The horizontal line shows the level at which the value was assumed to be statistically significant ($-\log_{10}(0.05)$)

14.2.3 **Supplementary Figure 3.** Gene-set Analysis of the Differentially Expressed Genes

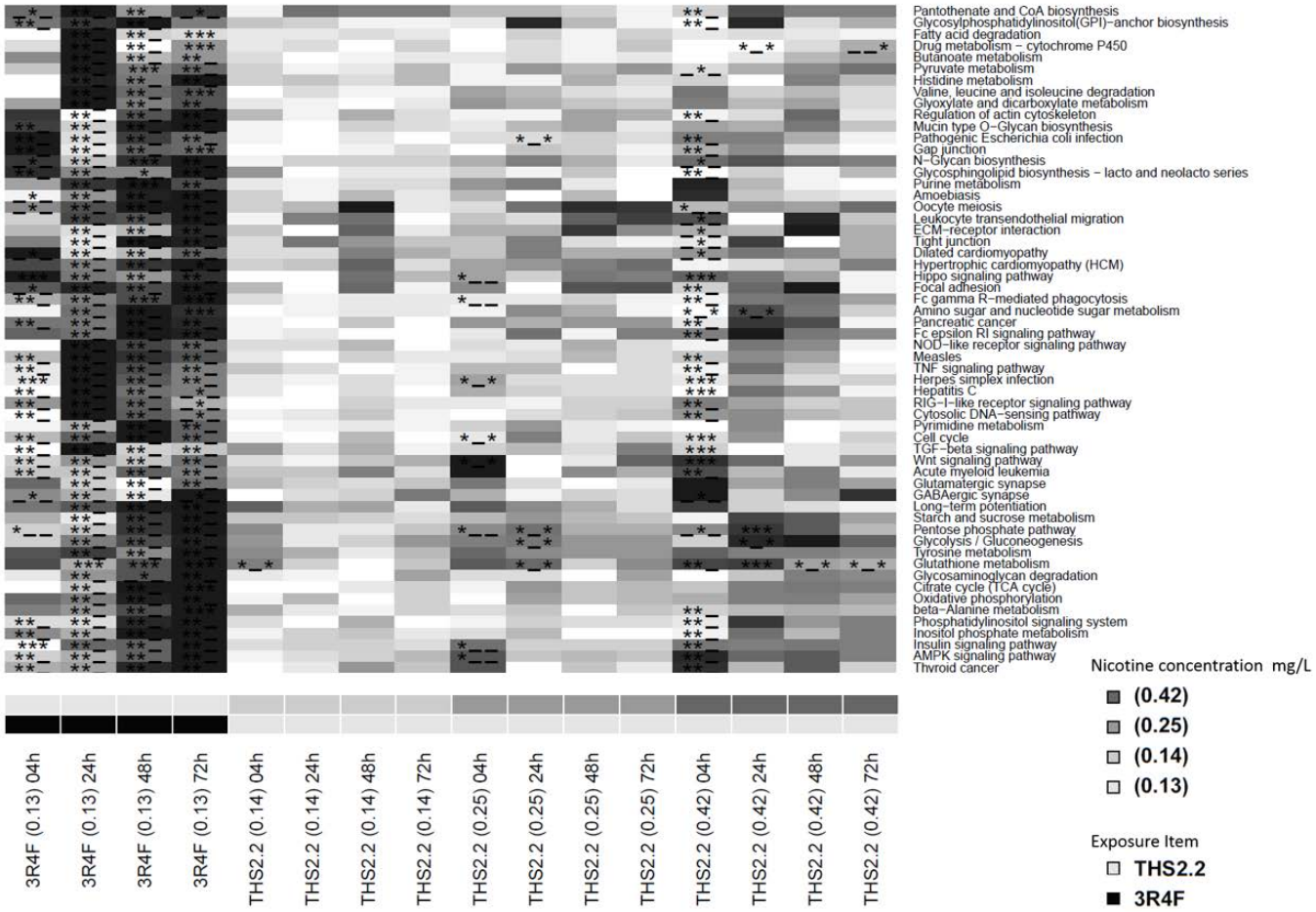


Figure continues

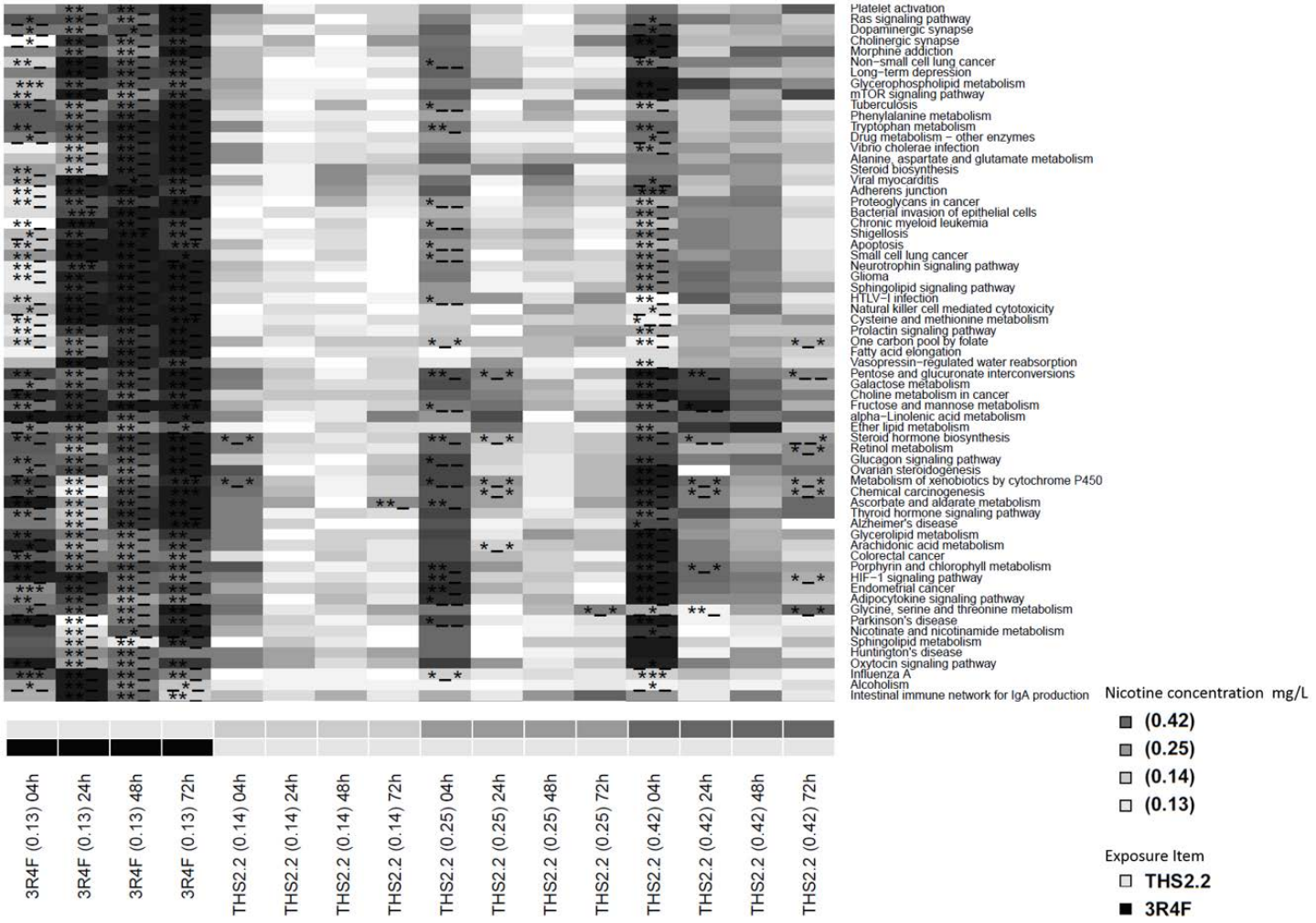


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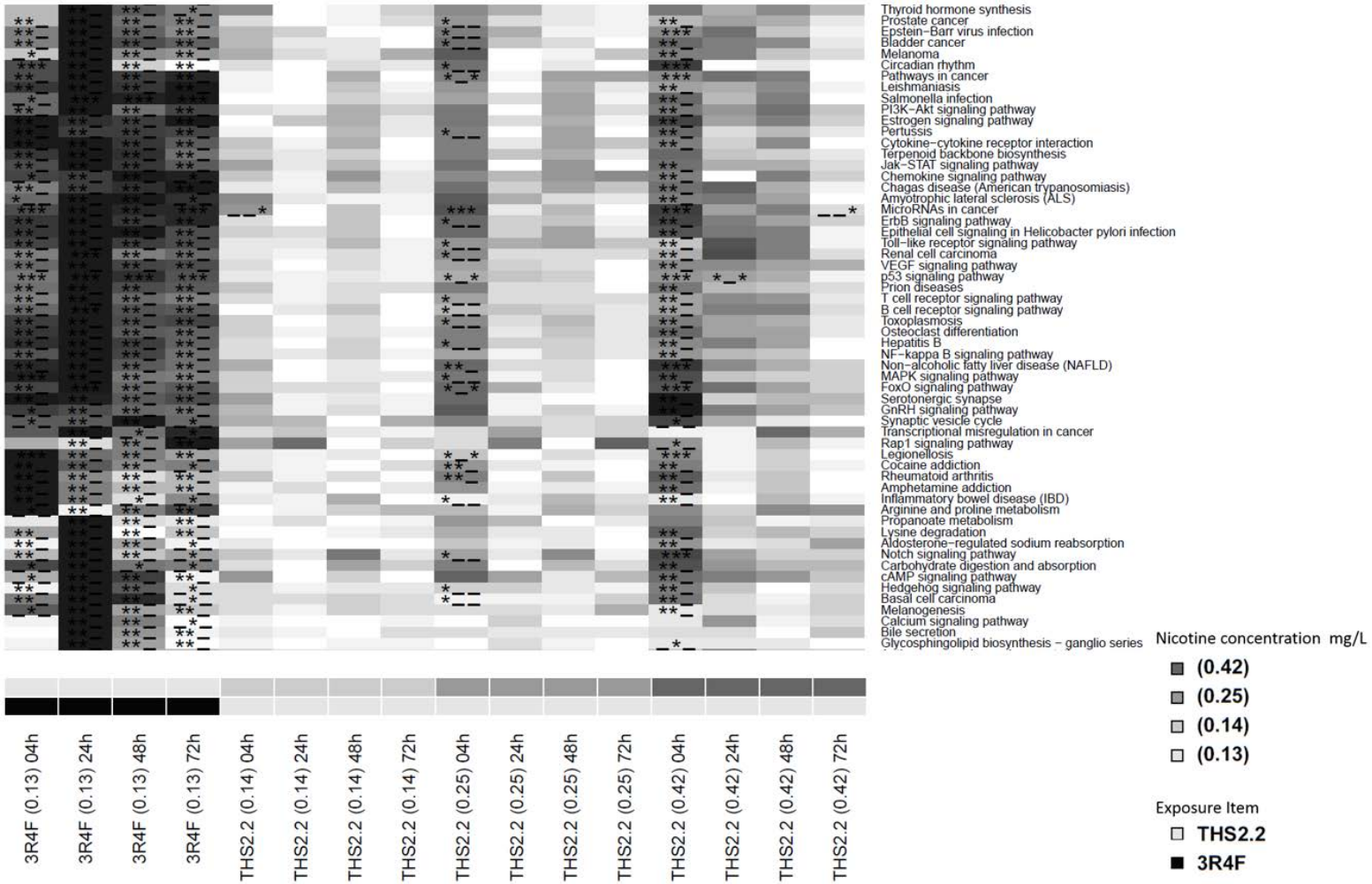
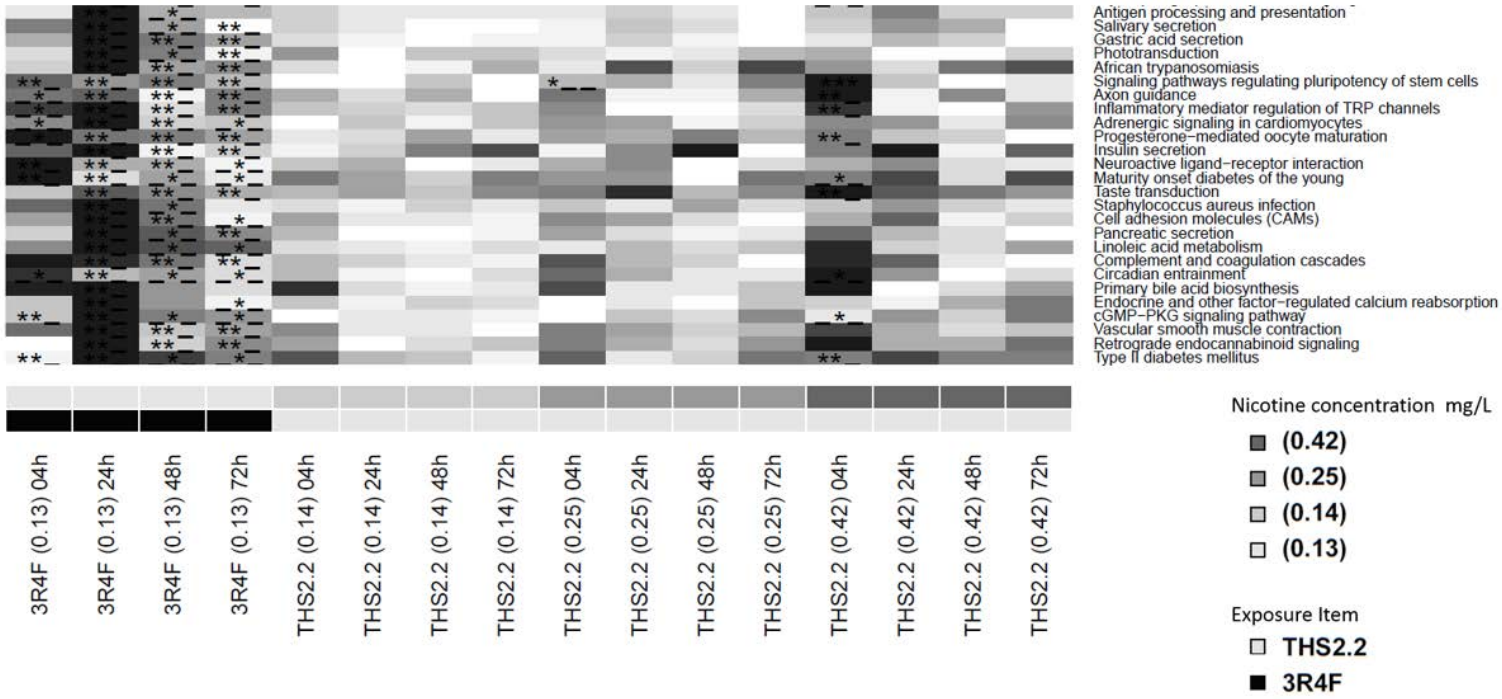


Figure continues



Supplementary Figure 3. Gene-set analysis results for the differentially expressed genes

Gene-set enrichment was assessed using over-representation analysis and gene-set analysis. Three types of significance (*) are marked for each annotation. The first character indicates the adjusted Fisher p-value, the second character indicates the gene-set analysis Q2 test, and the third character indicates the over-representation analysis test.

14.2.4 **Supplementary Figure 4.** Volcano Plots of the Differentially Expressed miRNA Results for All Experimental Groups Relative to the Air Controls

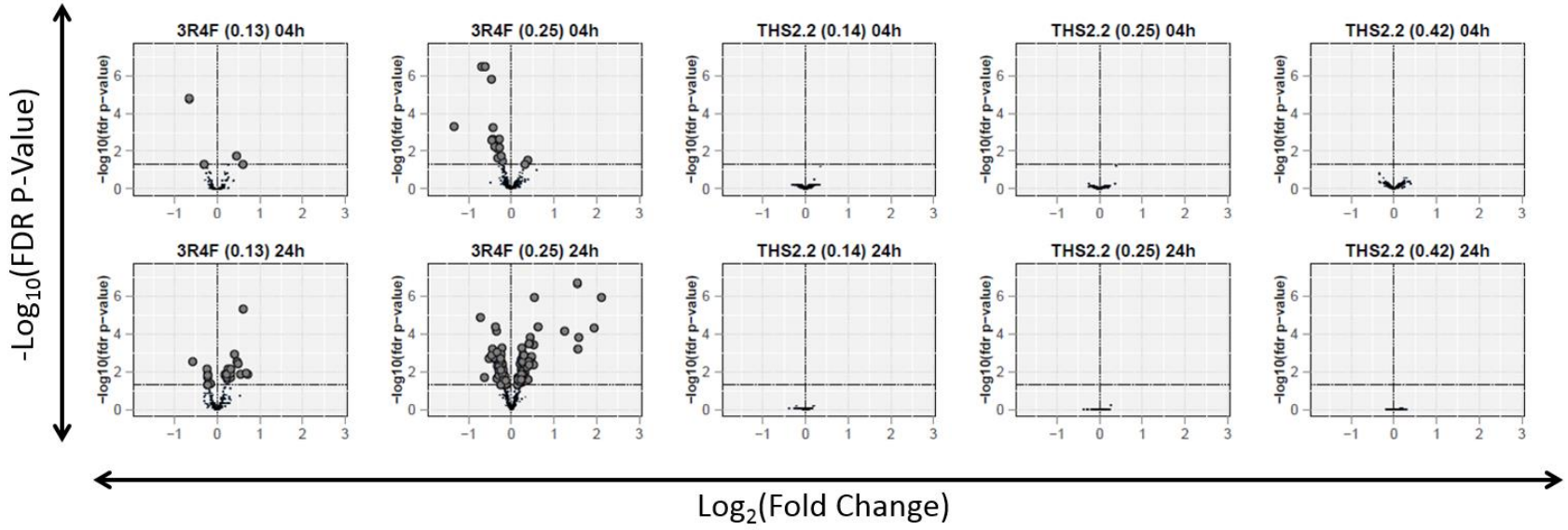
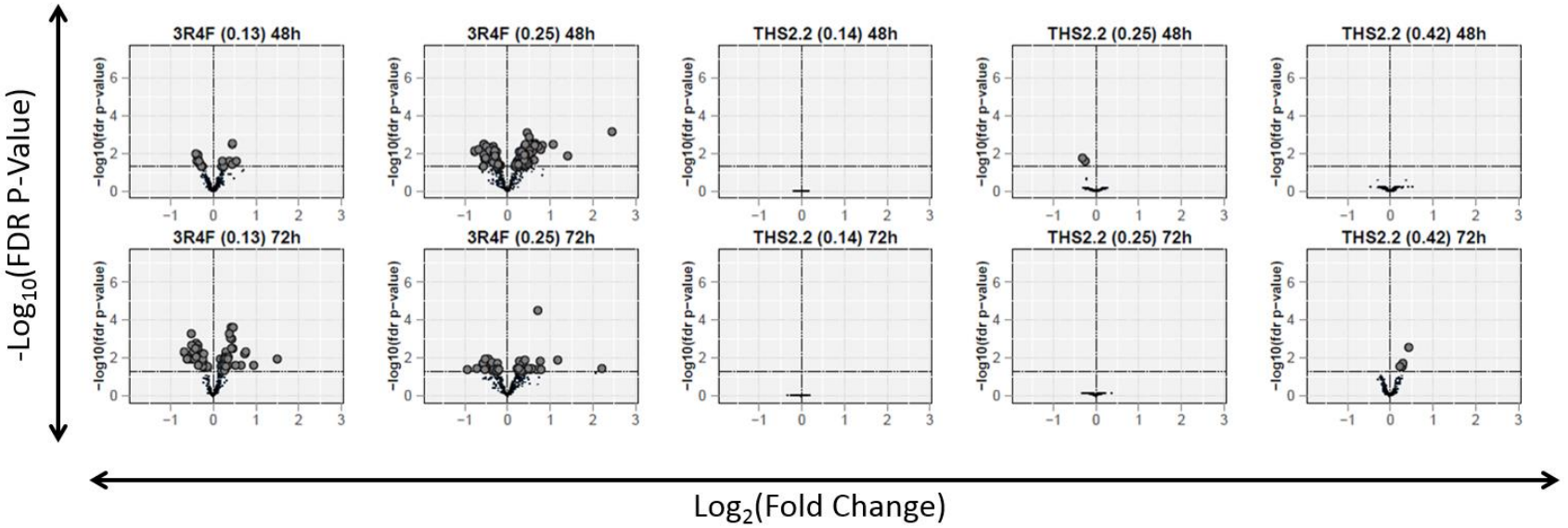


Figure continues



Supplementary Figure 4. Volcano plots of the differentially expressed miRNA results

Each dot represents the miRNA results produced in six experimental repetitions (N = 4 to 14). Changes in miRNA expression (on the *x*-axis) are expressed as $\log_2(fold\ change)$, which increased when the value was > 0 and decreased when the value was < 0. The statistical significance (on the *y*-axis) is shown as the negative $\log_{10}$(adjusted *p*-value for the false discovery rate). The horizontal line shows the level at which the value was assumed to be statistically significant ($-\log_{10}(0.05)$).

14.2.5 **Supplementary Figure 5.** Quantile–Quantile Plots of the Differentially Expressed miRNA Results for All Experimental Groups

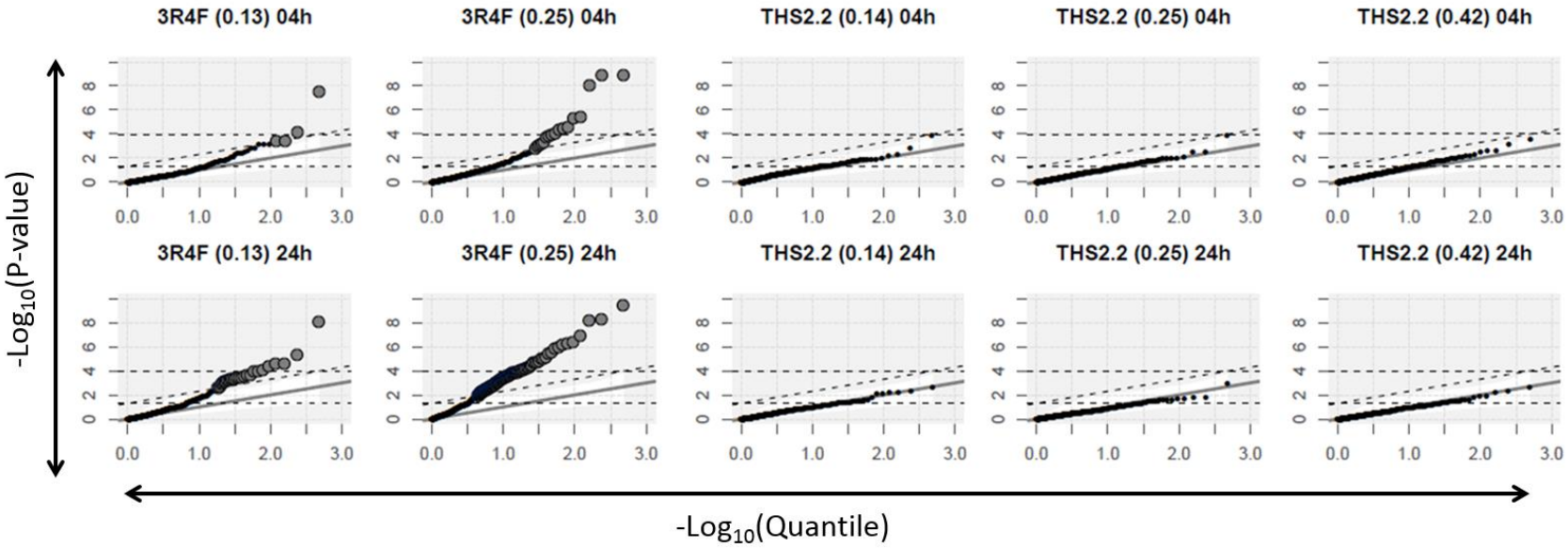
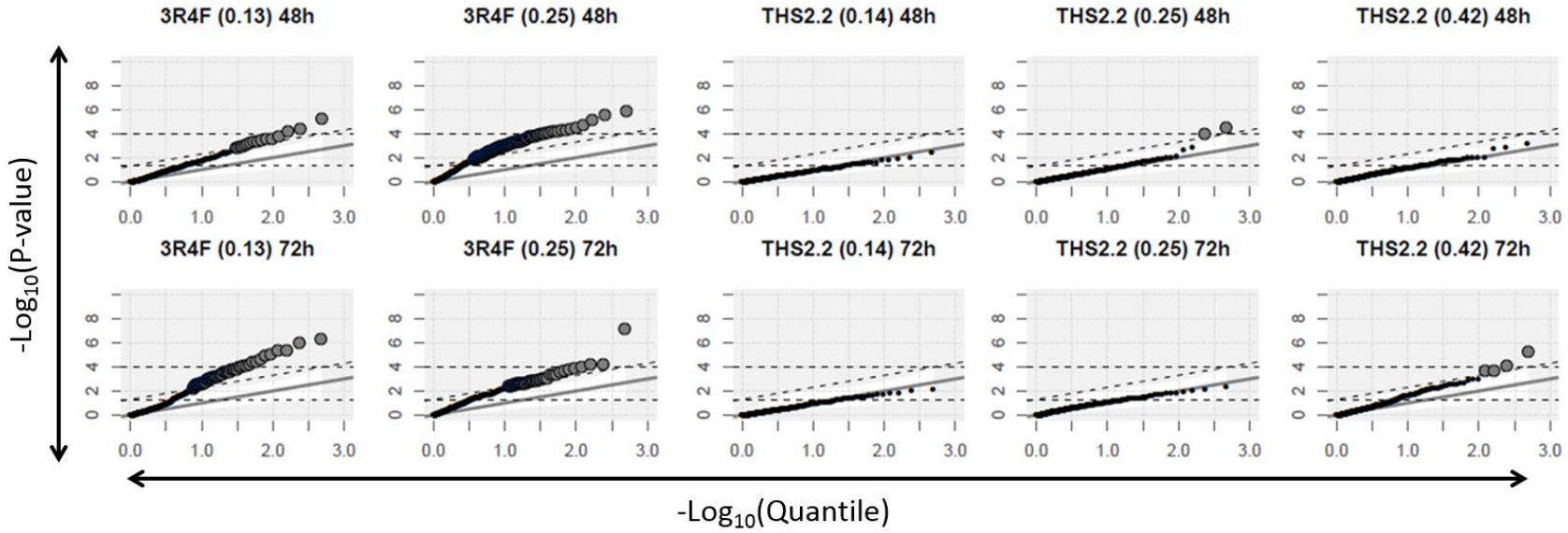


Figure continues



Supplementary Figure 5. Quantile-quantile plots of the significance profiles of the miRNA results

Each dot represents the miRNA results produced in six experimental repetitions (N = 4 to 14, except for the results for the single test that was performed using 3R4F smoke at a nicotine concentration of 0.20 mg/L, for which N = 3). The statistical significance (on the *y*-axis) is expressed proportionally as the negative $\log_{10}(raw\ p\ value)$. The quantiles are plotted on the *x*-axis. A miRNA was considered to be significantly altered if it fell above the oblique line (shown in grey).

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