STUDY REPORT

ASSESSMENT OF THE EFFECTS OF ACUTE EXPOSURE OF THS2.2 AEROSOL ON ORGANOTYPIC HUMAN BUCCAL CULTURES (STUDY NUMBER 174300)

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1. APPROVAL

1.1 Study Director's Statement of Compliance

Accountability for this study lies with the study director. The study director takes responsibility for the study having been executed as defined in the Study Plan (SP).
The laboratory heads take responsibility for the data generated in their labs.

Name	Date/Signature
Study Director: Filippo Zanetti	01-July 2016 Filippo Zanetti.
Lab Head, Cellular Research: Stefan Frentzel	01-Jul-2016 Stefan Frentzel
Lab 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: Alain Sewer	04.JUL.2016
Program Manager: Julia Hoeng	7-July 2016 Julia Hoeng

1.2 Quality Assurance Statement

Not Applicable. This study is a non-GLP study.

2. GENERAL INFORMATION

2.1 Names and Addresses

Sponsor	Manuel C. Peitsch Philip Morris Products SA Research & Development Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Philip Morris Products SA Research & Development PMI Product Testing Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Former Study Director(s)	- Experiment 1 (Repetition Phase (RP), also referred to as Main Phase 3): Anita Iskandar - Experiment 2 (also referred to as Main Phase 4): Anita Iskandar - Experiment 3 (also referred to as Main Phase 5): Anita Iskandar - Experiment 4 (also referred to as Main Phase 6): Filippo Zanetti - Dose Range Assessment (also referred to as DRA): Filippo Zanetti
Former Study Deputy Director(s)	- Experiment 1 (Repetition Phase (RP), also referred to as Main Phase 3): Carole Mathis - Experiment 2 (also referred to as Main Phase 4): Carole Mathis - Experiment 3 (also referred to as Main Phase 5): Carole Mathis, Filippo Zanetti - Experiment 4 (also referred to as Main Phase 6): Carole Mathis, Anita Iskandar - Dose Range Assessment (also referred to as DRA): Carole Mathis, Anita Iskandar

Other Scientists/Technicians involved in the study	Sam Ansari, Karine Baumer, Abdelkader Benyagoub, Flavia Buchler, Maica Corciulo, Remi Dulize, Philippe Alexandre Guy, Lola Hamied, Stephanie Johne, Samuel Kleinhans, Diana Kuehn, Shoaib Majeed, Maude Mayer, Celine Merg, Dariusz Peric, Fabio Talamo, Laura Ortega Torres, Gregory Vuillaume, Yang Xiang
Test Site Management (external analysis, delete if not applicable)	TPL Pathology
Principal Investigator for external analysis	Daniel Konrad

2.2 Study Schedule

Experiment start date:	25 April, 2014
Experiment completion date:	28 October, 2015

2.3 Test Guidelines

- Health Canada Intense 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 for the Vitrocell® system).
- International Organization for Standardization: International Standard ISO 3402, Tobacco and tobacco products – Atmosphere for conditioning and testing, 4th ed., 1999.

3. STUDY PLAN DEVIATIONS

The SP can be accessed at: 1743_Organotypic_Buccal_SP.
During the execution of study S174300, deviations from the SP occurred. They are summarized below **(Table 1)**.

Table 1. Study Plan Deviations

Number	Description	Justification of impact on study data and integrity
1	**Concerning the Test Item.** Deviation from the 1743_Organotypic_Buccal_SP, section 7.1.2. Conventional cigarette and Modified-risk Tobacco Product (MRTP). For the test item THS2.2, the batch of sticks mentioned in the SP was B-08164, but other batches were also tested during subsequent experimental phases. The reason for the change was having reached the expiration date of the batch of sticks. The batch information of the test item used in future experimental repetitions was updated accordingly when new batches were used. The reason for this change was to ensure the use of sticks before the expiry dates. The test item, also named in the SP ZRH_4.2 (Zurich) was also referred to as THS2.2 in the additional experimental repetitions. The reason for the change is to keep the terminology consistent across various studies.	The batch release certificates for each additional batch used in this study indicated no differences in the characteristics/features of the batch mentioned. No negative impact on data integrity.
2	**Concerning the Exposure System.** Deviation from the 1743_Organotypic_Buccal_SP, section 7.3.1. Exposure system. The smoking machine (SM) 2000 P1 in the Vitrocell Lab was replaced, because of technical problems, with the SM in the Aerosol Lab.	Both machines performed equally according to the quality controls standards. No negative impact on data integrity.
3	**Concerning the Test System.** Deviation from the 1743_Organotypic_Buccal_SP, section 7.4. Human Buccal organotypic culture. a. Donor G26 was chosen between the two tested in the DRF. The reason for the change was that donor G26 culture quality was good throughout the experimental week, while the cultures from the second donor were not. The overall impact of this change was that the study was conducted on donor G26. b. The procedure of producing the buccal culture was changed during the study's Main Phase 3 (referred as Experiment 1 in this report). The reason for the change was that cultures were aging too rapidly. The overall impact of the deviation was that the rapid aging was prevented, but Main Phase 1 and 2 were not incorporated in the final results. c. Greiner inserts (product 662640) replaced the inserts used previously (Corning, product 3413). The reason for the change was to eliminate the sub-membrane extra layer observed in the previous buccal inserts.	The changes were meant to improve the quality of the cultures, therefore no negative impact on data integrity.

Table continues

4	**Concerning the Exposure Design.** Deviation from the 1743_Organotypic_Buccal_SP, section 7.2.1 and from SP Amendment 1, section 2. The dilution of 3R4F cigarette smoke (CS) or THS2.2 aerosol mentioned in SP Amendment 1 was altered slightly during subsequent experimental phases to guarantee the same correlation between smoke/aerosol dilutions within the Vitrocell system. The reason for the change was the use of different batches of sticks over the whole study (see deviation 1, Test Item).	The adaptation of the smoke/aerosol dilution ensured the delivery of the same dose of nicotine and smoke/aerosol constituents throughout the study period, therefore not affecting the exposure conditions of the inserts.
5	**Concerning the Endpoints.** Deviation from the 1743_Organotypic_Buccal_SP, Appendix: Amendment 1, section 3. Main Phase. a. 48-h time point was added for adenylate kinase (AK) assay, multi-analyte profile (MAP), and gene expression (GEX) assessment. The reason for the change was to have the complete post-exposure (PE) time point analysis. Increasing the number of inserts together with the appropriate controls to accommodate endpoint analysis for an additional time point affects the organization/logistics of the smoke exposure, but not the integrity of the data. b. The CYP3A4 assay was excluded from the list of endpoints because it was not validated. The overall impact of this change was that the CYP3A4 assay is not included in the report. c. Most of the MAP analytes were either not detectable or were expressed at very low levels at the 4-h post-exposure time points. To avoid MAP analysis at an early post-exposure time point (which does not allow for a sufficient accumulation of analytes), samples collected 4 h after exposure were not analyzed after experimental phase 2. Low or undetectable levels do not permit conclusions regarding the early effects of acute exposure. Therefore, there was no impact on the study. d. Samples for histology were fixed for 1 h instead of 24 h as reported in the SP. The change was due to the same efficiency of 1-h and 24-h fixation time. This deviation resulted in no impact on the histology fixation. e. Histological evaluation of the cultures was shifted from the 24-h to the 48-h time point, because tissues did not show significant modifications at 24 h, but instead became visible from 48 h onwards. The impact of the deviation was to gain important information on the progression of histological modifications due to different smoke/aerosol exposures.	No negative impact on data integrity.

Table continues

6	**Concerning Study Timelines.** Deviating from the 1743_Organotypic_Buccal_SP (section 9.1)—in which the timeline of the study would be finished in 30-September-2014—, the completion of the study would be delayed because of the additional experimental repetitions that were conducted.	The additional experimental repetitions were planned to repeat the experiments and to increase the number of samples. Therefore, it was considered that these deviations would not have a negative impact on the data integrity.

End of the Table

4. ABSTRACT/EXECUTIVE SUMMARY

The oral mucosa is a membrane lining the oral cavity that interfaces anteriorly with the skin at the lips and posteriorly with the esophagus. Cigarette smoke (CS) exposure has been associated with cancer and inflammatory diseases of the oral cavity. As CS enters the organism through the mouth, the anatomical cytoarchitecture of the oral mucosa is essential in providing a barrier to counteract possible harmful consequences to the whole organism (Gualerzi 2012). For this reason, the use of an organotypic culture model, which retains the three-dimensional (3D) structure of the epithelium at the air–liquid interface (ALI) and resembles many of the features of the native oral human epithelium, is appropriate for *in vitro* research.

The current study aimed to investigate and compare the effects of mainstream smoke from reference cigarettes (3R4F) and aerosol from a Candidate Modified Risk Tobacco Product (MRTP), the Tobacco Heating System (THS) 2.2, on human oral organotypic epithelial cultures using well-known cell-based assays and systems toxicology approaches.

Human organotypic oral (buccal epithelium-derived) cultures were exposed to 3R4F CS and THS2.2 aerosol at comparable levels of nicotine for 28 minutes (acute exposure), and analyzed at different post-exposure time points for different endpoints. Histological examination, cytotoxicity, activity of cytochrome P450 (CYP) 1A1/1B1, and gene expression (mRNA and miRNA) were assessed, and data were generated at the various time points after exposure. A systems toxicological approach leveraging the gene expression data and biological network models was used to elucidate the biological impact of the exposure. In addition, a dose range assessment of THS2.2 was conducted to evaluate the likelihood and severity of adverse cytotoxic effects related to THS2.2 aerosol exposure.

The impact of THS2.2 aerosol at any of the concentrations tested was less than that of 3R4F CS. At the equivalent nicotine concentrations, adenylate kinase (AK) release-based cytotoxicity assays showed lower cytotoxicity after THS2.2 exposure compared with 3R4F exposure, which is consistent with the tissue histology evaluation. Additionally, greater numbers of secreted inflammatory mediators were found in the basolateral media of cultures exposed to 3R4F than in media of cells exposed to THS2.2 aerosol. Possible toxicity-related mechanisms associated with the exposure were investigated using a network approach and transcriptomics data. This analysis showed a greater perturbation of various biological networks in the 3R4F-exposed than in THS2.2-exposed cultures.

The results of the current study showed that THS2.2 exposure had less impact on the oral cultures than 3R4F exposure at equivalent nicotine concentrations. However, donor-specific factors associated with the culture model used in this study (e.g., genetic variations), that may have been undetected, yet could influence the outcome of the experiment, cannot be excluded from consideration, and could be addressed in future studies.

5. INTRODUCTION

The oral mucosa is involved in several functions in the mouth, including protection, sensation, secretion, and absorption. *In vivo* it is composed of two layers: the oral epithelium and a basal connective tissue layer termed the lamina propria. Many connective tissue papillae project into the epithelium at the interface between the two layers and function both as points of attachment and as part of the nutritive or sensory network of the mucosa. The epithelial surface is covered by a moist liquid layer, which is supplied by three major salivary glands, and contributes to the various functions of saliva such as lubricating food and surfaces during mastication and swallowing mediated by statherin, glycoproteins and mucins, digesting food polysaccharides (amylases), and supporting the barrier properties of the oral mucosa by its component mucins, histatins, and defensins—peptides with known antimicrobial and antifungal properties (Carpenter 2013).

Cigarette smoke (CS) exposure has been associated with oxidative stress, inflammation, and genetic and epigenetic alterations throughout the respiratory tract (Goldkorn 2014). For example, smoking was found to alter the expression of genes involved in xenobiotic metabolism, oxidant stress, eicosanoid synthesis, nicotine signaling, and cell adhesion (Boyle 2010). Multiple genes encoding enzymes (*CYP1A1, CYP1B1, AKRs, ALDH3A1, NQO1, UGTs*) involved in carcinogen metabolism were overexpressed in the oral mucosa of smokers (Boyle et al. 2010). Smoking and other forms of tobacco use are also associated with oral cancer (Winn 2001) and a plethora of different diseases, such as periodontal disease (e.g., gingivitis) (Johnson 2000). Smoking can also modify the number of cells with early and late apoptotic features in the oral epithelium (Michcik 2014). CS can induce changes in gene expression in oral epithelia similar to those seen in bronchial and nasal epithelia, suggesting a common airway response to smoking-induced injury (Sridhar 2008b). The "field of injury" hypothesis indicates common CS-related changes in gene expression that occur throughout the respiratory tract despite the frequent absence of noticeable histological/phenotypical changes (Gower 2011, Spira 2007, Sridhar 2008a, Steiling 2008). This hypothesis could potentially be used to develop non-invasive biomarkers of CS exposure in the easily accessible oral epithelia (Boyle 2010, Kupfer 2010, Paszkiewicz 2008, Spivack 2004).

As CS enters the organism through the mouth, the preservation of the anatomical cytoarchitecture of the oral mucosa is essential in providing a barrier to counteract possible harmful consequences to the whole organism (Gualerzi 2012). Organotypic three-dimensional (3D) cultures reconstituted from the cells of the oral cavity, such as 3D oral mucosal cultures (MatTek), have characteristics resembling the native oral epithelia. Thus, they are suitable for studying biological processes in the oral mucosa, including those related to innate immunity, that frequently occur in gingivitis, candidiasis, oral cancer, and inflammatory oral diseases (Andrian 2004, Ceder 2007, Hansson 2001, Klausner 2007, Mostefaoui 2002, Moyes 2010, Walle 2006, Wang 2001). These cultures are superior to conventional 2D oral epithelial cell cultures, because they retain the 3D structure typical of the native epithelium; they are grown on a permeable porous membrane, at the air–liquid interface (ALI), allowing for direct exposure to whole smoke (aerosol) at the apical side (whereas the basolateral side is submerged in culture medium). Moreover, a 3D system allows cells to more readily form natural cell-to-cell attachments with one another, including tight junctions, adherens junctions, and desmosomes that build up a paracellular barrier against uncontrolled diffusional substance influxes, as well as gap junctions that enable intercellular communication via exchange of

ions, small molecules, and electrical currents. Binding of surface adhesion molecules to relevant receptors is facilitated by close proximity of neighboring cells, which enables signaling that is important for proper cellular function, division, and differentiation. 3D culture conditions restored ability of cells to polarize, which altered proliferation rate, adhesion structures, and transcription of adhesion molecules.
In addition, the commercial availability of human-derived organotypic cultures (MatTek) from different donors in a high-throughput format guarantees reproducible use for long time periods (BeruBe 2009).
Compared with 2D cultures, 3D organotypic cultures provide a convenient, accessible, and more accurate representation of *in vivo* cellular processes, including proliferation, differentiation, adhesion, intracellular communication, and transcription, without the controversy and cost of *in vivo* animal studies.

The 3D EpiOral™ culture model (MatTek Corp.) used in this study consists of normal human oral epithelial cells. This model exhibits morphological and growth characteristics similar to the *in vivo* situation. The cells have been cultured to form multilayered, highly differentiated models of the human buccal mucosa, consisting of an organized basal layer and multiple non-cornified layers analogous to native human buccal epithelium (the full-thickness model containing the fibroblast layer was not used for this study). The EpiOral model expresses cytokeratin K13 in the upper part of the culture and cytokeratin K14 in the lower layers (Kimball 2006, Presland 2000). The EpiOra mucosal model has been used to measure viability and cytokine release measurements in response to oral care excipients and prototype formulations. They also produce the naturally occurring antimicrobial peptides, e.g., human beta defensins (HBD) such as HBD-1 and HBD-3, but not HBD-2. Lipid analysis showed the presence of only ceramide 2, similar to what is observed in the *in vivo* situation. The *in vivo*-like morphological and growth characteristics are uniform and highly reproducible (Kimball 2006, Klausner 2007, Mattek 2012). Klausner et al. reported that EpiOral cultures exhibited differential cellular responses such as cytokine release and altered viability following exposure to commercially available oral care products (Klausner 2007).
Importantly, these cells are cultured at the ALI, which mimics the aerosol exposure of the *in vivo* respiratory system (BeruBe 2009), making it a gold standard for aerosol/smoke and particle exposure studies (Klausner 2007).
In a previous publication (Schlage 2014), it was shown that EpiOral cultures responded to CS exposure at the ALI with increased secretion of inflammatory mediators, induction of cytochrome P450s activity, and an activation of gene expression patterns (mostly resembling those of the cell stress and senescence network) similar to those induced in buccal biopsies of smokers.

Collectively, these data indicate that human oral organotypic culture is a relevant *in vitro* model for assessment of new tobacco products using a systems toxicology approach.

6. OBJECTIVES

The objectives of the study were:

To determine the biological impact of THS2.2 aerosol exposure on a human organotypic 3D oral culture model, as compared with that of 3R4F smoke. The biological impact assessment was done by combining results obtained using well known cellular and functional assays, and those obtained 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 oral 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 oral cultures were observed.

All the above objectives were exploratory.

7. EXPERIMENTAL DESIGN

7.1 Test and Reference Items

7.1.1 Identification and Description

THS2.2 and 3R4F were regarded as test and reference items, respectively **(Table 2)**.

Table 2. Test and Reference Items

Short Name	Description	Type
THS2.2	ZRH/DDA1/C3/CAST LEAF– CL/Flavor/Reynaldo	Test
3R4F	Conventional cigarettes	Reference

Information on the test item characteristics, including composition, stability, and homogeneity, was provided by the sponsor.

The 3R4F reference cigarette was purchased from the University of Kentucky (Kentucky Tobacco Research & Development Center). The specifications of the test and reference items used in this study are described in **Table 3**.

Table 3. Reference and Test Items Used in Experimental Repetitions

Week of Experiment	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Dose Range Assessment
	13 April, 2015	15 June, 2015	13 July, 2015	10 August, 2015	10 August, 2015
Reference Item (3R4F)					
Stick Information on 3R4F					
Pack No.	V350Y61B5	V350Y61B5	V350Y61B5	V350Y61B5	V350Y61B5
Receiving Date	December 2014	December 2014	December 2014	December 2014	December 2014
Expiry Date	May 2017	May 2017	May 2017	May 2017	May 2017
Test Item (THS2.2)					
Stick Information on THS2.2					
Stick Batch No.	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
Receiving Date	24 June, 2014	24 June, 2014	24 June, 2014	24 June, 2014	24 June, 2014
Expiry Date	24 June, 2015	24 June, 2015	January, 2016*	January, 2016*	January, 2016*
Device Information on THS2.2					
Product Code	B-178731	B-178731	B-178731	B-178731	B-178731
Receiving Date	27 February, 2015	27 February, 2015	27 February, 2015	27 February, 2015	27 February, 2015
Specific Description	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. Aerosol generation.				

*The batch was re-tested in January 2016 to assess its stability. The Analytical Report for Batch Release No. B-13055 confirms that the batch was stable for the whole remaining experimental period. Information on the 3R4F reference item is provided by the supplier (Kentucky Tobacco Research & Development Center).

7.1.2 Item Storage

After the items were received, they were stored in a refrigerator at 5 ± 3°C with uncontrolled humidity conditions in their original packaging.

7.1.3 Item Conditioning

All test items were conditioned between 7 and 21 days under controlled conditions at a temperature of 22 ± 1°C and a relative humidity of 60 ± 3%, according to ISO 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 representative carbonyls deposited in the Base Module of the Vitrocell® 24/48 exposure system following exposure to the reference cigarette smoke and test item aerosols at the corresponding target nicotine concentrations used in this study (i.e., the concentration applied in the four experimental repetitions described in section Sample Generation: Experimental Repetitions) The descriptive statistics that report the values obtained in every sampling are given in **Supplementary Table 1–Supplementary Table 8**.

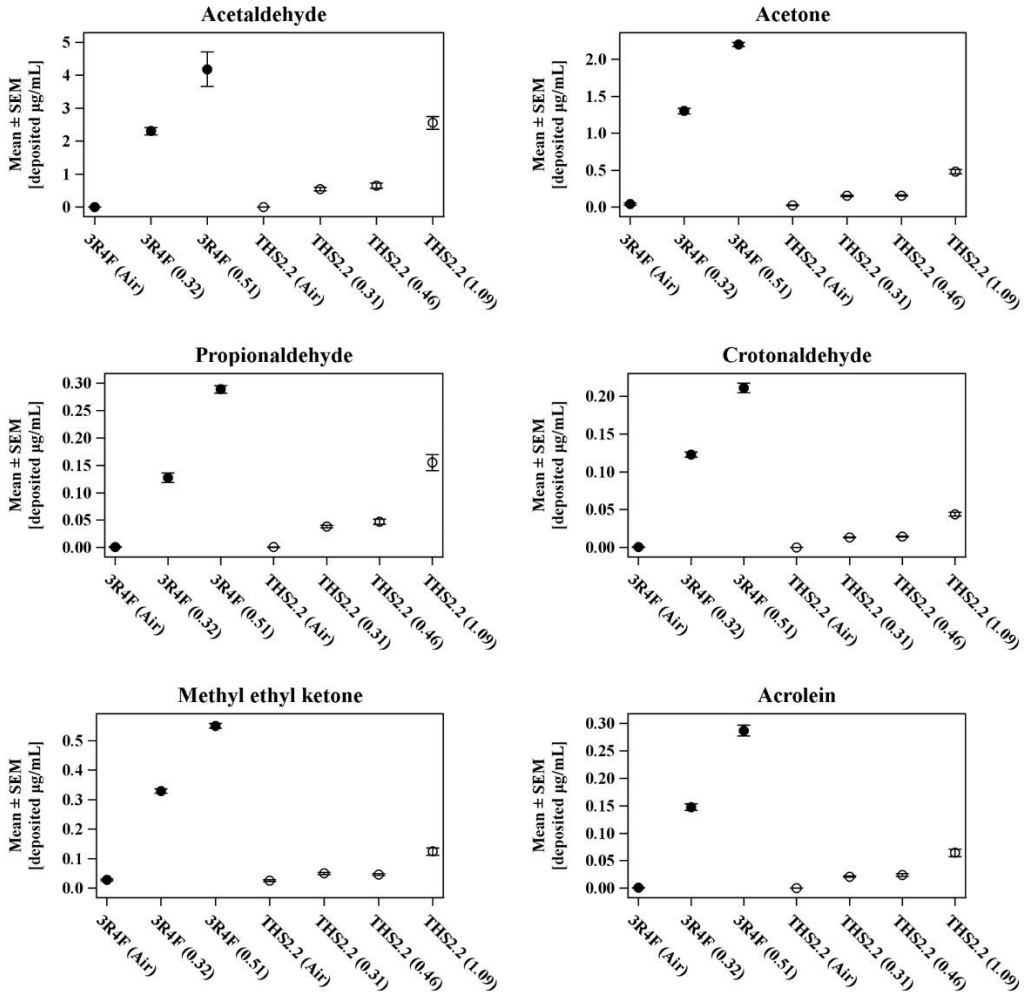


Figure 1. Concentrations of representative carbonyls deposited in the Cultivation Base Module of Vitrocell 24/48 exposure system following exposures to the reference cigarette smoke and test item aerosol.

The mean of a carbonyl in PBS-filled inserts (µg/mL) ± SEM (N = 9 samplings from 2014-2015) are shown. The PBS-filled inserts were placed in the Cultivation Base Module of Vitrocell 24/48 exposure system and exposed to 3R4F smoke or THS2.2 aerosol according to the concentrations used in this study. Representative carbonyls are shown. The descriptive statistics are given in **Supplementary Table 1–Supplementary Table 8**. Abbreviations: N, number of samples; SEM, standard error mean.

7.2 Test System

The human buccal organotypic EpiOral culture (product code ORL-200-PE6.5-7d, MatTek Corp., Ashland, MA, USA) was used as the test item for this study. The EpiOral cultures were grown in a 6.5-mm Transwell® insert with a 0.4-µm polyester sterile membrane (Corning, product 3413 for experiments 1 and 2; Greiner, product 662640 for experiments 3 and 4). They were differentiated upon arrival, over the weekend, in EpiOral differentiation media (roduct ORL-200-DM4a, MatTek Corp.), containing EpiOral media supplement (product ORL-200-SUPP, MatTek Corp.). Prior to exposure, the medium was replaced with a maintenance medium (product ORL-200-MM-SUPP, MatTek Corp.). The study was performed using cultures derived from the same donor. **Table 4** summarizes the information provided in the certificates from the supplier.

Table 4. Specifications of the different batches of oral culture used in the experimental repetitions

	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4 and Dose Range assessment**
Week of Experiment	**13 April, 2015**	**15 June, 2015**	**13 July, 2015**	**10 August, 2015**
Culture Delivery Date	10 April, 2015	12 June, 2015	10 July, 2015	7 August, 2015
Lot No.	21158	21178	21185	21193
Cell Type	NHOC/Oral	NHOC/Oral	NHOC/Oral	NHOC/Oral
Age of Donor	46	46	46	46
Sex of Donor	Male	Male	Male	Male
Origin of Donor	NA	NA	NA	NA
Smoking Status of Donor	Non-smoker	Non-smoker	Non-smoker	Non-smoker
Pathology Status of Donor	None reported	None reported	None reported	None reported
Sterility	Yes	Yes	Yes	Yes
Mycoplasma	NA	NA	NA	NA
Morphology	Normal (sub-membrane extra layer)	Normal (sub-membrane extra layer)	Normal	Normal
HIV-1	Negative	Negative	Negative	Negative
Bacteria, yeast, fungi, antimycotic-free culture	Negative	Negative	Negative	Negative
Hepatitis B	Negative	Negative	Negative	Negative
Hepatitis C	Negative	Negative	Negative	Negative
Certificate of Analysis (CoA)	CoA_Experiment 1	CoA_Experiment 2	CoA_Experiment 3	CoA_Experiment 4

On each delivery day, we monitored the quality of the buccal culture batch by analyzing hematoxylin and eosin-stained tissue sections of incubator controls (data not shown). Abbreviations: NHOC, normal human oral cells; NA, not available.

7.3 Sample Generation

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

The study to assess the biological impact of THS2.2 aerosol as compared with 3R4F smoke exposure on a human 3D oral culture model comprised of four experimental repetitions. During each of the four experimental repetitions, the cultures were exposed to the reference item 3R4F smoke or test item THS2.2 aerosol at similar nicotine concentrations in the 3R4F smoke/ THS2.2 aerosol **(Table 5)**. Smoke from the reference item (3R4F) was generated using a 30-port-carousel smoking machine (SM)-2000 (Philip Morris International, Neuchâtel, Switzerland). Aerosol from the test item (THS2.2) was generated using a modified 30-port carousel smoking machine SM-2000 THS2.2 (Philip Morris International). Each of the smoking machines was connected to a Vitrocell® 24/48 exposure system (Vitrocell Systems GmbH, Waldkirch, Germany), where the culture inserts were exposed.

Table 5. Target Nicotine Concentrations in the Smoke/Aerosol (mg nicotine/L) Applied in the Study

	Experiment Week				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	
	13 April, 2015	15 June, 2015	13 July, 2015	10 August, 2015	
Group name of 3R4F-exposed Cultures	**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	SM-2000
3R4F (0.32)	0.32	0.33	0.29	0.35	
3R4F (0.51)	0.54	0.48	0.49	0.54	
Exposure Run Number	3	3	3	3	
Group name of THS2.2-exposed Cultures	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**mg nicotine/L smoke**	**Smoking Machine**
Air Control (THS2.2)	0.00	0.00	0.00	0.00	SM-2000 THS2.2
THS2.2 (0.31)	0.29	0.34	0.27	0.32	
THS2.2 (0.46)	0.48	0.48	0.43	0.45	
THS2.2 (1.09)	1.08	1.02	1.14	1.13	
Exposure Run Number	3	3	3	3	

Abbreviations: SM, smoking machine.

The Vitrocell exposure system is equipped with a dilution system. To achieve the desired concentrations of nicotine, the smoke/aerosol was diluted with fresh air. **Figure 2** illustrates the setup of the exposure run for 3R4F and THS2.2, applying the nicotine concentrations described in **Table 5**.

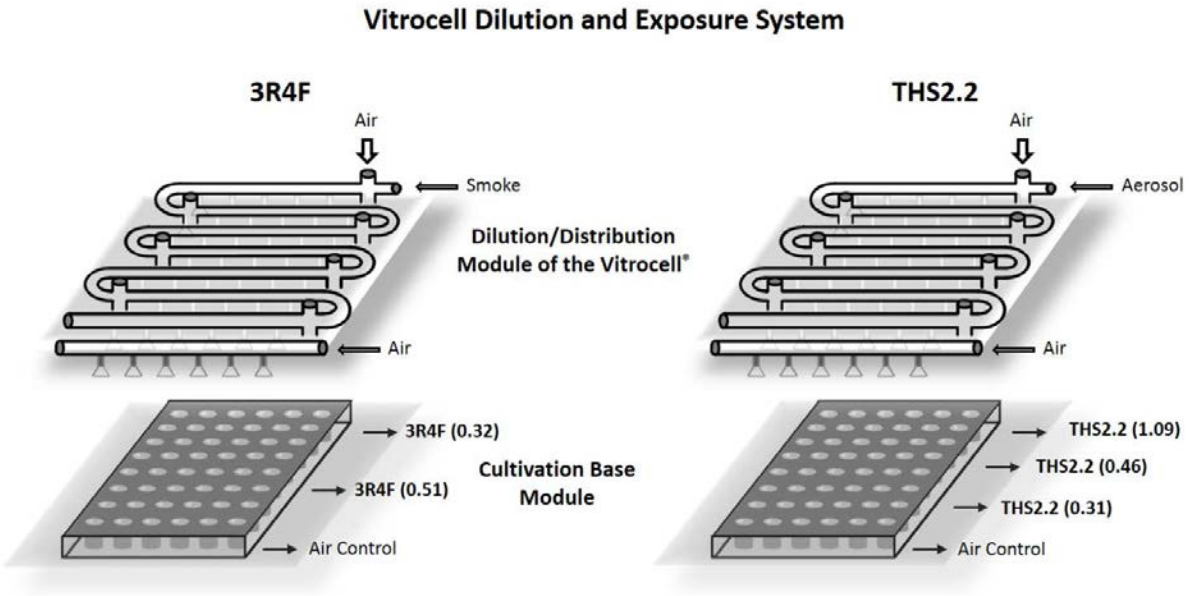


Figure 2. Schematic of an Exposure Run for 3R4F and THS2.2.

A Dilution/Distribution Module of the Vitrocell 24/48 exposure system located on top of a cultivation Base Module is illustrated. Each of the Vitrocell 24/48 exposure system was connected to a smoking machine (SM) ("SM-2000" for 3R4F smoke exposure and "SM-2000 THS2.2" for THS2.2 aerosol exposure). In the Base Module, up to 48 wells (culture inserts) can be simultaneously exposed in a single run. The delivery of the whole smoke/aerosols was achieved by delivering diluted smoke/aerosol through individual trumpets to each well. An exposure run consisted of the applied target concentration(s) paired with its air control (N =1 paired sample per run, for any given endpoint and post-exposure time point).

In each of the four experimental repetitions, various endpoints were measured at 4, 24, 48, and 72 h post-exposure, for each applied concentrations and air control **(Table 6)**. The results reported in this document were calculated from the aggregated data obtained from experiments 1–4.

Table 6. Post-Exposure Endpoints

Experimental Week		Cytotoxicity				Gene Expression Microarray				Secreted Pro-Inflammatory Mediators				CYP1A1/1B1 Activity				Histology			
		Post-exposure (h)				Post-exposure (h)				Post-exposure (h)				Post-exposure (h)				Post-exposure (h)			
		4	24	48	72	4	24	48	72	4	24	48	72	4	24	48	72	4	24	48	72
Exp 1	**13 April, 2015**	(3)	(3)	(3)	(3)	(3)	(3)	(3)*	(3)	NA	(3)	(3)	(3)	NA	(3)	(3)	NA	NA	NA	(3)	(3)
Exp 2	**15 June, 2015**	(3)	(3)	(3)	(3)	(3)	(3)	(3)*	(3)	NA	(3)	(3)	(3)	NA	(3)	(3)	NA	NA	NA	(3)	(3)
Exp 3	**13 July, 2015**	(3)	(3)	(3)	(3)	(3)	(3)	(3)*	(3)	NA	(3)	(3)	(3)	NA	(3)	(3)	NA	NA	NA	(3)	(3)
Exp 4	**10 August, 2015**	(3)	(3)	(3)	(3)	(3)	(3)	(3)*	(3)	NA	(3)	(3)	(3)	NA	(3)	(3)	NA	NA	NA	(3)	(3)

Numbers in parentheses indicate the total number of paired samples collected for the indicated endpoints per post-exposure time point at any given experimental repetition. *MicroRNA measured. One exposure run consisted of the applied target dose(s) paired with air controls (N = 1 paired sample per run, for any given endpoint and post-exposure time point). Abbreviations: CYP, cytochrome P450; Exp, experiment; h, hours; NA, not available (i.e., endpoints were not generated/collected at the indicated experimental repetition).

7.3.2 Dose Range Assessment of THS2.2

For the dose range assessment of the test item, various dilutions of the aerosol from THS2.2 were generated using the SM-2000 THS2.2 smoking machine **(Table 7)**. For a given endpoint, three independent exposure runs were conducted with one replicate per exposure run.

Table 7. Dilutions of Aerosol (%) Applied and Endpoints Tested in the Dose Range Assessment

Group	Dose Range Assessment Experiment Week: 10 August, 2015	Endpoint Measured at 72 h Post-Exposure		Smoking Machine
	Aerosol Concentration	Cytotoxicity (N)	Tissue Histology (N)	
Air Control (THS2.2)	-	(3)	(3)	SM-2000 THS2.2
THS2.2 Dilution 1	15%	(3)	(3)	
THS2.2 Dilution 2	25%	(3)	(3)	
THS2.2 Dilution 3	32%	(3)	(3)	
THS2.2 Dilution 4	50%	(3)	(3)	
THS2.2 Dilution 5	69%	(3)	(3)	
THS2.2 Dilution 6	80%	(3)	(3)	
THS2.2 Dilution 7	100%	(3)	(3)	

Numbers in brackets indicate the total number of paired samples collected for the indicated endpoints.
Abbreviation: N, number of replicates.

7.4 Testing Procedure

7.4.1 Aerosol/Smoke Generation and *In Vitro* Exposure Regimens

Reference smoke was generated from 3R4F reference cigarettes (Kentucky Tobacco Research & Development Center) using a 30-port carousel smoking machine (SM2000; Philip Morris International) connected to a Vitrocell 24/48 system (Vitrocell Systems GmbH). Another 30-port carousel smoking machine, SM2000 THS2.2, was used to generate the test aerosol from the THS2.2 sticks. 3R4F cigarettes and THS2.2 sticks were conditioned for at least 48 h and up to 21 days at 22 ± 1°C with a relative humidity of 60 ± 3% according to ISO standard 3402 (International Organization for Standardization 1999). For the exposure experiments, each 3R4F cigarette was smoked to a standard butt length (approximately 35 mm), and each THS2.2 stick was aerosolized up to 12 puffs per stick. For a 28-min exposure experiment, 10 3R4F cigarettes were smoked and 10 THS2.2 sticks were aerosolized using the dedicated smoking machines for a 55-mL puff over 2 s, puffed twice per min with an 8-s pump exhaust time (according to the Health Canada smoking regimen).

7.4.2 Nicotine Determination in Whole Smoke/Aerosol

The nicotine concentration in whole smoke or test aerosol delivered to the *in vitro* exposure systems was determined routinely (during each *in vitro* exposure experiment) to ensure that dosing of 3R4F smoke and THS2.2 aerosol were standardized to equal nicotine concentrations. For this, diluted whole 3R4F smoke or THS2.2 aerosol was trapped at the exhaust of the dilution/distribution module of the Vitrocell 24/48 system using EXtrelut® 3NT columns (Merck Millipore, Billerica, MA, USA) impregnated with 2 mL of 0.5 M H_2SO_4 solution (PMI_RD_WKI_001066). Whole smoke or aerosol from two sticks per item were trapped (10 puffs per stick, 5 min trapping duration), with each of them smoked/aerosolized by applying a modified Health Canada Intense

puffing protocol of a 55-mL puff over 2 s, puffed twice per min with an 8-s pump exhaust time. After trapping, EXtrelut columns were either stored at 2–8°C for a maximum of 4 weeks or processed immediately by determining nicotine levels by gas chromatography-flame ionization detection (GC-FID) (PMI_RD_WKI_000409). Briefly, 250 µL of isoquinoline (4 mg/mL), used as an internal standard, was added to the top of the column, and the trapped nicotine, including the internal standard, was eluted by adding 5 mL solution of 5% (v/v) triethylamine in N-butyl acetate for a total of three cycles (total volume, 15 mL). This was mixed well, and 1 mL of the dilution was transferred to an amber glass vial for analysis. One microliter of the eluent was injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) in split mode using a split ratio of 1:20 and injector temperature of 220°C. The separation was performed at an oven temperature of 140°C in isothermal mode (run time: 6 min) using a 15-m DB-5 fused silica capillary column (Agilent Technologies) with an internal diameter of 0.25 mm and 0.25-µm film thickness. Helium was used as the carrier gas, and the column flow was set to 2.25 mL/min. Peak detection, integration, and quantification of nicotine (according to an internal standard calibration curve) were performed automatically using the Agilent ChemStation software (Agilent Technologies).

7.4.3 Carbonyl Concentrations Deposited in the Vitrocell Base Module Following Whole Smoke/Aerosol Exposure

The deposition of representative carbonyls was measured in PBS following a 28-min exposure to whole smoke or test aerosol (10–12 sticks per item, each of them was smoked/aerosolized by applying a modified Health Canada Intense puffing protocol of 55-mL puff over 2 s, twice per min, with an 8-s pump exhaust time) (PMI_RD_WKI_001094). Briefly, before exposure, each row in the cultivation base module of the Vitrocell 24/48 system was filled with 18.5 mL PBS. Following exposure, an aliquot of 1.2 mL PBS-exposed sample (per row) was collected and mixed with 1.8 mL 2,4-dinitrophenylhydrazine solution (15 mM). Subsequently, 150 µL pyridine was added to quench the chemical derivatization. From this mixture, a 500 µL aliquot was placed into a liquid chromatography-mass spectrometry (LC-MS) amber glass vial. After 30 min, 485 µL acetonitrile and 15 µL internal standard mixture (24 µg/mL acetone-d6 and 21 µg/mL methyl ethyl ketone-d5) were added to the mixture. Finally, 5 µL of the mixture was injected into a high-performance liquid chromatography (HPLC) instrument coupled with a tandem MS (HPLC-MS/MS) to determine the quantity of the analytes using an isotope dilution technique.

7.4.4 Maintenance of EpiOral 3D Organotypic Culture (ALI Cultures)

Upon arrival, human EpiOral cultures (MatTek Corp., Ashland, MA, USA) were placed into differentiation medium (PMI_RD_WKI_001064). In brief, 24-well culture plates were prepared by adding 0.5 mL differentiation medium (ORL-200-DMa, supplemented with freshly added 45 µL ORL-200-SUPP for each 150 mL; MatTek Corp.) to each well and pre-warmed to 37°C. EpiOral cultures were then transferred from the shipping plates to fresh plates using sterile tweezers and incubated at 37°C (5% CO_2, 90% humidity) for 1 h. Afterwards, the cultures were re-transferred to 12-well plates with modified lids (HNG-TOP-2x, MatTek Corp.) containing 5.5 mL of differentiation medium, and incubated for 3 days at 37°C (5% CO_2, 90% humidity) until fully differentiated. After differentiation, EpiOral inserts were kept in 12-well plates containing 5.5 mL maintenance medium (ORL-200-MM-SUPP, supplemented with freshly added 75 µL ORL-200-

SUPP for each 250 mL; MatTek Corp.), at 37°C (5% CO_2, 90% humidity). For all *in vitro* exposure studies, EpiOral cultures grown in 6.5-mm ThinCert™ (Greiner Bio-One, Monroe, NC, USA) or 6.5-mm Transwell (Corning, Tewksbury, MA, USA) inserts were used, to match the dimensions of the Vitrocell 24/48 *in vitro* aerosol exposure system (Vitrocell GmbH). Throughout the studies, the cultures were regularly inspected microscopically, to check for potential morphological changes, as well as bacterial or fungal contamination.

7.4.5 Adenylate Kinase (AK) Cytotoxicity Assay

AK activity was measured in the basolateral medium of smoke and aerosol-exposed 3D organotypic cultures as a marker of cytotoxicity using a ToxiLight™ bioassay kit (Lonza, Rockland, MA, USA) (PMI_RD_WKI_001048). Briefly, 20 µL of basolateral medium was transferred to a well of a luminescence-compatible 96-well microtiter plate and mixed with 100 µL AK detection reagent. After 5 min of incubation, luminescence was measured using a FLUOstar Omega reader (BMG LABTECH GmbH, Ortenberg, Germany). For normalization of the cytotoxicity level, the AK assay was conducted in positive control samples. These positive controls were obtained from cultures treated with Triton X-100 at a 1% final concentration for 24 h added to the basolateral side to induce complete lysis of the cells (100% cytotoxicity).

7.4.6 Measurements of Secreted Inflammatory Mediators

Multi-analyte profiling of pro-inflammatory mediators (MAP analysis) was carried out by collecting 200 µL of basolateral medium from the organotypic cultures, taken at different post-exposure times (24, 48, and 72 h after whole smoke or aerosol exposure) (PMI_RD_SOP_000361; PMI_RD_WKI001032; PMI_RD_WKI_001274; PMI_RD_FOR_000803). Luminex® xMAP® technology was used for the analysis (Luminex, Austin, TX, USA), using commercially available assay panels (Milliplex®, EMD Millipore Corp., Schwalbach, Germany), as outlined in **Table 8**, according to the manufacturer's instructions. Briefly, 25 µL of diluted or non-diluted sample were used for each detection, and the analysis was run on a Luminex 200™ or FLEXMAP 3D® platform equipped with the xPONENT® software version 4.2 (Luminex). Data are represented as median fluorescent intensity (MFI) units using a five-parameter logistic or spline curve-fitting method for calculating the analyte concentrations in the basal medium sample. As a positive control, EpiOral cultures (N = 3) were treated for 24 h with tumor necrosis factor alpha (TNFA) and interleukin-1 beta (IL1B) (PMI_RD_FOR_000849), each added to the basolateral medium at a final concentration of 10 ng/mL, to induce (majority) mediators targeted by the MAP analysis.

Table 8. Summary of Milliplex Kits used in the Experimental Repetitions

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Experiment Week	**13 April, 2015**	**15 June, 2015**	**13 July, 2015**	**10, August 2015**
CFS3/G-CSF	A	A	A	A
CSF2/GM-CSF	A	A	A	A
IL1A	A	A	A	A
IL1B	A	A	A	A
CXCL10/IP10	A	A	A	A
VEGFA	A	A	A	A
TGFA	A	A	A	A
IL6	A	A	A	A
CXCL8/IL8	A	A	A	A
MMP-1	B	B	B	B

Milliplex kits were purchased from EMD Millipore Corp. **A**, custom panel HCYTOMAG-60K; **B**, custom panel HMMP2-55K. Abbreviations: CSF, colony-stimulating factor; CXCL, chemokine (C-X-C motif) ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; IP10, interferon gamma-induced protein 10; MMP, matrix metalloproteinase; TGFA, transforming growth factor alpha; VEGFA, vascular endothelial growth factor A.

7.4.7 Tissue Processing, Embedding, Sectioning, and Staining

Culture inserts were washed three times with PBS and fixed for 2 h in freshly prepared 4% paraformaldehyde (PMI_RD_WKI_001242). Upon completion of the fixation, the fixative was aspirated and the fixed organotypic cultures were washed three times with PBS both apically and basally at room temperature. Following this process, the fixed tissues were separated from the inserts by detaching the membrane from the plastic using forceps, and bisected at the midpoint prior to processing using a Leica ASP300 S tissue processor (Leica Biosystems Nussloch GmbH, Nussloch, Germany) (PMI_RD_WKI_001243). Subsequently, the two bisected pieces (per tissue sample) were placed in a cassette for embedding in paraffin (PMI_RD_WKI_001260). Microscopy sections of the paraffin block (5 µm thickness) were obtained using a microtome (PMI_RD_WKI_001262) and mounted on glass slides that were then transferred to a Leica ST5020 automated slide stainer (Leica Biosystems Nussloch GmbH) for staining with hematoxylin (Merck Millipore) and eosin (Sigma-Aldrich, St. Louis, MO, USA) (H&E) (PMI_RD_WKI_001266). Digital images of each slide (comprising the two bisected pieces per tissue sample) were generated using a Hamamatsu NanoZoomer 2.0 slide scanner (Hamamatsu Photonics K.K., Japan) (PMI_RD_WKI_001314).

7.4.8 Morphological Assessment of H&E-stained Tissue Sections

The digital images of the H&E-stained sections were independently assessed in a blinded manner by TPL Path Labs (Freiburg, Germany). Briefly, the two bisected pieces per tissue sample (per slide) were examined simultaneously, and one unique score was reported for one sample. The microscopic findings were recorded during the evaluation and entered manually into Excel spreadsheets. Various histological features were chosen for the systematic histological assessment of each section (**Table 9**). The grading system for these features is described in **Table 10**.

Table 9. Outline for the Systematic Assessment of Histological Features in Oral Epithelial Tissue Sections

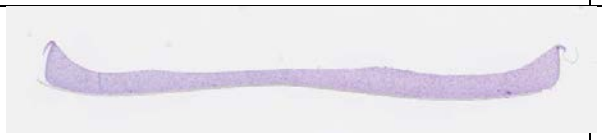
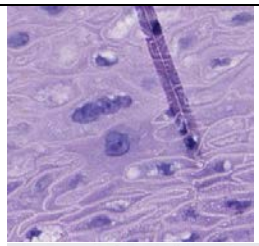
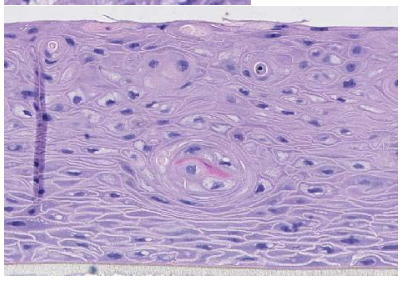
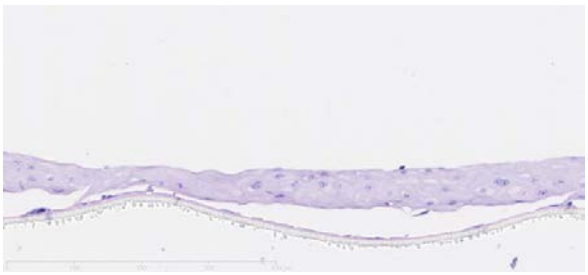
Applied Descriptive Finding	Explanation	Illustration	Scoring System
Tissue thickness inhomogeneity	Except for a neglected tissue border of 500 µm, inhomogeneously distributed findings lead to focal or regional variation in tissue thickness (e.g., by atrophy, degenerative changes, or cysts).		−, 1–5
Cellular alteration	Cellular alteration is synonymous with cellular/nuclear changes that make up dysplastic foci and dysplastic areas. In this context, tissue dysplasia is defined as findings not typical for a normal, stratified epithelium, including nuclear or cellular pleomorphy, shifted nuclear to cytoplasmic ratio, and multinucleation, including loss of polarity. May be accompanied by typically ectopic keratinization, necrosis, or the formation of whirls or laminated circular structures.	 	−, 1–5
Epithelial atrophy	The term describes the state of the tissue thickness and appearance. Generally, epithelial atrophy may be linked to trophic changes following irritants, or to an attempted tissue regeneration following tissue defect. Therefore, reduced		−, 1–5

Table continues

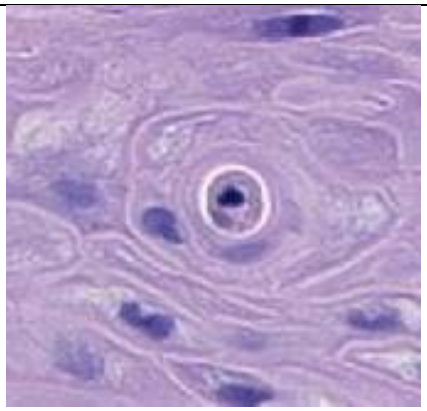
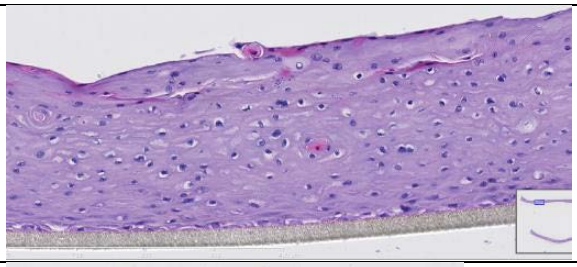
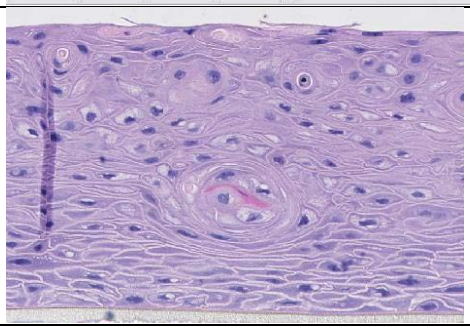
	tissue thickness following incomplete tissue repair could reflect atrophic changes of the epithelium.		
Single-cell necrosis/ apoptosis	The term “single-cell necrosis” was included to cover single, non-viable cells that do not formally meet the criteria for apoptosis. Apoptosis: Focally or multi-focally occurring single-cell lesions characterized by shrunken/rounded cells with eosinophilic cytoplasm and condensed and/or fragmented nuclear chromatin, including the formation of apoptotic bodies.		−, 1–5
Apical keratinization	Keratinization, located apically.		−, 1–5
Ectopic keratinization	Keratinization within the epithelium.		−, 1–5

Table continues

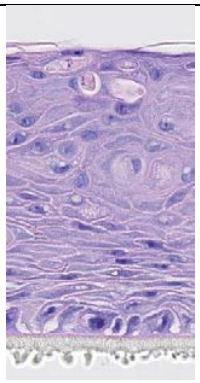
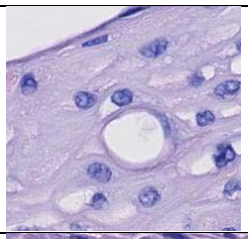
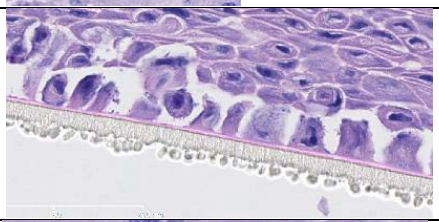
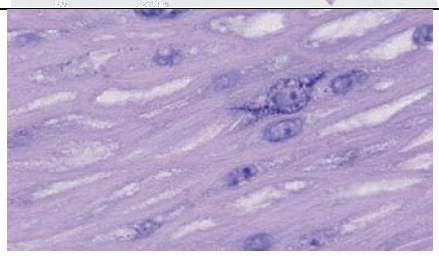
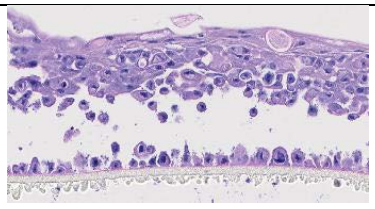
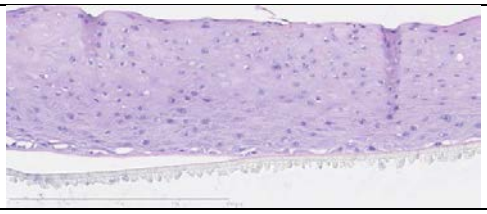
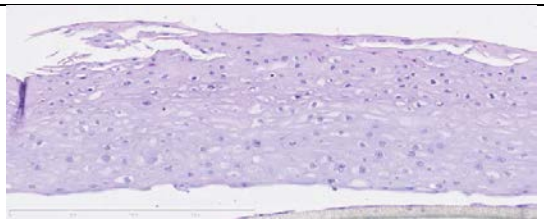
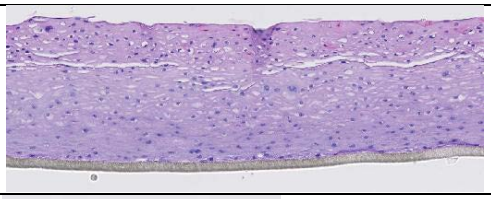
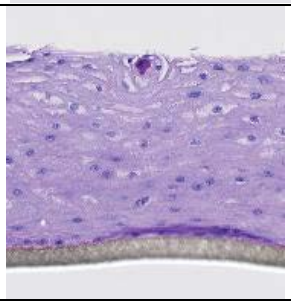
Increased cytoplasmic vacuolation	Cellular vacuolation.		−, 1–5
Cysts	Empty or mucus-filled round spaces, diameter approx. 4–5 cells across.		−, 1–5
Extracellular granules	Structures suggestive of extracellular bacteria.		−, 1–5
Increased intracellular fine granular basophilic material	Intracellular, basophilic granules, most likely related to cytoplasmic granules normally found in keratinized epithelium (stratum granulosum). Affected cells not confined to dedicated layer, focal to diffuse.		−, 1–5
Detachment of tissue above basal cell level	Epithelial detachment above basal cell layer points to a cytotoxic effect. Cytotoxicity within a planar section of the epithelium is likely to impair cellular adherence.		−, 1–5
Detachment of tissue below basal cell level	Epithelial detachment below basal cell layer, leaving no cells on the artificial insert membrane; promoted by compromised tissue integrity.		−, 1–5

Table continues

Increased desquamation	Focal to diffuse finding, confined to the epithelium, not extending to the substrate. Includes increased apical desquamation characterized by loosening of the apical-most epithelial layers of tissue and instances of (resulting) erosions. Exceeds the normally occurring grades of desquamation, therefore the finding is termed increased desquamation.		−, 1–5
Longitudinal cleft formation	Formation of longitudinal clefts within the tissue, sometimes showing keratinization.		−, 1–5
Mineralization	Focal areas of mineralization, originating from necrotic/apoptotic single cells with basophilic staining, only rarely occurring, may be neglected in this study.		−, 1–5

End of the table

Table 10. Histology Scoring System

Score grade	Description
Grade 0	No alteration visible
Grade 1	Minimal, very few, very small alterations
Grade 2	Slight, few, small alterations
Grade 3	Moderate, moderate number, moderate size alterations
Grade 4	Marked, many, large alterations
Grade 5	Severe, extensive number, extensive size alterations
Binary grading	Present/not present

7.4.9 Cytochrome P450 (CYP) 1A1/1B1 Activity Assay

The combined activity of CYP1A1/CYP1B1 was determined using the non-lytic P450-Glo™ assay (Promega, Madison, WI, USA), according to the manufacturer's instructions (PMI_RD_WKI_001049; PMI_RD_FOR_000846). Briefly, the luminogenic P450-Glo substrate luciferin-6′ chloroethyl ether (Luciferin-CEE), which is a substrate for both CYP1A1 and CYP1B1, was added to the basolateral medium 24 h prior to sample collection. The luciferin product of the CYP reaction was detected by mixing 50 µL of the collected medium with 50 µL of Luciferin Detection Reagent. After 30 min of incubation at room temperature, luminescence was measured in a FLUOstar Omega reader (BMG LABTECH GmbH). As a positive control for CYP1A1/B1 induction, three replicates of organotypic culture inserts were treated for 48 h with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Sigma-Aldrich, St. Louis, MO, USA), then added to the basolateral side at a final concentration of 30 nM. As a negative control, basolateral media from three replicates of untreated culture inserts were tested for CYP1A1/B1 activity.

7.4.10 RNA/MicroRNA Purification

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

7.4.11 mRNA Profiling Analysis using GeneChips

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

Purified cRNA (≥ 12 µg) was quantified, normalized, and quality-checked using a Fragment Analyzer™ (Advanced Analytical Technologies, Ames, IA, USA) (PMI_RD_WKI_001125) and chemically fragmented (PMI_RD_WKI_001109). The completion of fragmentation was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies). Then, 29.4 µL of fragmented and labeled cRNA was added to 190.6 µL of Hybridization Cocktail. After denaturation for 5 min at 95°C and 5 min at 45°C, followed by centrifugation at Vmax (16,000× g) for 1 min, 200 µL of the cRNA cocktail was hybridized to a GeneChip® Human Genome U133 Plus 2.0 Array (Agilent Technologies) (PMI_RD_WKI_001126). The arrays were incubated in a GeneChip® Hybridization Oven 645 (Affymetrix) for 16 hours at 45°C with a rotation speed of 60 rpm. Arrays were washed and stained on a GeneChip® Fluidics Station FS450 DX (Affymetrix) using the Affymetrix® GeneChip® Command Console® Software (AGCC software v 3.2) with protocol FS450_0001.

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

7.4.12 MiRNA Profiling

A FlashTag™ Biotin HSR kit (Affymetrix) was used to label the miRNA (PMI_RD_WKI_001123). Briefly, 200 ng of total RNA containing low molecular weight RNA was subjected to a brief tailing reaction, followed by ligation of the biotinylated signal molecule to the target RNA sample. After denaturation for 5 min at 99°C and 5 min at 45°C, followed by centrifugation at Vmax (16,000× g) for 1 min, 21.5 µL of the biotin-labeled sample was mixed with 110.5 µL hybridization cocktail. The arrays (miRNA version 4.0) were incubated in a GeneChip Hybridization Oven 645 (Affymetrix) for 16 h at 48°C with a rotation speed of 60 rpm. Arrays were washed and stained on a GeneChip Fluidics Station FS 450 DX (Affymetrix) using the Affymetrix GeneChip Command Console Software (AGCC software version 3.2) with protocol FS450_0002. Finally, the arrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix). Raw images from the scanner were saved as DAT files. The AGCC software automatically gridded the DAT file image and extracted probe cell intensities into a CEL file.

8. STATISTICAL AND COMPUTATIONAL METHODS

8.1 Statistical Methods

The analysis and graphical display of the data were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA).

8.1.1 Experimental Unit

For the exposure experiments using organotypic culture models and the Vitrocell 24/48 cultivation and exposure system, the experimental unit was the smoke exposure run. If a particular endpoint was measured several times in the same culture insert or in different inserts exposed to the same experimental condition (e.g., a specific dose of smoke/aerosol concentration at a specific post-exposure time) during the same smoke exposure run, then all the mean values of a given endpoint were calculated.

For the controls, endpoints were measured from culture inserts that were not exposed to smoke/aerosol in the Vitrocell 24/48 cultivation and exposure system but treated by reagents/chemicals. In this case, the experimental unit was the study phase, i.e., a single experimental repetition, and the mean values of a given endpoint were calculated from the measurements performed on culture inserts that were treated identically with the specific corresponding reagent/chemical.

8.1.2 Derived Variables

8.1.2.1 Normalized Cytotoxicity Levels

All values of the AK assay readout were normalized using the mean of the positive control (Triton X-100-treated culture inserts) and negative control (PBS-treated or untreated culture inserts [unexposed, incubator control]), and the percentage of cytotoxicity was calculated as follows:

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

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

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

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

8.1.2.2 Normalized CYP Activity (%)

All measured activity of CYP1A1/CYP1B1 was normalized using the mean of the positive control (TCDD-treated culture inserts) and negative control (luciferin substrate-treated, non-exposed culture inserts) as follows:

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

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

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

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

8.1.3 Data Transformation

All numerical values from the MAP analysis were transformed using the natural log transformation.

8.1.4 Statistical Comparison

Continuous variables were compared as follows:

- The comparison of an exposed sample versus the corresponding air control (i.e., the paired samples from the same exposure run at a given experimental repetition) was done using a paired *t*-test.
- The comparison of 3R4F-exposed versus THS2.2-exposed samples was done after subtracting the values of the corresponding air controls (i.e., the paired samples). The comparison was then done 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 (QC) of Raw CEL Files

The Affymetrix GeneChip Human Genome U133 Plus 2.0 Array was used for hybridization, which simultaneously probes the expression of thousands of genes. In accordance with PMI_RD_WKI_001228 and PMI_RD_SOP_000346, raw CEL files were background-corrected, normalized, and summarized using frozen robust multiarray analysis (fRMA) (McCall 2010).

Background correction and quantile normalization were used to generate microarray expression values from all arrays passing quality control checks, which were performed using the custom CDF environment HGU133Plus2_Hs_ENTREZG v16.0 (Dai 2005). A log-intensities plot, normalized unscaled standard error plot (NUSE), relative log expression (RLE) plot, median absolute value RLE (MARLE) and pseudo-images, 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 displaying 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

For each experimental factor combination item, dose and post-exposure, a model for estimating the treatment effect was fitted using the *limma* software (Smyth 2004), by including the covariate "smoking run" as a blocking variable to account for insert pairing during an exposure experiment (an exposure run comprises samples exposed to a reference or test item and air-exposed controls, see **Figure 2**). The *p*-values for each computed effect were adjusted across genes using the Benjamini–Hochberg false discovery rate (FDR) method. The results were displayed as volcano plots (x-axis representing the estimated effect and y-axis representing the $-\log_{10}$(FDR) for each gene). Differentially expressed genes (DEGs) were defined as the set of genes whose FDR was below 0.05.

Reproducibility was estimated by correlating the fold-changes (exposed samples *vs.* controls) across experimental repetitions. First, the gene expression data from experiments 1 and 2 were combined and the mean of the fold-changes was computed for each of the genes in the dataset. Similarly, the combined data from experiments 3 and 4 were used to compute the mean of the fold-changes. Correlation plots of the fold-changes for all genes in the dataset were then generated for each comparison.

8.2.3 Statistical Analysis: Network-level Analysis

8.2.3.1 Network Perturbation Amplitude

The collection of causal biological networks used in the study(s) was the human network suite CBN version 1.3 (Boue 2015). These networks contain from a few dozen to 200 nodes. The relevant network models considered in this study are summarized in **Table 11**. The network models can be organized into four major families, representing global biological processes: cell stress (CST), cell proliferation (CPR), inflammatory process network (IPN), and cell fate (CFA).

Table 11. Network and Network Models Used in the Analysis

No	Abbreviated Network Family Name	Network
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 Signaling
24	CST	Osmotic Stress
25	CST	Oxidative Stress
26	CST	Xenobiotic Metabolism Response
27	IPN	Epithelial Innate Immune Activation
28	IPN	Tissue Damage

Abbreviations: CFA, cell fate; CST, cell stress; CPR, cell proliferation; IPN, inflammatory process network; Hox, homeobox; 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; Wnt, wingless-type.

Network scoring exploits the network backbone nodes that are connected to downstream mRNA-abundance-nodes based on their known relationship reported in the literature. These signed relationships can be the backbone node increasing or decreasing the abundance of certain mRNAs, and are used to infer the activation of a backbone node using transcriptomics data. Because not all backbone nodes have downstream mRNA nodes, the network models need to be prepared for

scoring to improve the specificity and relevancy of the overall NPA score. Only backbone nodes that are on a directed path that starts and ends with a node that has downstream mRNA nodes are considered. After removing the nodes that do not satisfy the criteria above, the largest connected component is kept. Finally, the edges "causesNoChange" are disregarded for the scoring.

The Network Perturbation Amplitude (NPA) methodology (Hoeng 2014, Martin 2014, Martin 2012) aims at contextualizing the high dimensional transcriptomics data by combining gene expression ($\log_2$) fold-changes, β, into fewer differential node values (one value for each node of the network), f. The differential node values are determined by a fitting procedure that infers the values that best satisfy the directionality of the causal relationships (positive or negative) contained in the network model, while being constrained by the experimental data (the gene $\log_2$-fold-changes, which are described as downstream effects of the network itself):

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

where L is the signed weighted Laplacian of the network and the extra edges and nodes describing the downstream effects (to gene expression nodes); L3 is the sub-matrix of L for the nodes in the network, and L2 is the sub-matrix corresponding to the edges connecting the network nodes to the downstream gene expression nodes. The differential node values are in turn summarized as a single positive number, referred to as the amplitude of perturbation (NPA scores):

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

where E is the set of edges in the network; |E| is its size, and e_0 and e_1 denote the start and the end, respectively, of an edge e. The sum computing the NPA score can be expressed as fTQf, where Q is the signed Laplacian of the network when all of the edge signs have been reversed. All details of the methodology are described in a previous publication (Martin 2014).

For the NPA scores, a confidence interval accounting for experimental variation and associated *p*-values is computed. Additionally, companion statistics, derived to inform on the specificity of the NPA score with respect to the biology described in the network model, are shown as *O and K* if their *p*-values are below the significance level (0.05). A network is considered to be significantly affected if three values (the confidence interval, *O, and K* statistics) are below 0.05.

8.2.3.2 Biological Impact Factor (BIF)

The BIF method (Hoeng 2014, Thomson 2013) provides a unified and coherent framework for investigating mechanistic effects at each level of granularity of the biological processes represented in the network models. The method enables the derivation of a relative BIF, which is a measure of the overall biological impact across all network models. The BIF methodology allows an assessment of the exposures in an objective, systematic, and quantifiable manner, by computing a systems-wide and pan-mechanistic biological impact measure for a given substance, mixture, or test item.

An aggregation of the NPA should satisfy the following criteria:

a) *Credibility*: The BIF should be an intuitive and logical aggregation scheme.
b) *Fairness*: All network families are equally important.
c) *Understandable*: Each decision or step should have a defined rationale.

To fulfil the first criterion, network scores are aggregated via a weighted sum (because every scoring in NPA is additive). In addition, only networks that have *p*-values of the three statistics below 0.05 (*O, *, K*) are considered (denoted by N_i*). BIF is defined as:

$$BIF = \sum_{N_i^*} \omega_i \, NPA(N_i) = \sum_{N_i^*} \omega_i \sum_{e \epsilon E(N_i)} (f(e_0) + \sigma(e) f(e_1))^2$$

where f is the function of the network nodes that describe the differential node values; E(N_i*) represents the network edges, and e_0 and e_1 are the start and end, respectively, of an edge e.

For the second criterion, a *fair* BIF would represent the equality of the biological processes (networks) in a given system (e.g., 2D cultures of normal human oral epithelial cells, 3D organotypic cultures, or lung tissue samples from *in vivo* studies). If all the network models were fitted perfectly by the gene expression data, the contribution of each network to the BIF is assumed to be equal. Therefore, the BIF methodology considers that all specific processes represented in the network models are equally involved and important for a given network family. A perfect fit of the network will maximize the NPA value and be smooth over the network. Using the notation defined above, a "perfect" network response (differential node values) would both maximize $f^T Qf$ with $||f||_2 = 1$ and minimize $f^T L_3 f$ (the NPA criteria (Martin 2014)). Additionally, there is a constraint that $L_3 f$ vanishes for network nodes that do not have direct downstream gene expression nodes (Martin 2014, Martin 2012), because they are expected to be in the image of L^T_2.

Such nodes are denoted as NH. Therefore, the following optimization problem can be solved as:

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

To rebalance the network families, the mean over the networks will be considered.

For each edge $e = (e_0, \sigma(e), e_1)$, the number of occurrences of edge e in the set of networks used in the BIF calculation is denoted by $o(e)$. Therefore, the maximum occurrence-corrected amplitude of a network N_i can be defined as:

$$NPA_{max}(N_i) \doteq \sum_{e \epsilon E(N_i)} \frac{1}{o(e)} (f_{max}(e_0) + \sigma(e) f_{max}(e_1))^2$$

Finally, to normalize the NPAs, a correction factor of $|E(N_i)|/NPA_{max}(N_i)$ is applied. Considering all of these factors, the BIF is defined as the weighted sum (F denotes the network families), as follows:

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

Therefore, the contribution of each network F is:

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

8.2.4 Gene-set Analysis (GSA)

GSA of transcriptomics data was performed using the *Piano* package in the R statistical environment (Varemo 2013). Pathway maps were obtained from the Kyoto Encyclopedia of Genes and Genomes (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 GSA. 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 Raw CEL Files

The raw array data in the CEL files were pre-processed through a standard pipeline based on the generic documents PMI_RD_WKI_001228 and PMI_RD_SOP_000346. 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, R Core Team 2013). The quality of the array data was controlled using the *arrayQualityMetrics* package in Bioconductor (Kauffmann 2009), and examined according to the following four metrics:

a) Distances between arrays at the raw data level
b) Distances between arrays at the normalized data level
c) NUSE
d) Array intensity distributions.

Arrays that were found to be outliers in at least two of these metrics were discarded. The quality was iteratively re-examined for the remaining arrays until all were accepted. For the experimental repetitions, the QC procedure for the miRNA expression analysis was applied separately to each miRNA processing batch (each processing batch was composed of samples obtained from two consecutive experimental repetitions). This approach was used to avoid batch effects on the QC.

Subsequently, normalized probe-level data were obtained by applying robust multiarray (RMA) normalization and summarized using the median polish method at the probe-set level (Bolstad 2003, Irizarry 2003).

8.3.2 Probe Set Filtering

Affymetrix GeneChip miRNA 4.0 arrays were used for the hybridization. These arrays probe the expression of 30,424 mature miRNAs relevant for 203 species, among which 2,578 miRNAs correspond to human expression (Affymetrix, Inc., Affymetrix miRNA 4.0 Array). Using the annotation provided by Affymetrix and the latest miRNA nomenclature according to miRBase (released on 21 June 2014) (Kozomara 2013), only the probe sets pertaining to humans were kept in the expression matrix. Additionally, the probe sets not available on the Affymetrix GeneChip miRNA 3.0 arrays were discarded to make the miRNA results comparable across all the considered organotypic cultures (bronchial, nasal, and oral). The final expression matrix contained 1,728 miRNA probe sets.

According to the Affymetrix instructions, only the miRNA probe sets with significantly higher intensity values than their matched background probes (based on the GC content and sequence length) were considered to be "detected" (see the "Detection" section in (Affymetrix miRNA QCT Tool 2011)). A *p*-value threshold of 0.01 was chosen to determine the detection calls based on Wilcoxon tests. If a miRNA probe set was detected in more than 50% of the samples in at least one sample group, then it was kept for further analysis; otherwise, it was discarded. This pre-processing approach yielded 548 miRNA probe sets in the final expression matrix after exclusion of the control probe sets.

8.3.3 Calculation of Differential miRNA Expression

According to the experimental design, the experimental unit consisted of a number of pairwise treatment vs. control comparisons (i.e., an exposure run comprised samples exposed to a test item and samples exposed to air (control); see section 8.1.1. Experimental Unit). For each comparison, a sub-matrix was extracted from the global expression matrix by keeping only the samples that belonged to corresponding treatment or control groups, as well as the miRNA probe sets that were detected in more than 50% of the samples in at least one of these two groups. Linear models for differential expression were applied on all resulting sub-matrices using the moderated *t*-statistics implemented in the *limma* package (Ritchie 2015). The models included an additional variable that took into account the exposure runs, which improved the fitting procedure in *limma*. Subsequently, adjusted *p*-values were obtained following multiple testing corrections using the Benjamini–Hochberg FDR method (Benjamini 1995). The miRNAs below the FDR threshold of 0.05 were considered to be differentially expressed.

For a given comparison, the results were displayed as a volcano plot, where the x-axis represented the estimated treatment effect (differential expression) of each miRNA, and the y-axis represented the corresponding statistical significances given by −log10(FDR). Moreover, for a given comparison, quantile–quantile (QQ) plots were generated by first sorting all miRNAs according to their unadjusted (raw) *p*-values in ascending order. Subsequently, the −log10 transformed raw *p*-values of the resulting quantiles (i.e., the rank divided by total number of miRNAs) were displayed

on the x-axis, and the corresponding, −log10 transformed raw *p*-values were displayed on the y-axis. For a given statistical significance threshold α, the "Z"-shaped boundaries of the plot correspond to raw *p*-values below α (lower horizontal line), the oblique line parallel to the diagonal x = y corresponds to the FDR values below α, and the upper horizontal line corresponds to the Bonferroni adjusted *p*-value below the threshold α.

9. RESULTS

9.1 Nicotine Concentration in Smoke/Aerosol

The Vitrocell® 24/48 exposure system is equipped with a dilution system (referred to as the Dilution/Distribution Module, **Figure 2**). To achieve the target concentrations of nicotine in the 3R4F smoke or THS2.2 aerosol, certain dilutions were applied when exposing the organotypic cultures. The actual nicotine concentrations in the smoke/aerosol were measured following trapping of smoke/aerosol in EXtrelut® 3NT columns; at least three independent samples were collected per experimental repetition **(Table 12)**. Because the whole smoke/aerosol was trapped in the EXtrelut® 3NT columns at the first row of the Dilution/Distribution Module, further dilutions of the smoke/aerosol could not be applied during trapping measurements. Therefore, trapping of whole smoke/aerosol in EXtrelut® 3NT columns to measure nicotine concentrations could not be done at the same time when the cultures were exposed to 3R4F smoke or THS2.2 aerosol. The trappings were therefore done separately within the same experimental week.

Table 12 shows that the higher the percentages of 3R4F smoke and THS2.2 aerosol applied within the Dilution/Distribution Module, the higher the concentration of nicotine in the smoke/aerosol, as expected. The actual nicotine concentrations in the smoke/aerosol for a given concentrations of 3R4F and THS2.2 were consistent among the experimental repetitions.

Furthermore, according to the study design **(Table 5)**, at least two dose levels of the test aerosol THS2.2 would have comparable nicotine concentrations to those of the reference 3R4F CS. **Table 12** illustrates that to reach the comparable nicotine concentrations, the dilution of the THS2.2 aerosol was approximately 1.28 times that of the 3R4F CS.

Table 12. Target and Actual Nicotine Concentrations in Smoke/Aerosol

Group		Experimental Week			
		Experiment 1 13 March, 2015	Experiment 2 15 June, 2015	Experiment 3 13 July, 2015	Experiment 4 10 August, 2015
3R4F Applied Concentration 1	Target Nicotine (mg/L)	0.3	0.3	0.3	0.3
	Smoke concentration (%)	15	15	15	15
	Actual Nicotine (mg/L)	0.32 ± 0.010 (N = 3)	0.33 ± 0.011 (N = 3)	0.29 ± 0.011 (N = 3)	0.35 ± 0.004 (N = 3)
3R4F Applied Concentration 2	Target Nicotine (mg/L)	0.5	0.5	0.5	0.5
	Smoke concentration (%)	24	24	24	24
	Actual Nicotine (mg/L)	0.54 ± 0.016 (N = 3)	0.48 ± 0.018 (N = 4)	0.49 ± 0.013 (N = 3)	0.54 ± 0.009 (N = 3)
THS2.2 Applied Concentration 1	Target Nicotine (mg/L)	0.3	0.3	0.3	0.3
	Aerosol concentration (%)	25	25	25	25
	Actual Nicotine (mg/L)	0.29 ± 0.006* (N = 3)	0.34 ± 0.012 (N = 4)	0.27 ± 0.010 (N = 4)	0.32 ± 0.008 (N = 3)
THS2.2 Applied Concentration 2	Target Nicotine (mg/L)	0.5	0.5	0.5	0.5
	Aerosol concentration (%)	32	32	32	32
	Actual Nicotine (mg/L)	0.48 ± 0.017 (N = 3)	0.48 ± 0.006 (N = 4)	0.43 ± 0.067 (N = 4)	0.45 ± 0.045 (N = 3)
THS2.2 Applied Concentration 3	Target Nicotine (mg/L)	1.1	1.1	1.1	1.1
	Aerosol concentration (%)	69	69	69	69
	Actual Nicotine (mg/L)	1.08 ± 0.050 (N = 3)	1.02 ± 0.028 (N = 4)	1.14 ± 0.051 (N = 4)	1.13 ± 0.062 (N = 3)

Actual nicotine concentrations are expressed as mean ± SEM. Abbreviations: N, number of samples (trapped in the EXtrelut 3NT columns); SEM, standard error of the mean.

9.2 Histological Evaluation

The impact of exposure on the tissue morphology was evaluated by histological assessment at 48 and 72 h post-exposure.

9.2.1 Morphological Evaluation

Oral organotypic cultures were embedded in paraffin at 48 and 72 h after exposure, sectioned and stained with H&E. Representative H&E sections are shown in **Figure 3**. The images show similar tissue morphology at the 48- and 72-h post-exposure time points in air-exposed controls. At 48 h post exposure, the morphology of the tissues exposed to 3R4F was comparable to the air control. However, some cellular detachment above the basal cell layer was detectable in cultures exposed to 3R4F CS at both doses (0.32 and 0.51). At 72 h post-exposure to 3R4F CS, the tissues exhibited marked signs of damage, with detachment above the basal cell level, as well as signs of adaptation - e.g. apical keratinization, desquamation and intracellular granular structures. These effects were more prominent with the higher 3R4F CS concentration (0.51).

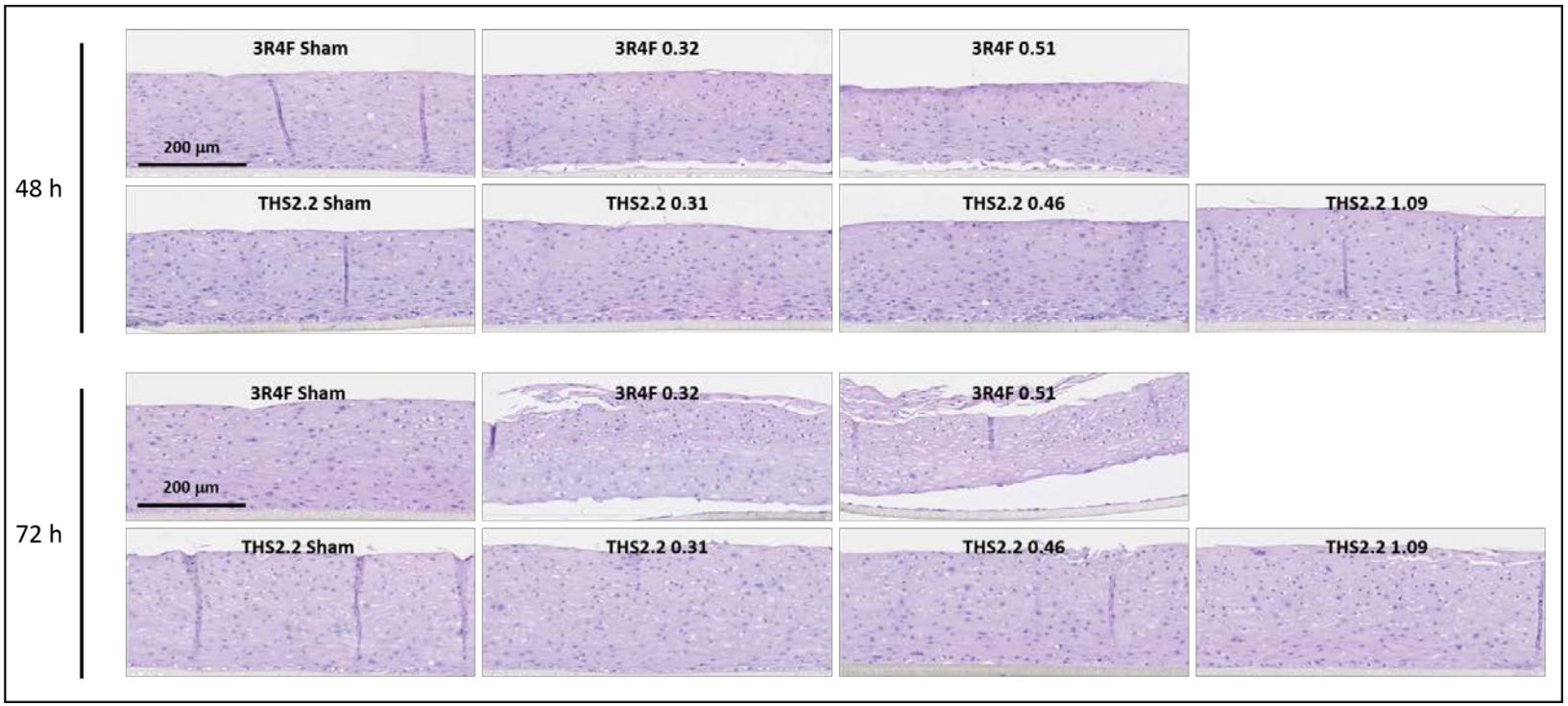


Figure 3. Histology Following Exposure of Organotypic Cultures to Smoke/Aerosol.

Representative images (10× magnification) of oral organotypic tissue sections stained with H&E, 48 h and 72 h after cultures were exposed for 28 min to 3R4F CS or THS2.2 aerosol at the indicated nicotine concentrations (mg/L). Abbreviations: H&E, hematoxylin and eosin; h, hour.

Gross morphological evaluation of all culture sections from the four experimental repetitions was conducted to assess various features observed following exposure (described in **Table 9**) in a blinded manner.

It should be noted that the evaluation of the culture slides was performed by a single pathologist, and has not been repeated so far. Moreover, an inherent variability of the culture models (as inferred from the variability in the scores for the culture inhomogeneity feature across the comparison groups) was observed. For these reasons, the observation was summarized and reported in a descriptive manner. The increased and decreased scores of each feature of the cultures exposed to 3R4F CS or THS2.2 aerosol were calculated compared with the air controls **(Table 13)**. Cells marked with black or grey indicate statistically significant increases or decreases, respectively, in the release of the pro-inflammatory markers. A comparison of 3R4F CS versus THS2.2 aerosol is represented in **Supplementary Table 22**.

The results suggest a different score pattern between the morphology of cultures exposed to 3R4F and THS2.2, relative to their corresponding air controls. The exposure to 3R4F CS resulted in ectopic and apical keratinization, detachment of culture above basal cell level, increased desquamation, intracellular fine granular basophilic material presence, and longitudinal cleft formation, present most prominently in the higher smoke concentration tested. In contrast, THS2.2 aerosol-exposed cultures showed a strongly attenuated response, with desquamation scores as the only relevant affected feature (only at 72 h for the 0.46 and 1.09 mg/L doses). Descriptive statistics capturing the scores for the individual feature are reported in **Supplementary Table 9**–**Supplementary Table 21**.

Table 13. Morphological Alterations Following Exposure Relative to Air Control

Morphological Finding		3R4F (0.32) vs. 3R4F (Air)		3R4F (0.51) vs. 3R4F (Air)		THS2.2 (0.31) vs. THS2.2 (Air)		THS2.2 (0.46) vs. THS2.2 (Air)		THS2.2 (1.09) vs. THS2.2 (Air)	
		48:00	72:00	48:00	72:00	48:00	72:00	48:00	72:00	48:00	72:00
Apical keratinization	Score	0.08	0.50	0.75	1.50	−0.17	−0.17	0.25	0.00	−0.17	−0.33
Cellular alteration	Score	−0.33	−0.33	0.25	−0.33	−0.25	0.00	−0.25	−0.25	−0.58	−0.25
Epithelial atrophy	Score	−0.17	0.42	-0.17	0.00	0.42	0.08	−0.08	0.00	−0.08	0.00
Cysts	Score	0.25	−0.58	-0.17	−0.75	0.17	−0.08	0.33	−0.25	0.00	−0.33
Ectopic keratinization	Score	0.08	0.50	0.08	0.42	−0.25	−0.08	−0.17	−0.17	0.00	−0.42
Detachment of tissue above basal cell level	Score	0.75	0.75	1.17	1.58	0.17	0.25	−0.17	0.08	0.08	0.25
Detachment of tissue below basal cell level	Score	0.50	0.25	-0.00	−0.42	0.17	0.08	0.33	−0.08	0.25	0.08
Extracellular granules	Score	0.00	0.00	-0.00	−0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cytoplasmic vacuolation	Score	0.08	−0.42	0.25	−0.42	−0.17	−0.08	0.33	0.00	−0.42	−0.33
Increased desquamation	Score	0.33	0.67	0.17	1.50	0.00	0.25	0.00	0.67	−0.17	0.67
Intracellular fine granular basophilic material	Score	0.25	0.50	0.67	1.00	0.00	0.00	0.00	0.00	0.00	0.00
Longitudinal cleft formation	Score	0.17	0.50	0.25	0.42	0.17	0.17	0.17	0.25	0.00	0.25
Single-cell necrosis/apoptosis	Score	−0.00	−0.08	-0.17	−0.25	0.00	0.17	−0.33	0.00	−0.17	−0.50
Tissue thickness inhomogeneity	Score	−0.33	−0.08	-0.42	−0.08	0.00	0.00	−0.17	0.08	0.08	0.00

Values indicate the difference between the means for a given feature of each exposure condition compared with air-exposed samples. Negative difference is indicated with the "–" sign. Mean differences between the contrasts that are below a raw *p*-value of 0.05 are marked with black or grey, for positive or negative differences, respectively.

9.3 Cytotoxicity

Cytotoxicity following exposure to 3R4F CS or THS2.2 aerosol exposure was assessed by measuring the activity of AK released from the cells into the basolateral media. **Figure 4** shows that 3R4F CS-exposed cultures exhibited an increased cytotoxicity compared with air controls (48 and 72 h post-exposure), although the very low increase of AK activity recorded (maximum ~6%) indicates a high resistance of the oral cultures to CS exposure. The cytotoxicity levels of the THS2.2 aerosol-exposed cultures were not different from those of the air controls, independently of dose or post-exposure time point.

All the THS2.2 values for the smoking run O, Experiment 2, were omitted from the AK result statistics. The amplitude of the AK signal was higher than Triton-X 100-exposed positive controls for some of the samples but no tissue damage was detected in the histological section corresponding to the same doses.

The elimination of one value (n=1) from the statistical analysis had no impact to the final statistical significance.

Descriptive statistics for these results are reported in **Supplementary Table 23**.

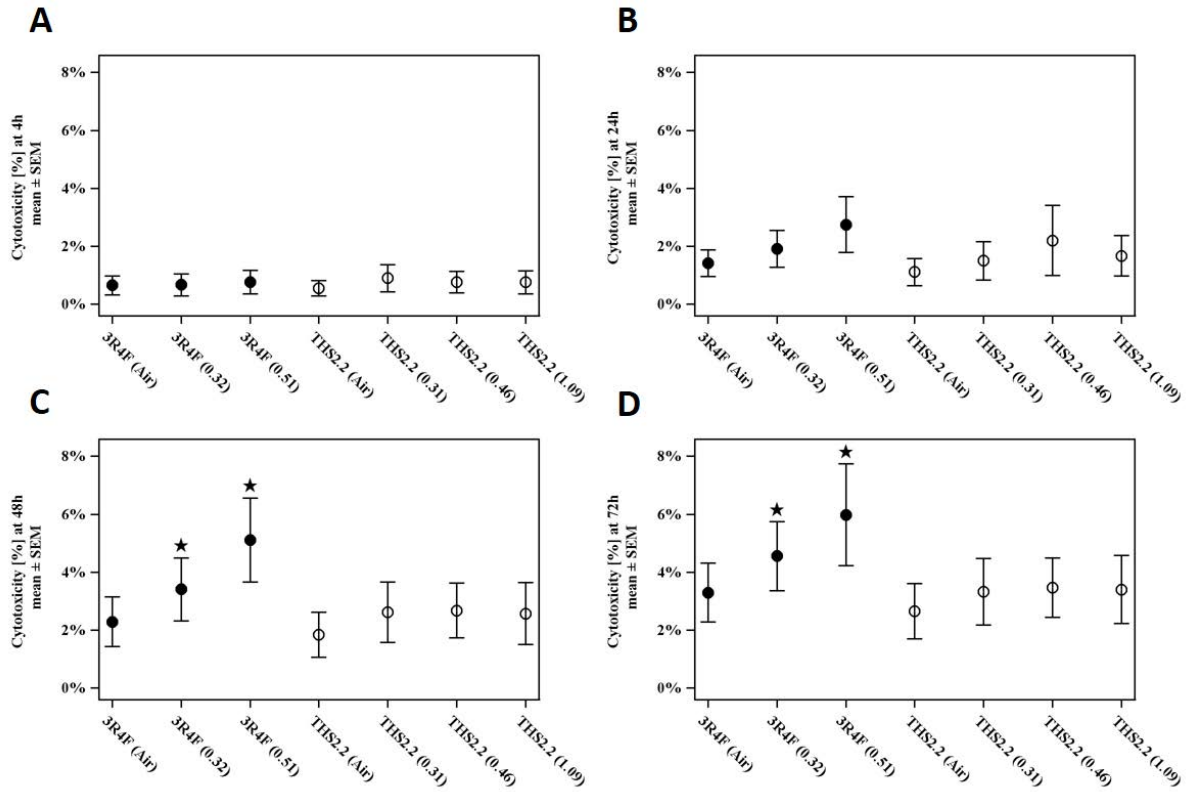


Figure 4. Cytotoxicity in organotypic cultures following 3R4F and THS2.2 exposure.

The release of adenylate kinase (AK) into the basolateral media of cultures was measured 4, 24, 48, and 72 h after exposure for 28 min to 3R4F CS or THS2.2 aerosol at the indicated nicotine concentrations (mg/L) (**A–D**). Data are presented as the mean of the normalized cytotoxicity relative to the positive control (Triton X-100-treated culture was taken as 100% cytotoxicity) ± SEM (N = 11–12, from four experimental repetitions with three exposure runs/repetition). ★indicates a significant difference compared with the corresponding air controls ($p < 0.05$).

9.4 Cytochrome P450 (CYP) 1A1/1B1 Activity

The combined activity of CYP1A1 and CYP1B1 enzymes was measured in organotypic oral cultures 24 and 48 hours following exposure.

Figure 5 shows that at 24 and 48 h post-exposure, there were no substantial changes in the CYP activity between the 3R4F CS-exposed inserts and the air controls. In contrast, a dose-dependent increase in CYP1A1/CYP1B1 activity was observed in cultures exposed to THS2.2 aerosol, at both post-exposure time points, reaching an approximate 15% increase. In addition, CYP activity was significantly higher for both comparable concentrations of THS2.2 versus 3R4F at 24 h, whereas at 48 h, this was only the case for the THS2.2 0.46 mg/L concentration. Descriptive statistics of these results are reported in **Supplementary Table 24**.

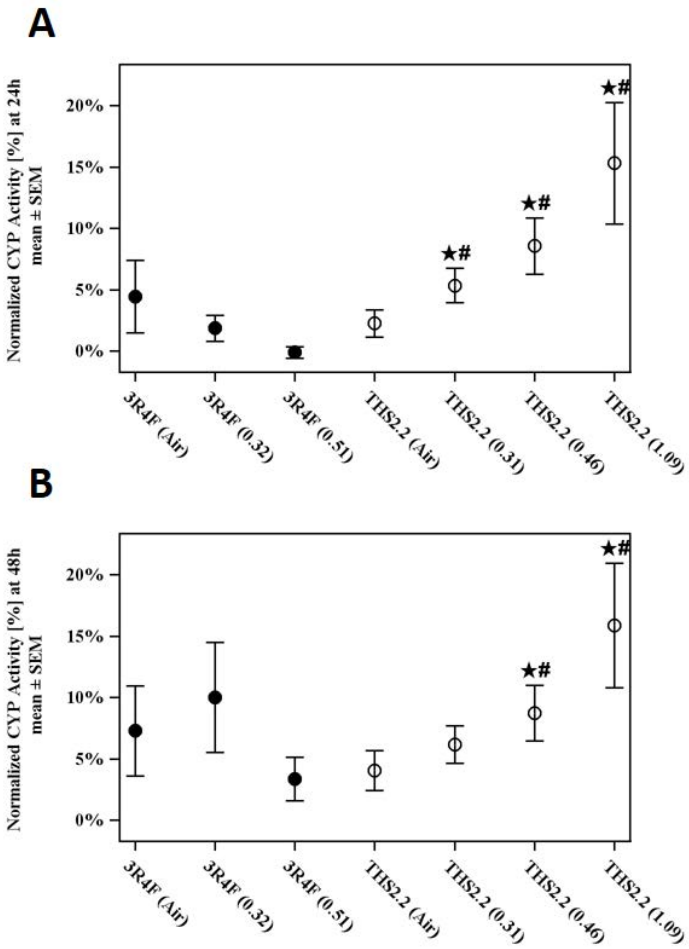


Figure 5. CYP1A1/CYP1B1 activity following exposure in the organotypic oral cultures.

The combined CYP1A1 and CYP1B1 activity was measured at 24 and 48 h after cultures were exposed for 28 min to 3R4F CS or THS2.2 aerosol at the indicated nicotine concentrations (mg/L) (**A–B**). Data are presented as the mean of the normalized activity relative to the positive control (2,3,7,8-tetrachlorodibenzo-p-dioxin-treated culture was considered as 100% induction) ± SEM (N = 12, from four experimental repetitions with three exposure runs/repetition). ★ indicates a significant difference as compared with the corresponding air controls ($p < 0.05$). # indicates a significant difference compared with 3R4F at the comparable nicotine concentration ($p < 0.05$).

9.5 Secretion of Pro-inflammatory Mediators

The inflammatory mediator release of the oral organotypic cultures following exposure was assessed by measuring the analytes secreted into the basolateral media at 24, 48 and 72 h post-exposure (from different cultures as cross-sectional data). VEGFA, TGFA, MMP-1, and IL1B levels in the medium were found to be significantly increased after 3R4F CS exposure (significant fold-changes are highlighted in black, **Figure 6A)**; THS2.2 aerosol at comparable nicotine concentrations also increased the level of these mediators significantly, although at a lower fold-change when compared with 3R4F CS-exposed samples. In contrast, CSF3 levels were significantly higher only after THS2.2 aerosol exposure. Some mediators were found to be downregulated by CS exposure, in particular CXCL8 (only at 72 h and 0.51 mg/L), CXCL10, IL6, and CSF2, with CXCL10 also being downregulated following THS2.2 aerosol exposure (0.46 mg/L exposure at 48 h, and at all PE time points for 1.09 mg/L).

Exposure to the highest THS2.2 aerosol concentration (1.09 mg/L, not comparable to 3R4F) led to a slight increase in the levels of all inflammatory mediators when compared with the lower THS2.2 aerosol concentrations (0.31, 0.46 mg/L), although fold-change was still lower than that in CS-exposed cultures. A statistical analysis assessing whether the 3R4F-induced levels of the mediators were different from the THS2.2-induced levels showed that levels of VEGFA, TGFA, MMP-1, IL1B, CXCL10, IL6, IL8, CSF2, and CSF3 levels in the medium after exposure were significantly different from 3R4F CS-exposed samples, even when 3R4F CS was compared with the highest THS2.2 aerosol concentration (1.09 mg/L) **(Figure 6)**.
Descriptive statistics of these results are reported in **Supplementary Table 25–Supplementary Table 34**; individual representations of the mediators are reported in **Supplementary Figure 1**.

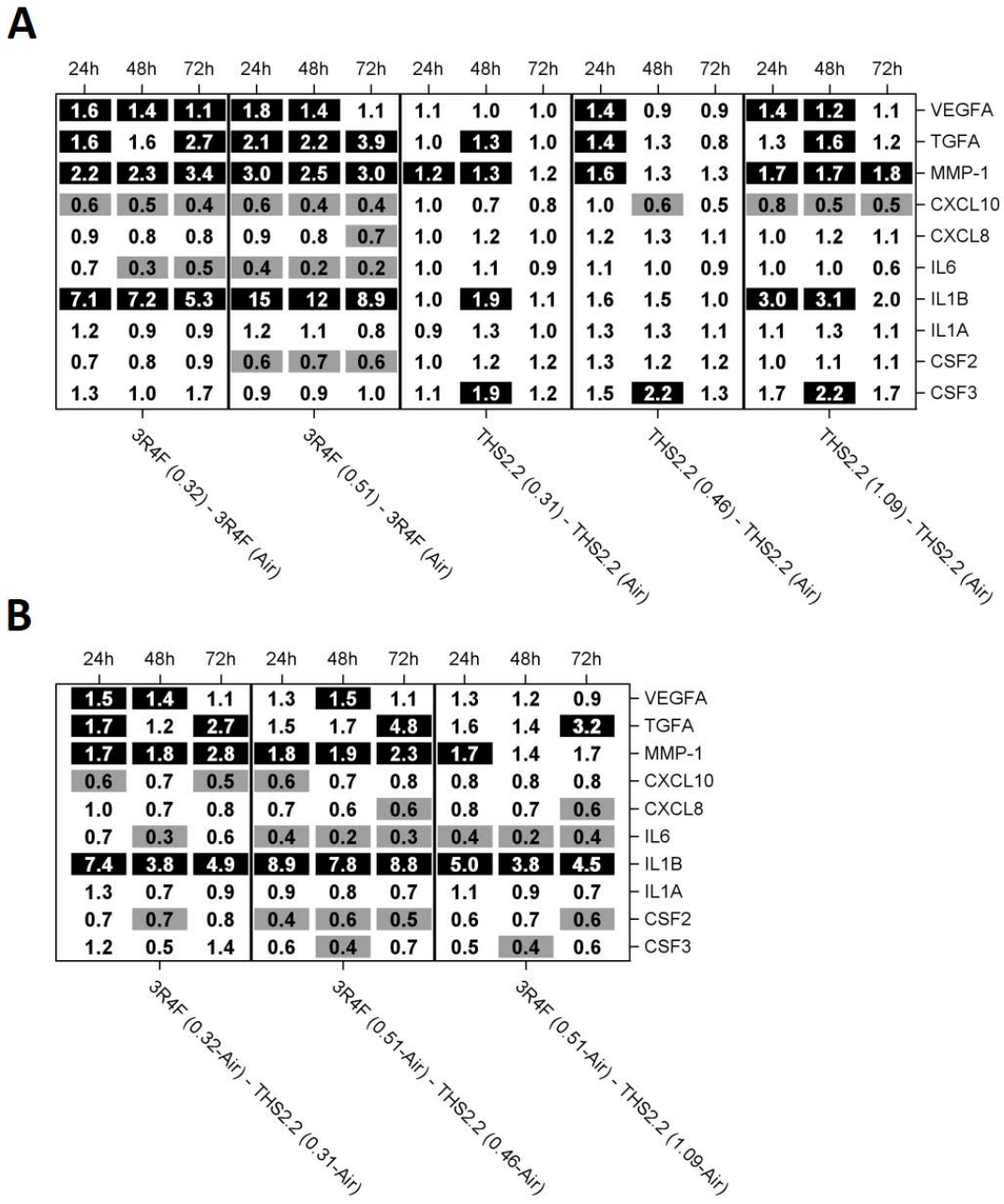


Figure 6. Fold changes of secretion levels of pro-inflammatory mediators into the basolateral medium of the organotypic oral cultures following exposure.

Fold-changes of the concentrations of mediators (listed on the y-axis, right) were measured in the basolateral media of organotypic cultures 24, 48, and 72 h after they were exposed to 3R4F CS or THS2.2 aerosol at the indicated nicotine concentrations (mg/L) for 28 min. The fold-changes are expressed relative to the air control (N = 12, from four experimental repetitions with three exposure runs/repetition). (**A**) Differences between 3R4F- and THS2.2-induced mediator levels are reported as the ratio of the fold-changes. (**B**) Dark and light grey shading indicates significant increases and decreases, respectively, between the comparisons ($p < 0.05$).

9.6 Transcriptome of Oral Epithelial Cultures

9.6.1 Differentially Expressed Genes

The investigation of possible toxicity-specific mechanisms associated with 3R4F and THS2.2 exposure was conducted in cultures that exhibited minimal damage. As reported in the previous sections, cytotoxicity **(Figure 4)** and tissue damage **(Figure 3)** observed in cultures exposed to 3R4F CS or THS2.2 aerosol were limited. Consequently, gene expression was evaluated using the transcriptomic data from all 3R4F CS and THS2.2 aerosol concentrations.

Gene expression profiles were generated from the oral culture samples collected 4, 24, 48, and 72 h post-exposure. Gene expression changes in the cultures exposed to 3R4F (0.32 mg/L), 3R4F (0.51 mg/L), THS2.2 (0.31 mg/L), THS2.2 (0.46 mg/L), and THS2.2 (1.09 mg/L) groups were compared with those of the corresponding control groups **(Figure 7)**. The volcano plots show that the 3R4F CS effect on gene expression was stronger than that of THS2.2 aerosol, leading to a sustained response in time and amplitude that could not be matched by any of the THS2.2 aerosol concentrations. At the highest and non-comparable nicotine concentration (1.09 mg/L), THS2.2 aerosol altered gene expression with a sustained effect over time, although at a much lower level than any 3R4F CS concentration.

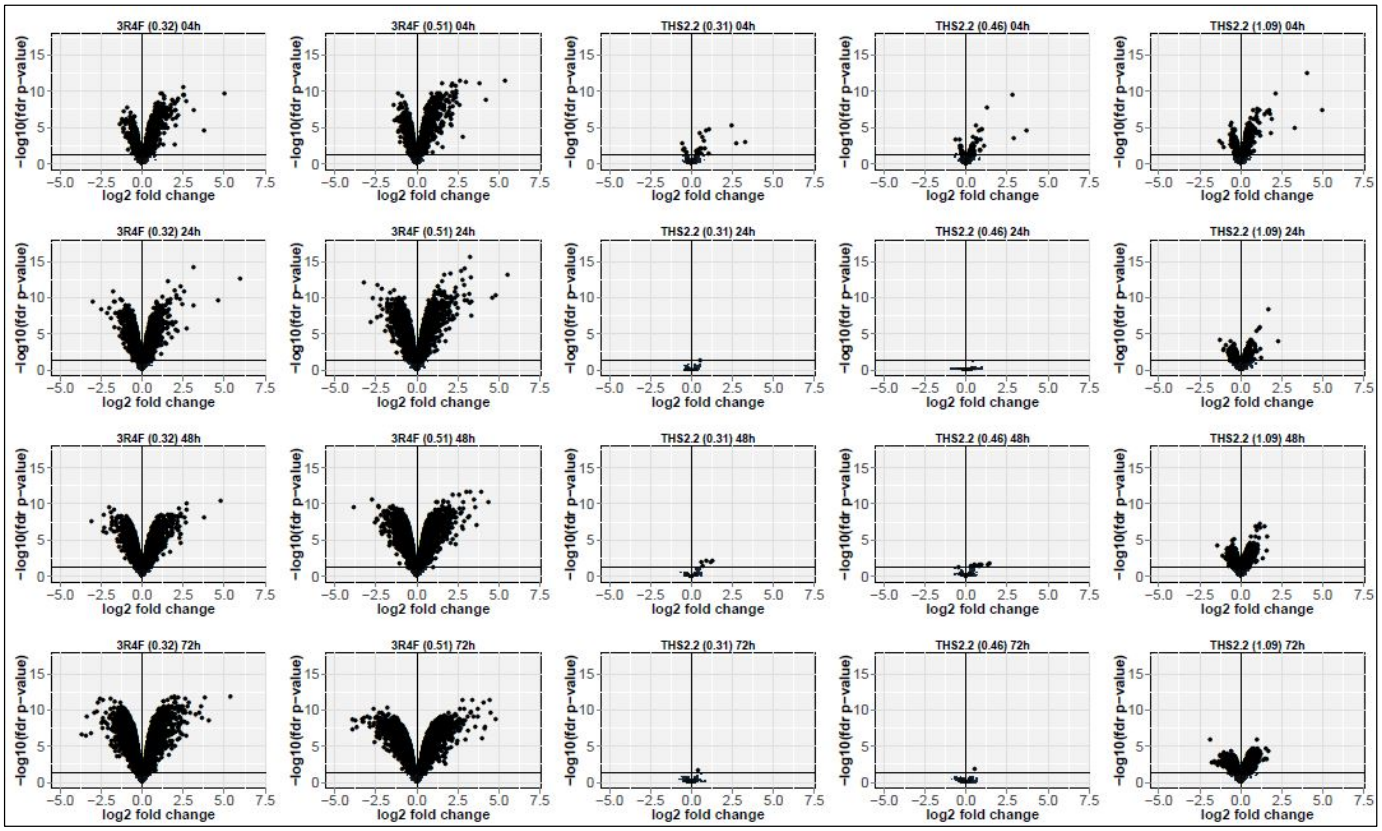


Figure 7. Volcano plots of the differentially expressed genes in oral epithelial cultures following exposure.

Each dot in a plot represents a gene. Gene expression changes (x-axis) are expressed as $\log_2(fold-change)$; increased and decreased when > 0 and < 0, respectively, compared with the corresponding air controls. The statistical significance (y-axis) is represented as $-\log_{10}$ (adjusted p-value FDR); N = 9–12, from four experimental repetitions with three exposure runs/repetition). The horizontal line represents statistical significance ($-\log_{10}(0.05)$).

9.6.2 Biological Impact Factor (BIF)

The overall systems impact (the aggregation of the network perturbation) is referred to as the biological impact factor (BIF) (Thomson 2013). **Figure 8** shows the overall biological impact, in the context of biology covered in the network models (**Table 11**), for each exposure condition compared with the respective air controls. At the 72-h post-exposure time point, 3R4F (0.32 mg/L) smoke exposure produced the highest impact on the cultures; thus, the relative BIF was set to 100% as the reference (REF). 3R4F (0.32 and 0.51 mg/L) exposure resulted in a high BIF at 4 h post-exposure, which initially declined during the post-exposure period, but rose again at 72 h post-exposure. For all THS2.2 concentrations, lower BIF values were observed at the different post-exposure time points compared with the corresponding 3R4F exposure groups.

The overall biological impact of each THS2.2 aerosol concentration was always lower than that of the corresponding 3R4F CS concentration.

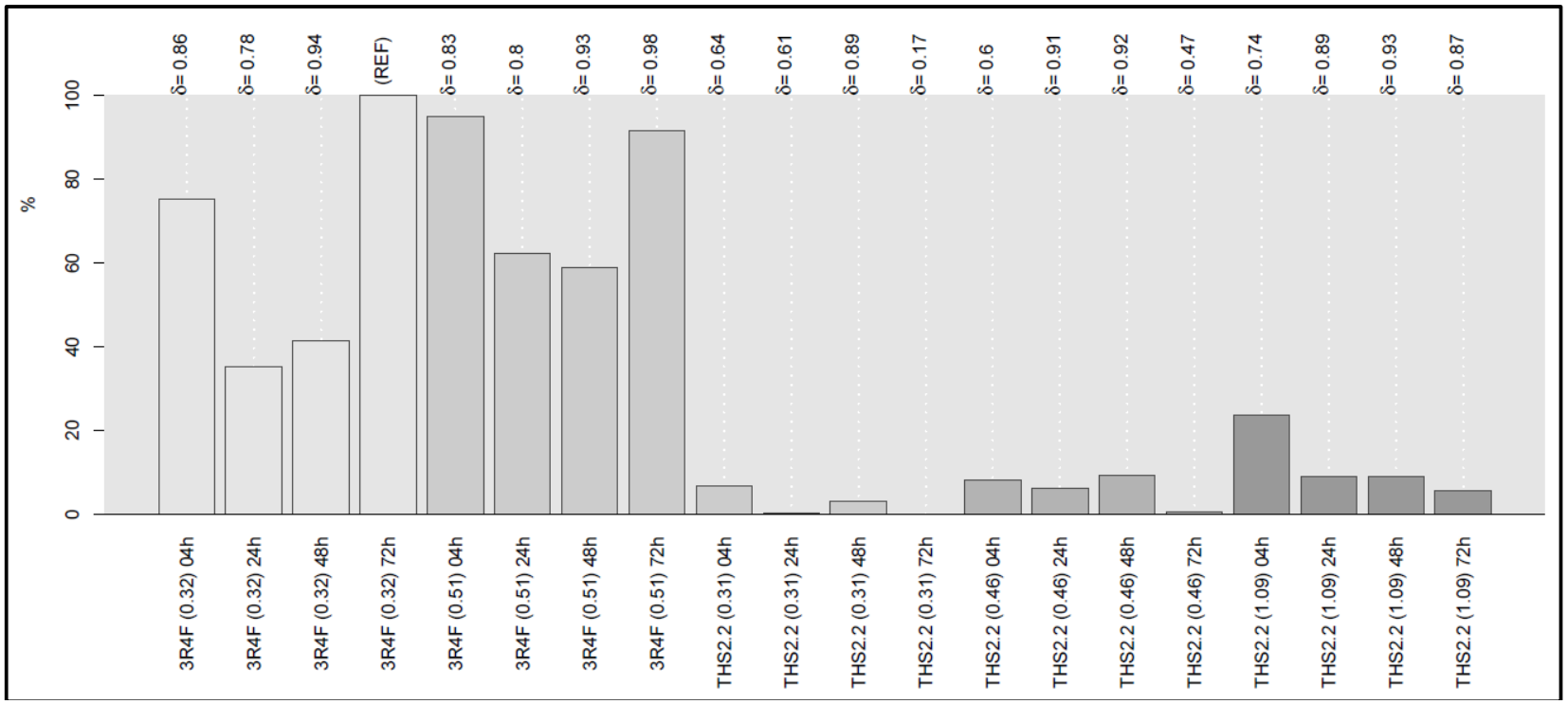


Figure 8. Relative biological impact factor (BIF) derived from cumulative network perturbations affected by 3R4F and THS2.2 exposure compared with air controls.

Percentage relative BIF is plotted on the y-axis. The highest BIF value, marked as REF and taken as 100%, and the δ values (δ in [-1,1]) given above the plot indicate how similar the underlying networks that are perturbed in a particular comparison are relative to those in the REF. A δ value of 0 indicates no similarity, and a δ value of 1 indicates that the networks are identical.

An overview of the different biological processes represented as network families that contributed to the overall BIF for each exposure is given in **Figure 9**. Pie charts are used to show the contribution of the perturbation of each network family to the BIF. The networks affected most by 3R4F CS exposure were cell fate (CFA), cell stress (CST), and inflammatory processes (IPN), compared with the air controls. The same networks were less affected by any of the THS2.2 aerosol concentrations analyzed, independent of the post-exposure time point.

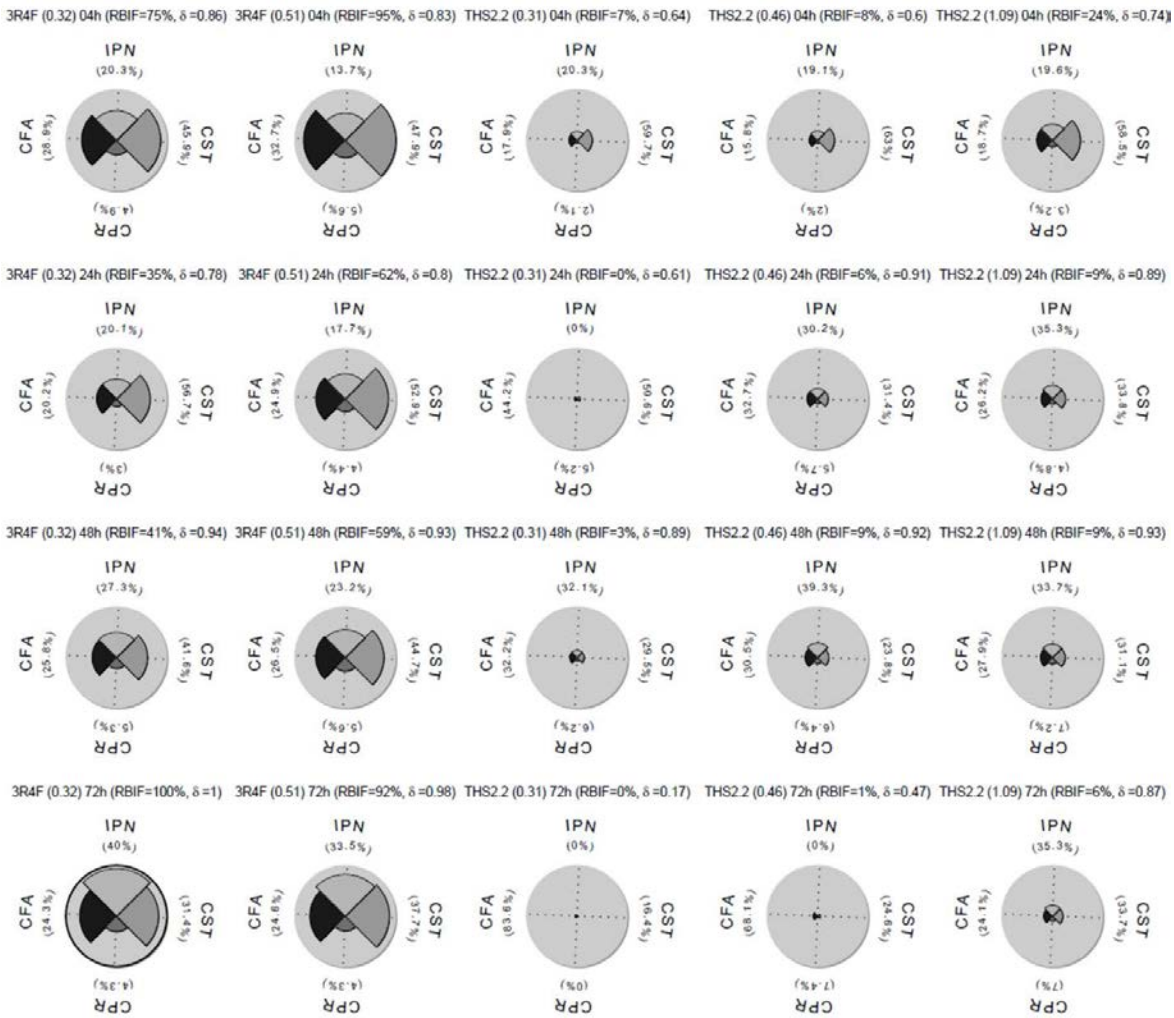


Figure 9. Contribution of Individual Network Perturbations to the Biological Impact Factor (BIF) Analysis.

Each pie chart represents the corresponding bar in the BIF plot in **Figure 8**. The charts show the subdivision of BIF into the different network families for each comparison. A pie slice indicates one of the CFA, CST, CPR, and IPN network families. The area of the slices indicates the relative contribution of each network perturbation to the BIF for a given comparison. The pie chart of the REF (the comparison with the highest BIF taken as 100%) is outlined with a thick black line. The relative BIF and the δ value for each comparison are given above each plot. δ values (δ in [-1,1]) illustrate the similarity of the underlying networks perturbed in a particular comparison, relative to those in the REF. A δ value of 0 indicates no similarity, and a δ value of 1 indicates that the networks are identical. Abbreviations: CFA, cell fate; CST, cell stress; CPR, cell proliferation; IPN, inflammatory process network; RBIF, relative BIF.

9.6.3 Network Perturbation Amplitude

Each network family comprises a set of network models, and the BIF can be decomposed down to network level. **Figure 10** shows the perturbation of each network across the comparisons.
The highest BIF, seen in the 3R4F (0.32 mg/L) cultures at the 72-h post-exposure time point, is reflected in the heatmap as the highest scores of perturbation in the majority of networks (network perturbation amplitude, NPA), compared with the other contrasts. Mirroring the trend of the BIF pattern, the impact of both 3R4F concentrations on the perturbation of the majority of the networks— particularly evident for CFA and CST network families—decreased at 24 h and 48 h post-exposure, and increased again at 72 h. Notably, the networks describing the biology of senescence, necroptosis, apoptosis, xenobiotic metabolism response, oxidative stress, osmotic stress, NFE2L2 signaling, culture damage, and epithelial innate immune activation belonging to the CFA, CST, and IPNs network families were most affected by CS. The CPR network family, in agreement with what is described in **Figure 9**, was less affected, showing less statistically significantly altered sub-networks, with only Wnt, PGE2, nuclear receptors, MAPK, JAK-STAT, and growth factors significantly affected.

In contrast, at the comparable nicotine concentrations, THS2.2 aerosol exposure did not result in substantial perturbations of these networks; although significant, the perturbations observed were much lower in terms of NPA scores with respect to the corresponding 3R4F CS concentrations. Moreover, it was only at the higher nicotine concentrations (0.46, 1.09 mg/L) that the THS2.2 aerosol-exposed cultures were affected at all post-exposure time points, as indicated by the NPA scores for the senescence, apoptosis, JAK-STAT, and NFE2L2 signaling networks. Nevertheless, the degree of impact (i.e., their NPA scores) was lower, compared with the impact of 3R4F (0.32 mg/L) at the corresponding post-exposure time points.

Among all network families of interest, only Hypoxic Stress and Calcium (only 0.46 mg/L, at 48 h) were specifically perturbed by THS2.2 aerosol but not 3R4F CS exposure.

A gene-set analysis was also conducted and the results are reported as a heatmap of the enrichment scores **(Supplementary Figure 2)**. The heatmap shows that the gene expression data from the cultures exposed to 3R4F had higher enrichment scores for the various biological annotations, compared with the data from the cultures exposed to all three nicotine concentrations of THS2.2 aerosol at any given post-exposure time point.

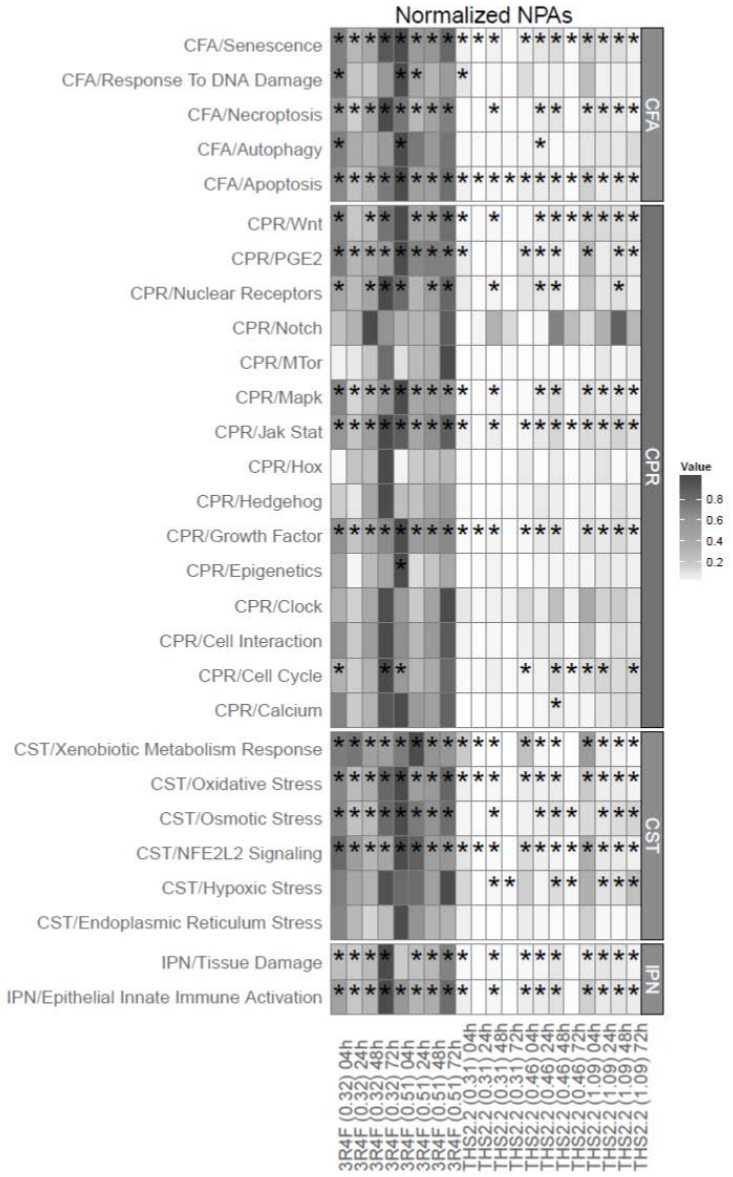


Figure 10. Heatmap of network perturbation amplitude (NPA) scores of biological networks affected by 3R4F and THS2.2 exposure.

The network names are listed on the left side of the heatmap, with the corresponding network family on the right. All networks that were significantly affected in at least one comparison are listed. The shading gradient of the NPA score was normalized to the maximum NPA score per network. A network was considered 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 each network. * indicates significant NPA scores with respect to the experimental variation and with significant O and K statistics. Abbreviations: CFA, cell fate; CST, cell stress; CPR, cell proliferation; IPN, inflammatory process network.

9.6.4 Alterations in Expression Levels of Genes Regulating Cellular Stress and Inflammation

In addition to the network approach analysis, alterations in the expression levels of genes known to regulate cell stress and inflammatory responses are displayed in **Figure 11** and **Figure 12**, respectively. In the 3R4F CS-exposed cultures, expression levels of almost half of the genes involved in stress responses had a constant differential expression, whereas the others increased/decreased with time, or displayed a biphasic behavior.

However, in the THS2.2 aerosol-exposed cultures, expression levels of only a few genes implicated in oxidative and xenobiotic stress were found to be altered (at the 4-h post-exposure time point only), while exposure to the highest concentration of THS2.2 aerosol (1.09 mg/L) resulted in alterations lasting up to 72 h, although at a lower fold-change compared with 3R4F exposure.

Increased expression of many of the genes regulating oxidative stress response was observed in 3R4F CS-exposed cultures at all post-exposure time points: *SLC7A11*, *HMOX1*, *GCLM*, *GCLC*, *TXNRD1*, *NQO1* and *GPX2*. In contrast, other genes were found to be downregulated: *MT1X*, *SLC40A1*, *XDH*, *CAT*, *GSTA4*, *SOD1*, *ID3* and *MAF* (**Figure 11**, upper right panel). The expression of these genes was affected mainly at the highest concentration of THS2.2 aerosol (1.09 mg/L), although to a lesser extent than their 3R4F-exposed counterparts.

Furthermore, the expression levels of many genes involved in xenobiotic stress (**Figure 11**, lower right panel) were increased in 3R4F CS-exposed cultures. At all post-3R4F-exposure time points, the expression levels of *CYP1A1* and *CYP1B1* increased more than the expression levels of the genes listed above. Additionally, 3R4F CS-exposed cultures showed increased levels of *TIPARP*, *ALDH1A3*, *AKR1C1*, *AKR1C2*, and *AKR1C3* at all post-exposure time points, and decreased levels of *AHR* and *ARNT*. In contrast, following exposure to THS2.2 aerosol at the comparable nicotine concentrations (0.31 and 0.46 mg/L), the expression levels of these genes (except *AKR1C1*) were increased only for the 4-h post-exposure time point. Moreover, the fold-changes in the expression levels of these genes were lower in THS2.2-exposed cultures (at all concentrations) than those in 3R4F-exposed cultures.

The expression levels of genes related to DNA damage, endoplasmic reticulum (ER) stress, heat shock, and hypoxic stress (**Figure 11**, left panels) were altered in 3R4F CS-exposed cultures, but not in THS2.2 aerosol-exposed cultures at comparable nicotine concentrations. THS2.2 aerosol exposure altered levels of genes regulating DNA damage, ER stress, heat shock, hypoxic stress, and osmotic stress only at the highest (and not comparable) nicotine concentration (1.09 mg/L).

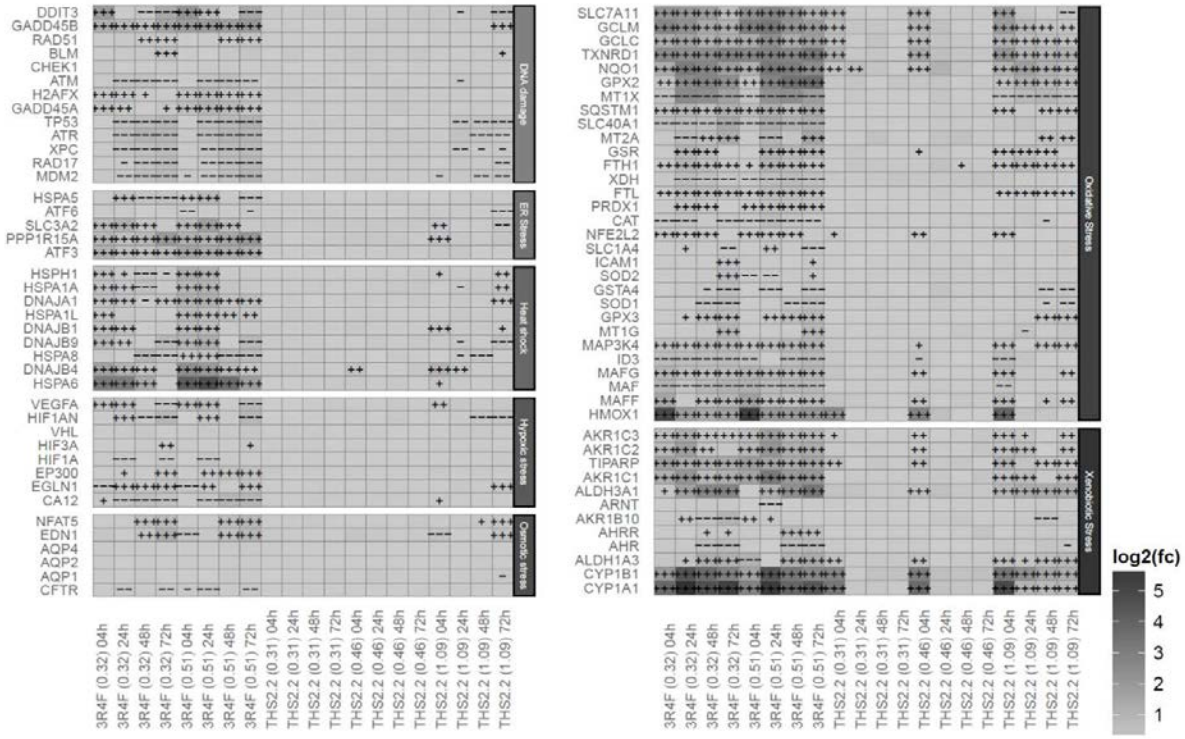


Figure 11. Altered expression levels of genes regulating cellular stress after 3R4F and THS2.2 exposure.

The heatmap shows changes in gene expression expressed as $\log_2(fold-change)$. Up- and downregulated gene expression levels compared with the corresponding air controls are marked with + and − signs, respectively (+++ and −−− indicate adjusted $p < 0.01$; ++ and −− indicate adjusted $p < 0.05$; + and − indicate adjusted $p < 0.1$). Gene symbols are listed on the left side of the heatmap. Stress-related processes are marked on the right side of the heatmap. Abbreviation: fc, fold-change.

3R4F CS-exposure substantially altered the expression levels of genes important for the epithelial inflammatory response **(Figure 12)**. In contrast, at the comparable nicotine concentrations, THS2.2 aerosol exposure minimally influenced the expression of these genes, if at all, and significant changes were observed only for *MMP1*, *MMP10* (48 h), *IL1A*, and *IL1B* (4 h). More genes were altered after exposure to the higher nicotine concentrations of THS2.2 aerosol (1.09 mg/L), although at a lower fold-change compared with all 3R4F CS-exposed cultures.

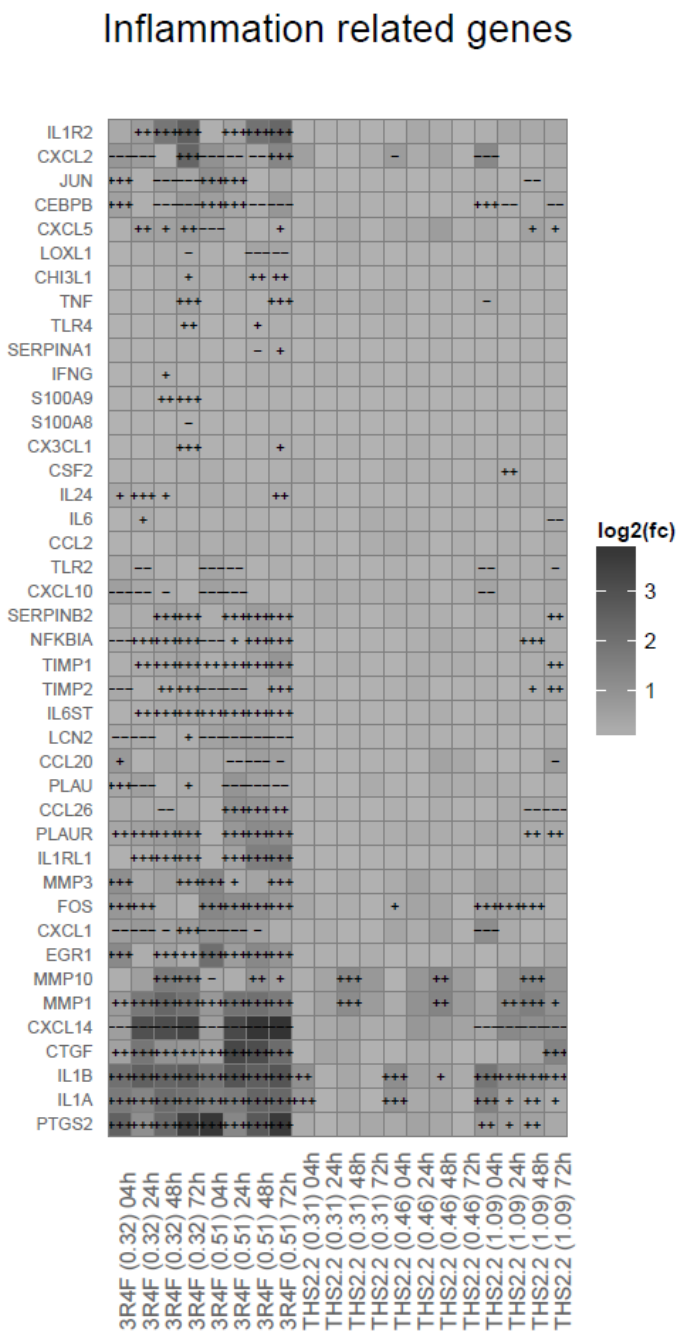


Figure 12. Altered Expression Levels of Genes Regulating Inflammatory Processes after 3R4F and THS2.2 Exposure.

The heatmap shows the changes in gene expression expressed as $\log_2(fold - change)$. Up- and downregulated gene expression levels compared with the corresponding air controls are marked with + and − signs, respectively (+++ and −−− indicate adjusted $p < 0.01$; ++ and −− indicate adjusted $p < 0.05$; + and − indicate adjusted $p < 0.1$). Gene symbols are listed on the left side of the heatmap. Abbreviation: fc, fold-change.

9.7 MiRNA Transcriptome of the Oral Epithelial Cultures

As regulators of gene expression, miRNAs influence many biological processes (Pritchard 2012). Possible mechanisms associated with the 3R4F and THS2.2 exposure responses were further inferred from the miRNA transcriptome data from the oral epithelial cultures exposed to 3R4F (0.32 mg/L), 3R4F (0.51 mg/L), THS2.2 (0.31 mg/L), THS2.2 (0.46 mg/L), and THS2.2 (1.09 mg/L), and analyzed 48 hours post-exposure.

Relative to the respective air controls, a greater number of miRNAs was altered following 3R4F CS exposure than THS2.2 aerosol exposure, at all concentrations tested **(Figure 13)**.

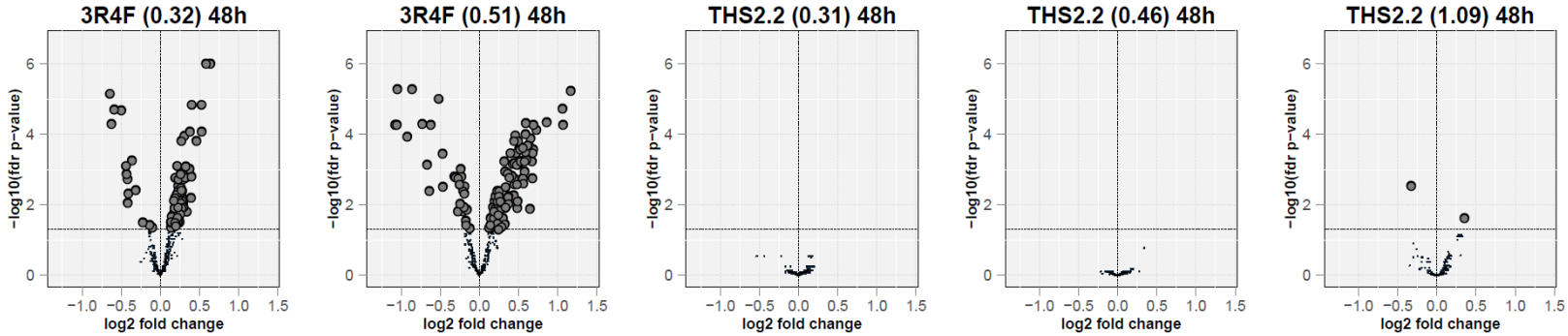


Figure 13. Volcano Plots Representing Differentially Expressed miRNA.

Each dot in a plot represents a miRNA. The change in miRNA expression (x-axis) is expressed as $\log_2(fold-change)$; increased when > 0 and decreased when < 0. The statistical significance (y-axis) is expressed as the $-\log_{10}(adjusted\ p-value)$ (N = 9–12 from four experimental repetitions with three exposure runs/repetition). The threshold line of statistical significance ($-\log_{10}(0.05)$) is drawn as a solid line (y-axis = 1.3).

Another representation of the global miRNA changes is illustrated as QQ plots **(Supplementary Figure 3)**. When differentially expressed miRNAs are not markedly visible (as in the volcano plots of the adjusted *p*-values in **Figure 13)**, QQ plots can provide an additional depiction of the miRNA alterations by comparing the similarity of the detected miRNA changes with those of randomly permuted miRNA expression matrices. The QQ plots suggested that 3R4F exposure may be related to significant global effects on miRNA expression levels. In contrast to the effects of 3R4F, THS2.2 aerosol exposure at all concentrations tested had a reduced effect on miRNA expression profiles. However, miR-375 was found to be significantly altered following both 3R4F (0.32, 0.51 mg/L) and THS2.2 (only highest concentration, 1.09 mg/L) exposure. The miRNAs that were significantly altered in at least one comparison are listed in **Figure 14**. At the comparable nicotine concentrations, THS2.2 did not affect the expression levels of these miRNAs.

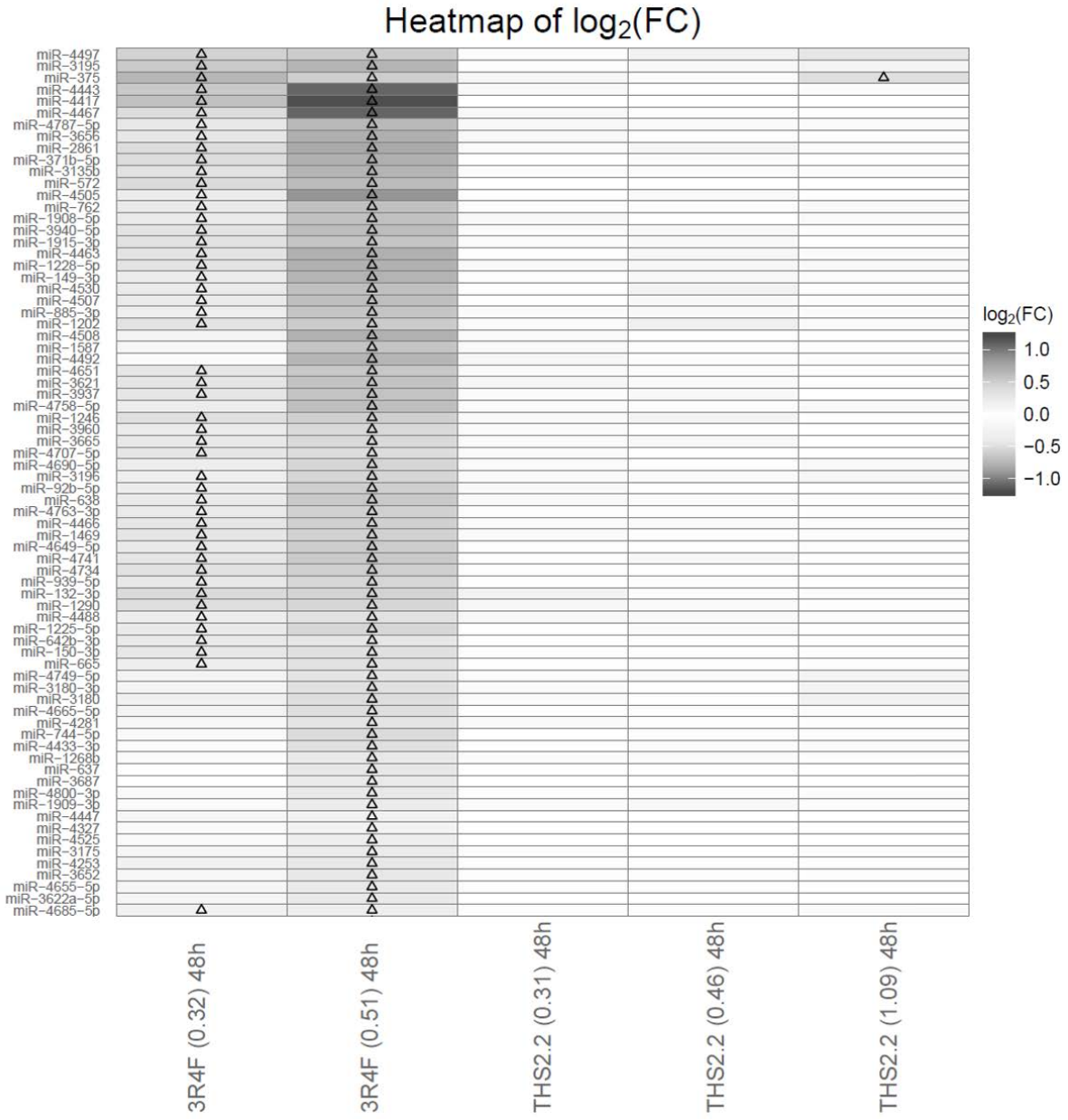
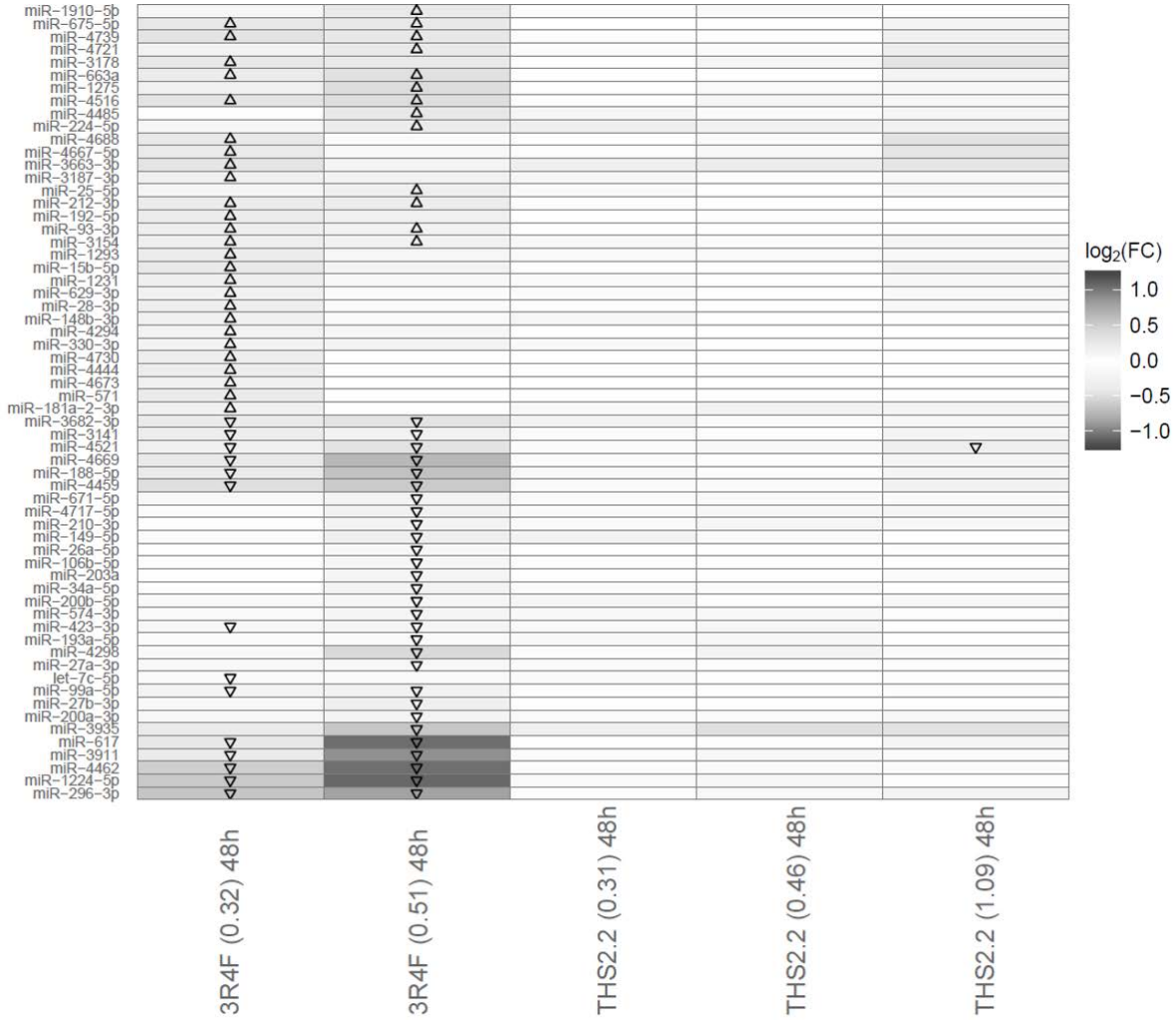


Figure continues



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Figure 14. Heatmap of differentially expressed miRNAs after 3R4F and THS2.2 exposure.

The heatmap shows changes in miRNA expression levels expressed as $\log_2(fold-change)$; increased expression when > 0 and decreased when < 0, compared with the corresponding air controls. The and signs indicate increased and decreased expression levels, respectively (N = 9–12/group from four experimental repetitions with three exposure runs/repetition). The miRNAs that were significantly altered in at least one comparison are listed on the left side of the heatmap. The shading indicates the extent of the absolute fold-changes compared with the controls.

9.8 Correlation of the Outcomes across the Experimental Repetitions

9.8.1 Correlation of Cytotoxicity, CYP1A1/1B1 Activity, and Inflammatory Mediators Results across 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. 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. 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 1 and 2 (the x-axis, **Figure 15)** and those obtained in experimental repetitions 3 and 4 (the y-axis, **Figure 15)**. The plots are separated into stimulus exposure type. They illustrate that these averaged values obtained from the experimental repetition 1 and 2, were correlated to those obtained from the experimental repetition 3 and 4. Greater correlations were observed for 3R4F CS exposure as compared with THS2.2 aerosol exposure. This is consistent with the greater biological impact upon 3R4F exposure (greater cytotoxicity and greater secretion of pro-inflammatory mediators) than upon THS2.2 exposure.

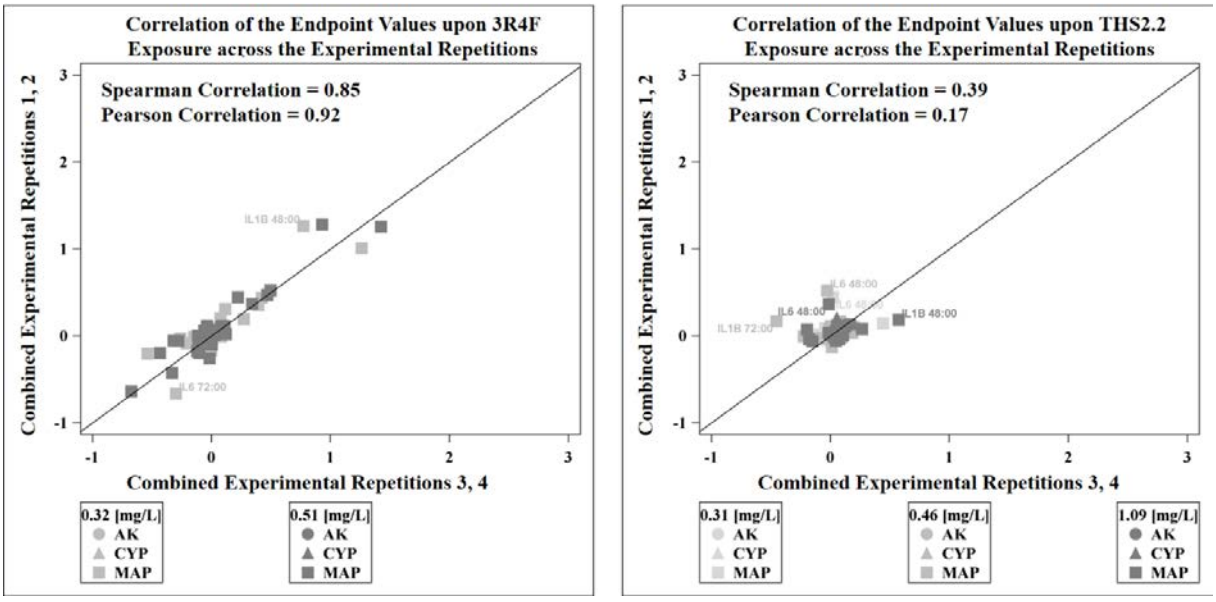


Figure 15. Correlation plots of the cytotoxicity, CYP1A1/1B1 activity, and pro-inflammatory mediators across the experimental repetitions.

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 and 4 (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). 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; MAP, multianalyte profiling.

9.8.2 Correlation of the Gene Expression Fold-Changes across the Experimental Repetitions

Correlation plots were generated to assess the reproducibility of the gene expression changes obtained from the different experimental repetitions. We first combined the gene expression data from experiments 1 and 2 and generated the mean of the fold-changes for each gene in the dataset. Similarly, we combined the gene expression data from experiments 3 and 4 and generated the mean of the fold-changes for each gene in the dataset. Finally, correlation plots for the different comparisons were generated, with each gene represented by a dot **(Figure 16)**.

The plots show that at the different post-exposure time points, the gene expression changes in cultures exposed to 3R4F (0.32 mg/L and 0.51 mg/L) were highly correlated between those obtained in experiments 1 and 2 and those obtained in experiments 3 and 4; the correlation values ranged from 0.72 to 0.91). The correlations between the gene expression fold-changes in THS2.2 aerosol-exposed cultures (especially 0.31 and 0.46 mg/L) were reduced; this finding was expected because the expression profiles of only a small number of genes were altered following THS2.2 exposure **(Figure 7)**.

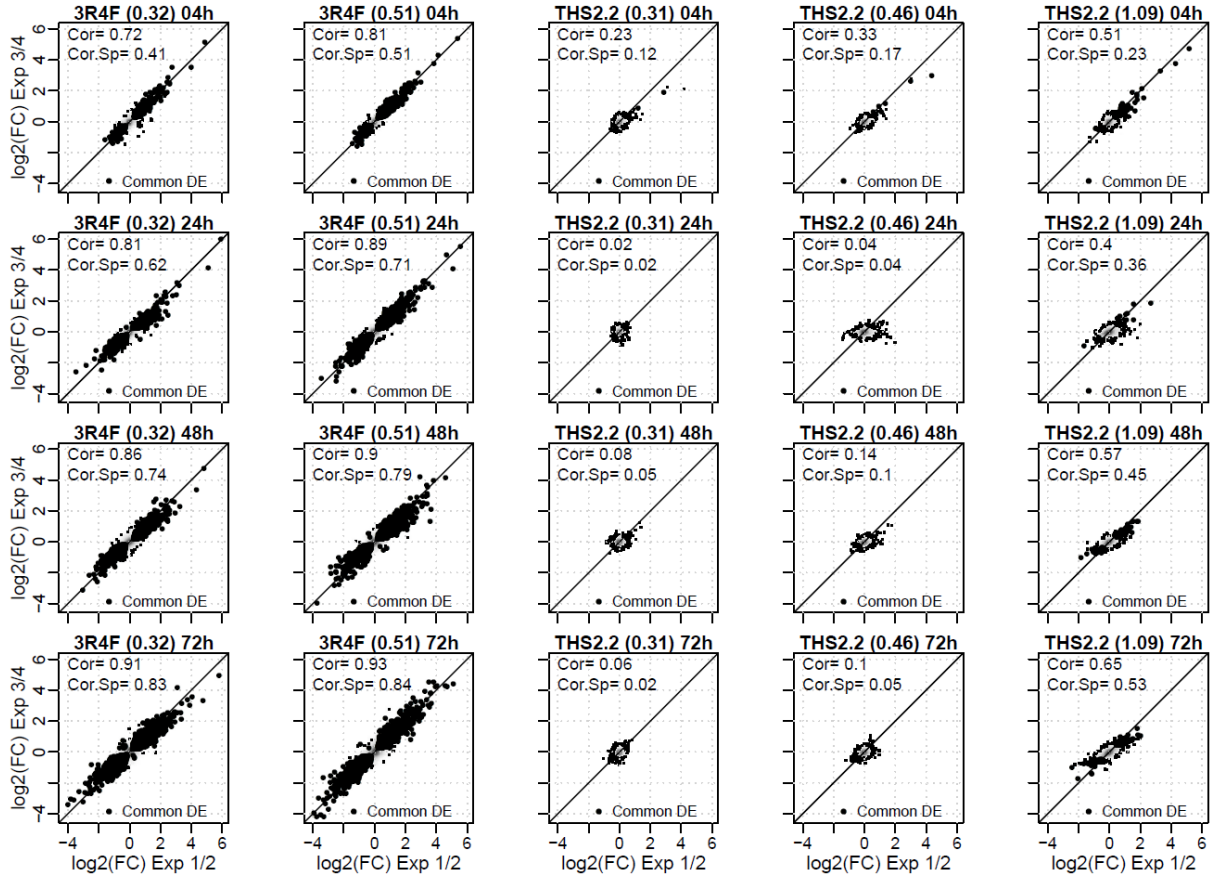


Figure 16. Correlation plots of gene fold-changes generated from the microarray analysis.
Each dot represents a gene. The fold-change of a given gene from the combined experiments 1 and 2 (x-axis; N = 6; each experiment performed in triplicate) was compared with the fold-change of the same gene from the combined experiments 3 and 4 (y-axis; N = 6; each experiment performed in triplicate). Abbreviations: Cor, correlation; Sp, Spearman correlation; DEG, differentially expressed gene.

9.8.3 Reproducibility of the miRNA Expression Fold-changes

A similar approach (to the one for assessing the reproducibility of the mRNA changes) was used to assess the reproducibility of the miRNA expression data from the different experimental repetitions at 48 h post-exposure. The miRNA expression data from experiments 1 and 2 were combined and used to compute a single value of fold-changes for each of the detected miRNAs. Similarly, the combined data from experiments 3 and 4 were used to compute a single value of the fold-changes of the miRNA. Finally, correlations between the fold-changes for all detected miRNAs in the datasets were computed for each comparison **(Figure 17)**.

Good correlations of the miRNA fold-changes were obtained across the experimental repetitions from the cultures exposed to 3R4F CS compared with the corresponding air controls; the correlation values ranged from 0.73 and 0.81). Correlation data demonstrated that when the levels of miRNA expression were greatly altered following the exposure, the signals (fold-changes of the miRNAs) were highly reproducible across the experimental repetitions, especially when the number of altered miRNAs was high.

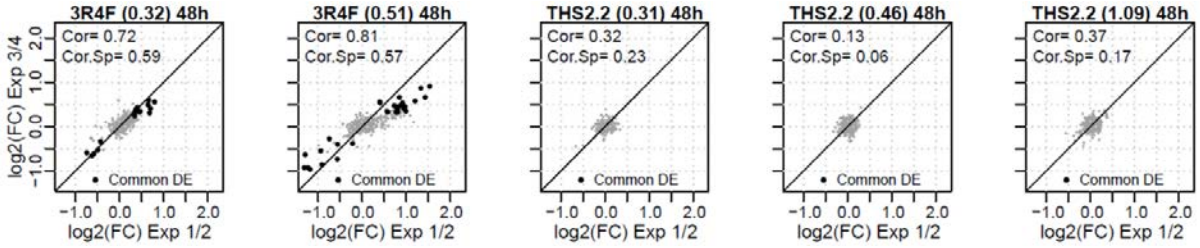


Figure 17. Correlation plots of miRNA fold-changes generated from miRNA array analysis.
Each dot represents a miRNA. The fold-change of a given miRNA from the combined experiments 1 and 2 (x-axis; N = 6; with triplicates per experiment) was compared with the fold-change of the same gene from the combined experiments 3 and 4 (y-axis; N = 6; with triplicates per experiment). Abbreviations: Cor, correlation; Sp, Spearman correlation.

9.9 Dose Range Assessment of THS2.2 for Cytotoxicity and Culture Histology

In addition to the concentrations of THS2.2 aerosol used in the above experiments, the effects of a wider range of THS2.2 concentrations on cytotoxicity and culture morphology were examined. We assessed the effects at 72 h post-exposure, because the greatest impact on cytotoxicity and overt alterations of the culture morphology were expected at the later time point.

Various dilutions of the THS2.2 aerosol were applied within the Dilution/Distribution Module of the Vitrocell system to assess the impact of the aerosols. **Table 14** shows the actual nicotine concentrations measured in the aerosol at the various dilutions that were tested in the dose range assessment. Three independent exposure runs were conducted for this dose range assessment.

Values corresponding to additional applied nicotine concentrations (0.14, 0.55, 1.13 mg/L) were not included in the results. Since the nicotine values for these samples were not proportional with the aerosol dilution and with the reported samples, these results were omitted.

Table 14. Actual Nicotine Concentrations in THS2.2 Aerosol from Various Dilutions

Group	Dose Range Assessment (11 August, 2015)	Actual Nicotine Concentrations in the Aerosol
	Aerosol Dilution	Mean (mg/L) ± SEM
Air Control (THS2.2)	-	NA
THS2.2 Dilution 1	25%	0.336 ± 0.014(N = 3)
THS2.2 Dilution 2	32%	0.45 ± 0.078 (N = 3)
THS2.2 Dilution 3	80%	1.024 ± 0.256 (N = 3) *
THS2.2 Dilution 4	100%	1.786 ± 0.096 (N = 3) *

The actual nicotine concentrations for a given aerosol dilution were obtained from three independent samplings (shown as the mean ± SEM). Abbreviation: SEM, standard error of the mean. *The values reported belong to a different set of control experiments performed on 8 July, 2015.

Figure 18 shows that no cytotoxicity was present at 72 h post-exposure in cultures exposed to THS2.2 aerosol at any of the nicotine concentrations tested. Descriptive statistics for the cytotoxicity are reported in **Supplementary Table 35**. This observation was also consistent with the absence of culture damage observed in the histological sections of cultures collected 72 h after exposure **(Figure 19)**.

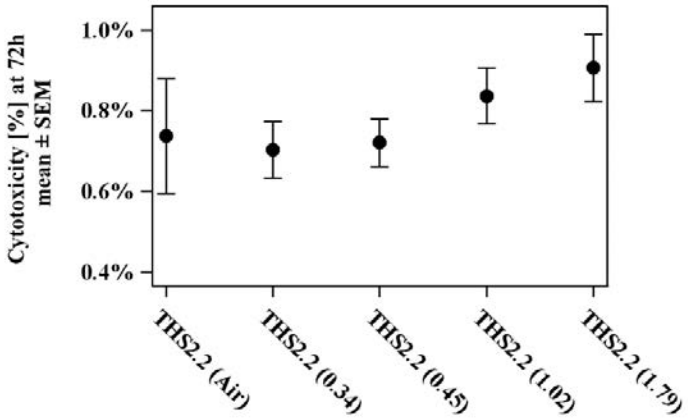


Figure 18. Cytotoxicity following exposure to various dilutions of THS2.2 aerosol.
The release of adenylate kinase (AK) into the basolateral media at 72 h post-exposure to THS2.2 aerosol for 28 min at the indicated nicotine concentrations (mg/L) was measured to determine cytotoxicity. The mean cytotoxicity (normalized cytotoxicity relative to the positive control (Triton X-100-treated culture taken as 100% cytotoxicity)) ± SEM (N = 3 independent exposure runs; one replicate per exposure run) is shown. Abbreviation: SEM, standard error of the mean.

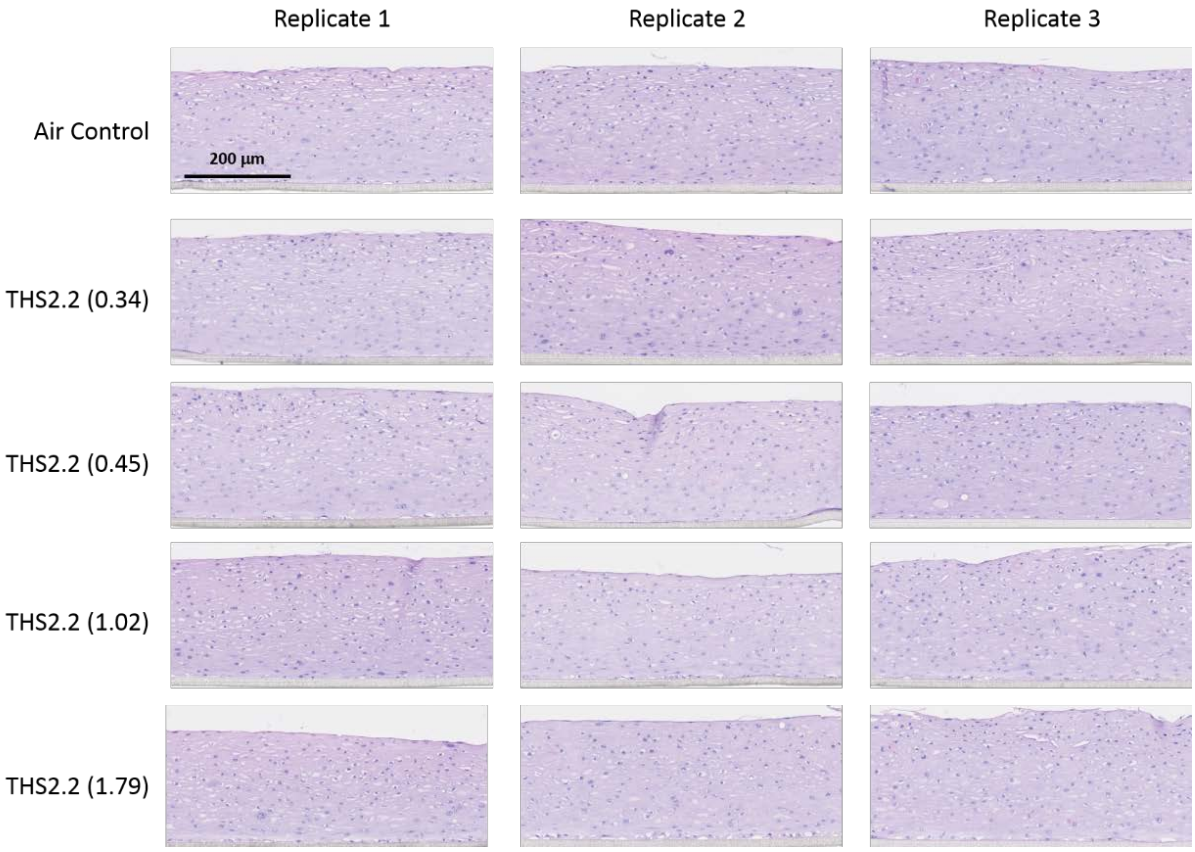


Figure 19. Histology following exposure to various concentrations of THS2.2 aerosol.
Culture sections stained with H&E at 72 h post-exposure to THS2.2 aerosol for 28 min at the indicated nicotine concentrations (mg/L). Abbreviation: H&E, hematoxylin and eosin

10. Discussion and Conclusion

This study examined the biological effects of exposure to THS2.2 aerosol compared with exposure to 3R4F CS at comparable nicotine concentrations on organotypic oral epithelial cultures. To reduce the variability of the biological effects of exposure, the study used oral epithelial cultures derived from a single donor. Moreover, to obtain robust data that could be leveraged to understand the exposure effects, four experimental repetitions were conducted. Within each of the experimental repetitions, three independent exposure runs were carried out. The results reported here are the aggregated data obtained from the four experimental repetitions. In addition, a dose range assessment of THS2.2 aerosol was conducted, in which higher concentrations of THS2.2 aerosol were tested.

3R4F CS and THS2.2 aerosol were generated using two independent smoking machines connected to two different Vitrocell exposure systems, each dedicated to either 3R4F or THS2.2. The Vitrocell system has a base module, in which the oral organotypic cultures were exposed to smoke or aerosol at the ALI. The suitability of the Vitrocell system for the assessment of smoke exposure has been reported previously (Adamson 2013, Majeed 2014). An exposure in an ALI permits exposures that are physiologically relevant to an actual smoking situation. The Vitrocell system also has a dilution module in which various dilutions of 3R4F CS or THS2.2 aerosol can be administered simultaneously. To compare the biological impact of the test aerosol relative to the reference smoke, two of the tested doses of THS2.2 aerosol were set at nicotine concentrations that were comparable to those of 3R4F CS. Nicotine measurements in smoke/aerosol samples trapped in the exposure system confirmed that the dilutions of the smoke/aerosol had successfully reached the target nicotine concentrations.

The impact of THS2.2 aerosol exposure on cytotoxicity at any of the concentrations tested was lower than that of 3R4F CS exposure. At the equivalent nicotine concentrations, AK release as a biomarker of cytotoxicity was lower after THS2.2 aerosol exposure compared with the levels after 3R4F CS exposure at the 48- and 72-h post-exposure time points. This is consistent with the findings of the histological evaluation. In addition, we obtained largely similar results from our previous study, which employed buccal organotypic cell cultures with a fibroblast layer and a repeated exposure design, where buccal cultures elicited a weak adaptive response to CS exposure (Schlage 2014). The response observed in the previous study implied an increase in culture thickness and keratinization; in the current study we found no difference in thickness but a significantly high score for apical/ectopic keratinization in 3R4F CS-exposed cultures, which is in agreement with the observations of Schlage et al. Furthermore, the increased number of intracellular fine granular material observed in the cultures might be indicative of an induced differentiation process moving towards the formation of cornified layers; indeed, these structures resemble the morphology of granules present in the stratum granulosum of keratinized epithelia (Muller 1980, Plackova 1975, Singh 1979). However, apart from the proportional increase of the latter morphological finding with CS concentration, it must be noted that intracellular fine granular material could also be observed in some incubator controls (but not in sham controls), making further analysis necessary to determine their treatment specificity. 3R4F CS induced other relevant histopathological modifications in the oral cultures: epithelial detachment above basal level, longitudinal cleft formation, and desquamation; these were more prominent at 72 h post-exposure, regardless of the nicotine concentration. Interestingly, detachment of the epithelium above basal cell

level is a feature that we observed in other 3D culture types exposed to CS (data not shown). It is deemed to be related to impaired cellular adherence; hence, further analysis of the status of the cellular junctions (i.e., E-cadherin) in this model could help to understand the mechanism behind this finding, particularly because it was also observed in untreated controls. Conversely, THS2.2 aerosol-exposed cultures did not show any particular signs of culture damage when compared with air-exposed controls, with only increased desquamation found as a significant feature, although with a much lower score than 3R4F-exposed cultures. Nonetheless, the assessment of the study made by a single pathologist plus the variability observed within the cultures (that may also be ascribed to the different insert brands used for experiments 1–2 and 3–4) make further verification of these observations necessary.

The combined activity of two phase I xenobiotic metabolizing enzymes, CYP1A1 and 1B1, involved in the metabolism of tobacco smoke constituents (Anttila 2011, Port 2004), was not enhanced by CS, although a strong transcriptional upregulation was observed, which is in agreement with our previous observations (Schlage 2014). THS2.2 aerosol caused a concentration-proportional increase in the activity of CYP1A1/1B1 at 24 and 48 h post-exposure.
It is likely that constituents present in 3R4F CS, but not in THS2.2 aerosol, elicit a culture-specific mechanism that interferes with the translation or post-translational processing of the CYP1A1/1B1 polypeptides, increases their degradation, or eventually inhibits their activity. It has been shown that alpha-tocopherol can inhibit benzo[a]pyrene-induced CYP1A1 activity in rat liver microsomes, likely by acting at the post-translational level (Sidorova 2005). Moreover, it has been shown that metal-induced heme oxygenase is a potent inhibitor of TCDD-induced CYP1A1 enzyme activity in human and rat hepatocytes (Amara 2010, Anwar-Mohamed 2012, Korashy 2012). In this study, notably, heme oxygenase (HMOX1) transcription was also strongly induced by the 3R4F CS exposure at all time points, but only transiently (4 h) by the THS2.2 aerosol (see **Figure 11**). Finally, other prototypic heat-not-burn products have been observed to induce CYP1A1 expression in rat lungs (Kogel 2014), as did an aerosol of nicotine in PBS (Phillips 2015). A deeper analysis of the contribution of various CS constituents and their specific pathophysiological effects on buccal keratinocytes will be needed to fully understand the differential regulation of CYP by 3R4F CS or THS2.2 aerosol.

Altered concentrations of secreted inflammatory mediators were found in the basolateral media of cultures following 3R4F CS exposure, including increased levels of IL1B, MMP-1, VEGFA, and TGFA, and decreased levels of CSF2, CXCL8 (only at 72 h – 0.51 mg/L), CXCL10 and IL6 compared with air-exposed controls. These results correspond with observations by Schlage et al., with the exception of IL6, which did not show any alteration (Schlage 2014). This discrepancy may be due to the presence of fibroblasts underneath the membrane in the oral culture model used for that study; indeed, we recently demonstrated that fibroblasts co-cultured with organotypic bronchial cultures may hamper the secretion of mediators in the basolateral medium (Iskandar 2015). The exposure to THS2.2 aerosol at comparable nicotine concentrations led to a much lower secretion of inflammatory mediators when compared with 3R4F CS-exposed samples.

The present study aimed to deduce the exposure-related mechanisms associated with 3R4F CS and THS2.2 aerosol by evaluating the expressions of global mRNA and miRNA in the cultures. We used a network-based systems biology approach to assess the biological impact of the exposures using mRNA profiles. This approach has been used successfully to investigate the impact of CS

exposure on various organotypic epithelial culture models (Iskandar 2015, Schlage 2014, Talikka 2014). We found that the impact of the exposure was higher and sustained in time for 3R4F CS-exposed cultures, whereas the response following THS2.2 exposure was lower and markedly decreased with longer post-exposure time points.

While a significantly enhanced NPA for cell stress was also observed for 3R4F in our previous study (Schlage 2014), the increased NPA scores for cell stress, cell cycle, and senescence were all below the significance threshold. These data were based on a different algorithm, and therefore some possible differences stemming from the computational approach must be taken into account. Moreover, the presence of fibroblasts and Langerhans cells in Schlage et al. may account for the difference in the network response profile. However, despite these differences, the overall BIF profile for the common time points analyzed (4, 24, 48 h post-exposure) was comparable, indicating an overall common perturbation of the system in the different studies.

We reported the impact of CS on several genes involved in oxidative stress and xenobiotic metabolism. Interestingly, some of these were reported to be upregulated in human buccal mucosa biopsies of smokers (i.e., *CYP1A1*/*1B1*, *NQO1*, *GPX2*, *ALDH3A1*, *AKR1C1*) (Boyle 2010).
Gene sets related to adherens junctions appeared to be more strongly downregulated by 3R4F CS than THS2.2 aerosol. Tight junctions are reported to be disrupted by CS exposure (Coppe 2008); in our gene-set analysis, we showed that the network corresponding to tight junctions was upregulated, probably because of an adaptive response to CS. Pathways related to cancer (pathways in cancer, chemical carcinogenesis) were also upregulated by CS exposure, but not by THS2.2 aerosol at the comparable nicotine concentrations. Furthermore, the tumor suppressor factor p53 pathway, after an initial period of upregulation (4 h, possibly an adaptive response), was ultimately downregulated by 3R4F CS; an effect not observed following THS2.2 exposure. Apoptotic pathways were upregulated by 3R4F CS, despite the lack of correspondence with the histological analysis. It is likely that CS compounds were activating the apoptotic molecular machinery without leading to an overt apoptotic phenotype, as already described (Zanetti 2014). The strong impact of CS observed for lipid metabolism (ether lipid, fatty acids, arachidonic acid) and signaling and the reduced effect of THS2.2 aerosol are in line with what we demonstrated previously in mice lungs (Titz 2016). In particular, the current study shows a CS-induced upregulation of the signaling pathway related to sphingolipids, which are key signaling mediators regulating the immune response, as well as apoptosis, survival, and differentiation (Gilroy 2010, Lee 2015). This effect was not observed for THS2.2 aerosol-exposed cultures. Upregulation of drug-metabolizing cytochrome P450 was observed at all time points tested (except 4 h PE), in agreement with previous observations (Schlage 2014).
In our study, the impact of THS2.2 aerosol on these genes was generally negligible at the 3R4F-comparable nicotine concentrations, or limited to the early post-exposure time point (4 h).

A dose range assessment of THS2.2 aerosol was conducted to evaluate the likelihood and severity of adverse effects in oral cultures. The dose range assessment aimed to evaluate the toxicity related to a broader range of THS2.2 aerosol concentrations than was used in the previous experiments, and to allow for an observation of critical doses where the cultures exhibited damage. Neither cytotoxic effects nor a major histopathological effect were observed at any tested THS2.2 aerosol concentration.

Finally, reproducibility testing was conducted to assess the consistency of the observations across the four experimental repetitions for cytotoxicity, CYP1A1/1B1 activity, secreted inflammatory mediators, and fold-changes of mRNA/miRNA expression. The reproducibility tests indicated that most of the observations were minimally affected by the experimental repetitions, suggesting that the design of the study was considerably robust.

Although in this study, donor-specific factors cannot be excluded and could be associated with the exposure response of the cultures that we used (e.g., undetected genetic variations influencing the outcome of the experiments), the overall findings show a substantially reduced impact of THS2.2 aerosol exposure on oral cultures compared with 3R4F CS exposure at comparable nicotine concentrations. Future studies using a larger number of donors could confirm the observations reported here.

11. ARCHIVING

After completion of the study, the following will be archived: Study Plan with any amendment, test/reference item retention samples, all raw data, report with any amendment, and all study-related records needed to reconstruct the study. These will be retained for at least 10 years according to the applicable archiving procedures. Paper records will be stored 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.

12. REFERENCES

12.1 Literature

GeneChip miRNA 3.0 Array: 902019. Available at: http://www.affymetrix.com/catalog/131473/AFFY/miRNA-Array#1_1 Affymetrix, Inc.

Ackermann M, Strimmer K. A general modular framework for gene set enrichment analysis. BMC bioinformatics. 2009;10(1):47. PubMed PMID: doi:10.1186/1471-2105-10-47.

Adamson J, Thorne D, Dalrymple A, Dillon D, Meredith C. Assessment of cigarette smoke particle deposition within the Vitrocell(R) exposure module using quartz crystal microbalances. Chem Cent J. 2013;7:50. Epub 2013/03/19. doi: 10.1186/1752-153X-7-50
1752-153X-7-50 [pii]. PubMed PMID: 23497606; PubMed Central PMCID: PMC3635897.

Affymetrix miRNA 4.0 Array. Affymetrix miRNA 3.0 Array. GeneChip miRNA 3.0 Array: 902019. Available at: //affymetrix.com/catalog/131473/AFFY/miRNA-Array#1_1.

Affymetrix miRNA QCT Tool. Affymetrix miRNA QCTool: User's Guide. Available at: //media.affymetrix.com/support/downloads/manuals/mirna_qctool_user_manual.pdf. 2011.

Amara IE, Anwar-Mohamed A, El-Kadi AO. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. Toxicology letters. 2010;199(3):225-33. doi: 10.1016/j.toxlet.2010.09.003. PubMed PMID: 20837117.

Andrian E, Grenier D, Rouabhia M. In vitro models of tissue penetration and destruction by Porphyromonas gingivalis. Infection and immunity. 2004;72(8):4689-98. doi: 10.1128/IAI.72.8.4689-4698.2004. PubMed PMID: 15271930; PubMed Central PMCID: PMC470627.

Anttila S, Raunio H, Hakkola J. Cytochrome P450-mediated pulmonary metabolism of carcinogens: regulation and cross-talk in lung carcinogenesis. Am J Respir Cell Mol Biol. 2011;44(5):583-90. Epub 2010/11/26. doi: 10.1165/rcmb.2010-0189RT
2010-0189RT [pii]. PubMed PMID: 21097654.

Anwar-Mohamed A, Klotz LO, El-Kadi AO. Inhibition of heme oxygenase-1 partially reverses the arsenite-mediated decrease of CYP1A1, CYP1A2, CYP3A23, and CYP3A2 catalytic activity in isolated rat hepatocytes. Drug metabolism and disposition: the biological fate of chemicals. 2012;40(3):504-14. doi: 10.1124/dmd.111.042564. PubMed PMID: 22159698.

Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300. doi: 10.2307/2346101.

BeruBe K, Aufderheide M, Breheny D, Clothier R, Combes R, Duffin R, et al. In vitro models of inhalation toxicity and disease. The report of a FRAME workshop. Altern Lab Anim. 2009;37(1):89-141. Epub 2009/03/19. PubMed PMID: 19292579.

Bolstad B, Collin F, Brettschneider J, Simpson K, Cope L, Irizarry R, et al. Quality assessment of Affymetrix GeneChip data. Bioinformatics and computational biology solutions using R and bioconductor: Springer; 2005. p. 33-47.

Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003;19(2):185-93. Epub 2003/01/23. PubMed PMID: 12538238.

Boue S, Talikka M, Westra JW, Hayes W, Di Fabio A, Park J, et al. Causal biological network database: a comprehensive platform of causal biological network models focused on the pulmonary and vascular systems. Database (Oxford). 2015;2015:bav030. Epub 2015/04/19. doi: 10.1093/database/bav030
bav030 [pii]. PubMed PMID: 25887162; PubMed Central PMCID: PMC4401337.

Boyle JO, Gumus ZH, Kacker A, Choksi VL, Bocker JM, Zhou XK, et al. Effects of cigarette smoke on the human oral mucosal transcriptome. Cancer prevention research. 2010;3(3):266-78. doi: 10.1158/1940-6207.CAPR-09-0192. PubMed PMID: 20179299; PubMed Central PMCID: PMC2833216.

Carpenter GH. The secretion, components, and properties of saliva. Annual review of food science and technology. 2013;4:267-76. doi: 10.1146/annurev-food-030212-182700. PubMed PMID: 23464573.

Carvalho BS, Irizarry RA. A framework for oligonucleotide microarray preprocessing. Bioinformatics. 2010;26(19):2363-7. Epub 2010/08/07. doi: 10.1093/bioinformatics/btq431. PubMed PMID: 20688976; PubMed Central PMCID: PMC2944196.

Ceder R, Merne M, Staab CA, Nilsson JA, Hoog JO, Dressler D, et al. The application of normal, SV40 T-antigen-immortalised and tumour-derived oral keratinocytes, under serum-free conditions, to the study of the probability of cancer progression as a result of environmental exposure to chemicals. Alternatives to laboratory animals : ATLA. 2007;35(6):621-39. PubMed PMID: 18186669.

Coppe JP, Boysen M, Sun CH, Wong BJ, Kang MK, Park NH, et al. A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion. Molecular cancer research : MCR. 2008;6(7):1085-98. doi: 10.1158/1541-7786.MCR-08-0062. PubMed PMID: 18644973; PubMed Central PMCID: PMC2768668.

Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, et al. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res. 2005;33(20):e175. Epub 2005/11/15. doi: 10.1093/nar/gni179. PubMed PMID: 16284200; PubMed Central PMCID: PMC1283542.

Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20(3):307-15. Epub 2004/02/13. doi: 10.1093/bioinformatics/btg405. PubMed PMID: 14960456.

Gilroy DW. Eicosanoids and the endogenous control of acute inflammatory resolution. The international journal of biochemistry & cell biology. 2010;42(4):524-8. doi: 10.1016/j.biocel.2009.12.013. PubMed PMID: 20026423.

Goldkorn T, Filosto S, Chung S. Lung injury and lung cancer caused by cigarette smoke-induced oxidative stress: Molecular mechanisms and therapeutic opportunities involving the ceramide-generating machinery and epidermal growth factor receptor. Antioxid Redox Signal. 2014;21(15):2149-74. Epub 2014/04/02. doi: 10.1089/ars.2013.5469. PubMed PMID: 24684526; PubMed Central PMCID: PMCPMC4215561.

Gower AC, Steiling K, Brothers JF, 2nd, Lenburg ME, Spira A. Transcriptomic studies of the airway field of injury associated with smoking-related lung disease. Proceedings of the American Thoracic Society. 2011;8(2):173-9. Epub 2011/05/06. doi: 10.1513/pats.201011-066MS. PubMed PMID: 21543797; PubMed Central PMCID: PMCPMC3159071.

Gualerzi A, Sciarabba M, Tartaglia G, Sforza C, Donetti E. Acute effects of cigarette smoke on three-dimensional cultures of normal human oral mucosa. Inhalation toxicology. 2012;24(6):382-9. doi: 10.3109/08958378.2012.679367. PubMed PMID: 22564096.

Hansson A, Bloor BK, Haig Y, Morgan PR, Ekstrand J, Grafstrom RC. Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. Oral oncology. 2001;37(5):419-30. Epub 2001/05/30. PubMed PMID: 11377230.

Hoeng J, Talikka M, Martin F, Sewer A, Yang X, Iskandar A, et al. Case study: the role of mechanistic network models in systems toxicology. Drug discovery today. 2014;19(2):183-92. Epub 2013/08/13. doi: 10.1016/j.drudis.2013.07.023. PubMed PMID: 23933191.

Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. Nat Methods. 2015;12(2):115-21. Epub 2015/01/31. doi: 10.1038/nmeth.3252. PubMed PMID: 25633503; PubMed Central PMCID: PMC4509590.

International Organization for Standardization. Tobacco and tobacco products -- Atmosphere for conditioning and testing, Switzerland, http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=28324. 1999.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 2003;31(4):e15. Epub 2003/02/13. PubMed PMID: 12582260; PubMed Central PMCID: PMC150247.

Iskandar AR, Xiang Y, Frentzel S, Talikka M, Leroy P, Kuehn D, et al. Impact Assessment of Cigarette Smoke Exposure on Organotypic Bronchial Epithelial Tissue Cultures: A Comparison of Mono-Culture and Coculture Model Containing Fibroblasts. Toxicological sciences : an official journal of the Society of Toxicology. 2015;147(1):207-21. doi: 10.1093/toxsci/kfv122. PubMed PMID: 26085348; PubMed Central PMCID: PMC4549394.

Johnson NW, Bain CA. Tobacco and oral disease. EU-Working Group on Tobacco and Oral Health. British dental journal. 2000;189(4):200-6. PubMed PMID: 11036748.

Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res. 2014;42(Database issue):D199-205. Epub 2013/11/12. doi: 10.1093/nar/gkt1076. PubMed PMID: 24214961; PubMed Central PMCID: PMC3965122.

Kauffmann A, Gentleman R, Huber W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. Bioinformatics. 2009;25(3):415-6. Epub 2008/12/25. doi: 10.1093/bioinformatics/btn647. PubMed PMID: 19106121; PubMed Central PMCID: PMC2639074.

Kentucky Tobacco Research & Development Center. 3R4F Preliminary Analysis. Available at: //ctrp.uky.edu/resources/pdf/webdocs/3R4F%20Preliminary%20Analysis.pdf.

Kentucky Tobacco Research & Development Center. Kentucky Tobacco Research & Development Center. 3R4F Preliminary Analysis. Available at: //ctrp.uky.edu/resources/pdf/webdocs/3R4F%20Preliminary%20Analysis.pdf.

Kimball JR, Nittayananta W, Klausner M, Chung WO, Dale BA. Antimicrobial barrier of an in vitro oral epithelial model. Archives of oral biology. 2006;51(9):775-83. doi: 10.1016/j.archoralbio.2006.05.007. PubMed PMID: 16815238; PubMed Central PMCID: PMC2376809.

Klausner M, Ayehunie S, Breyfogle BA, Wertz PW, Bacca L, Kubilus J. Organotypic human oral tissue models for toxicological studies. Toxicology in vitro : an international journal published in association with BIBRA. 2007;21(5):938-49. doi: 10.1016/j.tiv.2007.01.024. PubMed PMID: 17383851.

Kogel U, Schlage WK, Martin F, Xiang Y, Ansari S, Leroy P, et al. A 28-day rat inhalation study with an integrated molecular toxicology endpoint demonstrates reduced exposure effects for a prototypic modified risk tobacco product compared with conventional cigarettes. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2014;68:204-17. doi: 10.1016/j.fct.2014.02.034. PubMed PMID: 24632068.

Korashy HM, El-Kadi AO. Transcriptional and posttranslational mechanisms modulating the expression of the cytochrome P450 1A1 gene by lead in HepG2 cells: a role of heme oxygenase. Toxicology. 2012;291(1-3):113-21. doi: 10.1016/j.tox.2011.11.006. PubMed PMID: 22120038.

Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic acids research. 2013. doi: 10.1093/nar/gkt1181.

Kupfer DM, White VL, Jenkins MC, Burian D. Examining smoking-induced differential gene expression changes in buccal mucosa. BMC medical genomics. 2010;3:24. doi: 10.1186/1755-8794-3-24. PubMed PMID: 20576139; PubMed Central PMCID: PMC3161387.

Lee J, Yeganeh B, Ermini L, Post M. Sphingolipids as cell fate regulators in lung development and disease. Apoptosis : an international journal on programmed cell death. 2015;20(5):740-57. doi: 10.1007/s10495-015-1112-6. PubMed PMID: 25753687; PubMed Central PMCID: PMC4376961.

Majeed S, Frentzel S, Wagner S, Kuehn D, Leroy P, Guy PA, et al. Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke. Chem Cent J. 2014;8(1):62. Epub 2014/11/21. doi: 10.1186/s13065-014-0062-3
62 [pii]. PubMed PMID: 25411580; PubMed Central PMCID: PMC4236458.

Martin F, Sewer A, Talikka M, Xiang Y, Hoeng J, Peitsch MC. Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models. BMC bioinformatics. 2014;15:238. doi: 10.1186/1471-2105-15-238. PubMed PMID: 25015298; PubMed Central PMCID: PMC4227138.

Martin F, Thomson T, Sewer A, Drubin D, Mathis C, Weisensee D, et al. Assessment of network perturbation amplitudes by applying high-throughput data to causal biological networks. BMC Systems Biology. 2012;6(1):54. doi: 10.1186/1752-0509-6-54. PubMed PMID: doi:10.1186/1752-0509-6-54.

Mattek. EpiOral and EpiGingival. In: Corporation M, editor. Ashland, MA2012.

McCall MN, Bolstad BM, Irizarry RA. Frozen robust multiarray analysis (fRMA). Biostatistics. 2010;11(2):242-53. doi: 10.1093/biostatistics/kxp059.

Michcik A, Cichorek M, Daca A, Chomik P, Wojcik S, Zawrocki A, et al. Tobacco smoking alters the number of oral epithelial cells with apoptotic features. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society. 2014;52(1):60-8. doi: 10.5603/FHC.2014.0007. PubMed PMID: 24802962.

Mostefaoui Y, Claveau I, Ross G, Rouabhia M. Tissue structure, and IL-1beta, IL-8, and TNF-alpha secretions after contact by engineered human oral mucosa with dentifrices. Journal of clinical periodontology. 2002;29(11):1035-41. PubMed PMID: 12472997.

Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of Candida albicans in epithelial cells. Cell host & microbe. 2010;8(3):225-35. doi: 10.1016/j.chom.2010.08.002. PubMed PMID: 20833374; PubMed Central PMCID: PMC2991069.

Muller W, Schroeder HE. Differentiation of the epithelium of the human hard palate. Cell and tissue research. 1980;209(2):295-313. PubMed PMID: 6156766.

Paszkiewicz GM, Timm EA, Jr., Mahoney MC, Wallace PK, Sullivan Nasca MA, Tammela TL, et al. Increased human buccal cell autofluorescence is a candidate biomarker of tobacco smoking. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2008;17(1):239-44. doi: 10.1158/1055-9965.EPI-07-0162. PubMed PMID: 18199730.

Phillips B, Esposito M, Verbeeck J, Boue S, Iskandar A, Vuillaume G, et al. Toxicity of aerosols of nicotine and pyruvic acid (separate and combined) in Sprague-Dawley rats in a 28-day OECD 412 inhalation study and assessment of systems toxicology. Inhalation toxicology. 2015;27(9):405-31. doi: 10.3109/08958378.2015.1046000. PubMed PMID: 26295358.

Plackova A, Skach M. [Fine structural study of keratinization of the filiform papillae in the tongue in humans]. Zeitschrift fur mikroskopisch-anatomische Forschung. 1975;89(2):305-18. PubMed PMID: 1224766.

Port JL, Yamaguchi K, Du B, De Lorenzo M, Chang M, Heerdt PM, et al. Tobacco smoke induces CYP1B1 in the aerodigestive tract. Carcinogenesis. 2004;25(11):2275-81. Epub 2004/08/07. doi: 10.1093/carcin/bgh243 bgh243 [pii]. PubMed PMID: 15297370.

Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: function in health and disease. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2000;11(4):383-408. PubMed PMID: 11132762.

Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet. 2012;13(5):358-69. doi: http://www.nature.com/nrg/journal/v13/n5/suppinfo/nrg3198_S1.html.

R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/. 2013.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47. Epub 2015/01/22. doi: 10.1093/nar/gkv007 gkv007 [pii]. PubMed PMID: 25605792; PubMed Central PMCID: PMC4402510.

Sales G, Calura E, Cavalieri D, Romualdi C. graphite - a Bioconductor package to convert pathway topology to gene network. BMC bioinformatics. 2012;13(1):20. PubMed PMID: doi:10.1186/1471-2105-13-20.

Schlage WK, Iskandar AR, Kostadinova R, Xiang Y, Sewer A, Majeed S, et al. In vitro systems toxicology approach to investigate the effects of repeated cigarette smoke exposure on human buccal and gingival organotypic epithelial tissue cultures. Toxicol Mech Methods. 2014;24(7):470-87. Epub 2014/07/22. doi: 10.3109/15376516.2014.943441. PubMed PMID: 25046638; PubMed Central PMCID: PMC4219813.

Sidorova YA, Grishanova AY. Inhibitory effect of alpha-tocopherol on benzo(a)pyrene-induced CYPA1 activity in rat liver. Bulletin of experimental biology and medicine. 2005;140(5):517-20. PubMed PMID: 16758613.

Singh BB, Schuster GS, Merchant VA, Michelich VJ. Ultrastructural and autoradiographic observations of hamster cheek pouch epithelium grown in vitro. In vitro. 1979;15(11):865-72. PubMed PMID: 232058.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3. Epub 2006/05/02. doi: 10.2202/1544-6115.1027. PubMed PMID: 16646809.

Spira A, Beane JE, Shah V, Steiling K, Liu G, Schembri F, et al. Airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer. NatMed. 2007;13(3):361-6.

Spivack SD, Hurteau GJ, Jain R, Kumar SV, Aldous KM, Gierthy JF, et al. Gene-environment interaction signatures by quantitative mRNA profiling in exfoliated buccal mucosal cells. Cancer research. 2004;64(18):6805-13. doi: 10.1158/0008-5472.CAN-04-1771. PubMed PMID: 15375000.

Sridhar S, Schembri F, Zeskind J, Shah V, Gustafson AM, Steiling K, et al. Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. BMCGenomics. 2008a;9:259.:259.

Sridhar S, Schembri F, Zeskind J, Shah V, Gustafson AM, Steiling K, et al. Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. BMC genomics. 2008b;9:259. doi: 10.1186/1471-2164-9-259. PubMed PMID: 18513428; PubMed Central PMCID: PMC2435556.

Steiling K, Ryan J, Brody JS, Spira A. The field of tissue injury in the lung and airway. Cancer prevention research. 2008;1(6):396-403. doi: 10.1158/1940-6207.CAPR-08-0174. PubMed PMID: 19138985; PubMed Central PMCID: PMC2705781.

Talikka M, Kostadinova R, Xiang Y, Mathis C, Sewer A, Majeed S, et al. The response of human nasal and bronchial organotypic tissue cultures to repeated whole cigarette smoke exposure. Int J Toxicol. 2014;33(6):506-17. Epub 2014/10/10. doi: 10.1177/1091581814551647. PubMed PMID: 25297719.

Thomson TM, Sewer A, Martin F, Belcastro V, Frushour BP, Gebel S, et al. Quantitative assessment of biological impact using transcriptomic data and mechanistic network models. Toxicology and Applied Pharmacology. 2013;272(3):863-78. doi: http://dx.doi.org/10.1016/j.taap.2013.07.007.

Titz B, Boue S, Phillips B, Talikka M, Vihervaara T, Schneider T, et al. Effects of Cigarette Smoke, Cessation, and Switching to Two Heat-Not-Burn Tobacco Products on Lung Lipid Metabolism in C57BL/6 and Apoe-/- Mice-An Integrative Systems Toxicology Analysis. Toxicological sciences : an official journal of the Society of Toxicology. 2016;149(2):441-57. doi: 10.1093/toxsci/kfv244. PubMed PMID: 26582801; PubMed Central PMCID: PMC4725611.

Varemo L, Nielsen J, Nookaew I. Enriching the gene set analysis of genome-wide data by incorporating directionality of gene expression and combining statistical hypotheses and methods. Nucleic Acids Res. 2013;41(8):4378-91. Epub 2013/02/28. doi: 10.1093/nar/gkt111. PubMed PMID: 23444143; PubMed Central PMCID: PMC3632109.

Walle T, Walle UK, Sedmera D, Klausner M. Benzo[A]pyrene-induced oral carcinogenesis and chemoprevention: studies in bioengineered human tissue. Drug Metab Dispos. 2006;34(3):346-50.

Wang Y, Rotem E, Andriani F, Garlick JA. Smokeless tobacco extracts modulate keratinocyte and fibroblast growth in organotypic culture. Journal of dental research. 2001;80(9):1862-6. PubMed PMID: 11926249.

Winn DM. Tobacco use and oral disease. Journal of dental education. 2001;65(4):306-12. PubMed PMID: 11336115.

Zanetti F, Giacomello M, Donati Y, Carnesecchi S, Frieden M, Barazzone-Argiroffo C. Nicotine mediates oxidative stress and apoptosis through cross talk between NOX1 and Bcl-2 in lung epithelial cells. Free radical biology & medicine. 2014;76:173-84. doi: 10.1016/j.freeradbiomed.2014.08.002. PubMed PMID: 25151121.

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

The procedures and instructions followed to perform the study are listed below:

PMI_RD_WKI_001066
EXtrelut trapping for nicotine measurement

PMI_RD_WKI_000409
Perform Analysis: Determination of Nicotine in diluted aerosol

PMI_RD_WKI_001094
Sample preparation for Carbonyl measurements

PMI_RD_WKI_001064
Handling and maintenance of EpiOral cultures

PMI_RD_WKI_001048
Adenylate kinase assay for 3D cultures

PMI_RD_SOP_000361
Luminex

PMI_RD_WKI_001032
Calibration and Maintenance of LMX200

PMI_RD_WKI_001274
Procedure for Luminex assay (FM3D)

PMI_RD_FOR_000803
Luminex Assay Working Sheet

PMI_RD_FOR_000849
Treatment with controls for MAP analysis

PMI_RD_WKI_001242
Histology Fixation procedure

PMI_RD_WKI_001243
Culture Processing using LEICA ASP300S

PMI_RD_WKI_001260
Culture Paraffin Embedding
PMI_RD_WKI_001262
Sectioning Paraffin Blocks using Microtome

PMI_RD_WKI_001266
Haematoxylin & Eosin with or without Alcian Blue Staining Procedure for Formalin- Fixed Paraffin Embedded and Frozen Cultures Sections

PMI_RD_WKI_001314
Slides scanning with Nanozoomer

PMI_RD_WKI_001049
CYP assay for 3D cultures

PMI_RD_FOR_000846
Treatment and substrate preparation for CYP1A1/1B1 activity assay

PMI_RD_WKI_001117
Isolation of total RNA including microRNAs from Culture and Organotypic culture inserts using miRNeasy mini kit protocol

PMI_RD_WKI_000978
Quality controls following RNA extractions

PMI_RD_WKI_001109
Affymetrix IVT PLUS Protocol

PMI_RD_WKI_001125
Quality controls assessment of the Affymetrix IVT and Nugen amplification products using the Fragment Analyzer

PMI_RD_WKI_001126
Affymetrix-3'array-Cartridge-Hybridization (IVT_PLUS, NugenWB, PicoV2)

PMI_RD_WKI_001123
MiRNA labeling using the FlashTag Biotin HSR kits

PMI_RD_WKI_001228
Computational Practices for Omics (mRNA and miRNA) Analyses

PMI_RD_SOP_000346
Computational Processing for mRNA and miRNA Affymetrix Data

12.3 Philip Morris International (PMI) Internal Documents

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

PMI Internal Document. Unpublished data. Email Correspondence Regarding the Batch Issue. \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Analytical_Report_Signed_02-Mar-2016_ReTest_Stick_BatchNo_B-13056.pdf

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

PMI Internal Document. Device Information of THS2.2 Code No. B-178731 \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Batch_Certificates_CellSB_activities\P1_Device_Information_B-18731_Release_Date_Feb_2015.pdf

PMI Internal Document. Unpublished data. Certificate of buccal EpiOral CoA_Experiment 1 \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Acute human buccal - ZRH - S174300\Main Phase_CW16_2015_Part 3\Certificate of Analysis\S174300_MP3_CW16_2015_CoA_Donor 1_G26.pdf

PMI Internal Document. Unpublished data. Certificate of buccal EpiOral CoA_Experiment 2 \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Acute human buccal - ZRH - S174300\Main Phase_CW25_2015_Part 4\Certificate of Analysis\S174300_MP4_CW25_2015_CoA_Donor 1_G26.pdf

PMI Internal Document. Unpublished data. Certificate of buccal EpiOral CoA_Experiment 3 \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Acute human buccal - ZRH - S174300\Main Phase_CW29_2015_Part5\Certificate of Analysis\S174300_MP5_CW29_2015_CoA_Donor 1_G26.pdf

PMI Internal Document. Unpublished data. Certificate of buccal EpiOral CoA_Experiment 4-DRA \\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\Acute human buccal - ZRH - S174300\Main Phase_CW33_2015_Part 6\Certificate of Analysis\S174300_MP6_CW33_2015_CoA_Donor 1_G26.pdf

13. ABBREVIATIONS

Abbreviation/Term	
AK	Adenylate kinase
ALI	Air–liquid interface
BIF	Biological impact factor
cDNA	Complementary DNA
CFA	Cell fate
CoA	Certificate of Analysis
Cor	Correlation
CPR	Cell proliferation
cRNA	Complementary RNA
CS	Cigarette smoke
CSF	Colony-stimulating factor
CST	Cellular stress
CXCL	Chemokine (C-X-C motif) ligand
CYP	Cytochrome P450
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
DRA	Dose range assessment
FC	Fold-change
FDR	False discovery rate
fRMA	Frozen robust multiarray analysis
GC-FID	Gas chromatography–flame ionization detection
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSA	Gene-set analysis
H_2SO_4	Sulfuric acid

Abbreviation/Term	
H&E	Hematoxylin and eosin
mHCI	Modified Health Canada Intense
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
IL	Interleukin
IPN	Inflammatory process network
ISO	International Organization for Standardization
JAK	Janus kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCLM	Lower confidence limit of the mean
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
Limma	Linear Models for Microarray Data
MAD	Median absolute deviation
MAP	Multi-analyte profiling
MAPK	Mitogen-activated protein kinase
MARLE	Median absolute value relative log expression
mRNA	Messenger RNA
miRNA	Micro RNA
MMP	Matrix metalloproteinase
MRTP	Modified-risk tobacco product
mTOR	Mechanistic target of rapamycin
NA	Not available
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NHOC	Normal human oral cells
NPA	Network perturbation amplitude
Nrf2	See NFE2L2

Abbreviation/Term	
NUSE	Normalized-unscaled Standard Error
ORA	Over-representation analysis
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PE	Post-exposure
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
QC	Quality control
QQ	Quantile–quantile
RBIF	Relative BIF
REF	Reference
RLE	Relative log expression
RMA	Robust multiarray
RNA	Ribonucleic acid
SEM	Standard error of the mean
SM	Smoking machine
SOP	Standard Operating Procedure
STAT	Signal transducer and activator of transcription
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TGFA	Transforming growth factor alpha
THS	Tobacco Heating System (also referred to as ZRH)
TNFα	Tumor necrosis factor alpha
UCLM	Upper confidence limit of the mean
Untr	Untreated
WKI	Work instruction
Wnt	Proto-oncogene protein

Abbreviation/Term	
w/v	Weight/volume
v/v	Volume/volume
VEGFA	Vascular endothelial growth factor alpha
ZRH	See THS

14. TABLES AND SUPPLEMENTARY FIGURES

14.1 Supplementary Tables

14.1.1 Descriptive Statistics: Levels of Various Carbonyls

14.1.1.1 **Supplementary Table 1.** Acetaldehyde levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Acetaldehyde (deposited µg/mL)														
Year. Calendar week	**3R4F (Air)**		**3R4F (0.32)**		**3R4F (0.51)**		**THS2.2 (Air)**		**THS2.2 (0.31)**		**THS2.2 (0.46)**		**THS2.2 (1.09)**	
	M	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**
2014.14	0.00	0.00	1.93	0.17	NA	NA	0.00	0.00	0.75	0.13	NA	NA	NA	NA
2014.19	0.00	0.00	2.04	0.04	NA	NA	0.00	0.00	0.27	0.01	NA	NA	NA	NA
2014.25	0.00	0.00	1.95	0.13	NA	NA	0.00	0.00	0.18	0.02	0.45	0.01	NA	NA
2015.11	0.00	0.00	2.32	0.03	NA	NA	0.00	0.00	0.53	0.01	0.61	0.02	NA	NA
2015.12	0.00	0.00	3.47	0.47	NA	NA	0.00	0.00	1.03	0.09	1.17	0.23	NA	NA
2015.15	0.00	0.00	2.95	0.12	4.18	0.53	0.00	0.00	0.83	0.03	1.08	0.05	2.78	0.32
2015.16	0.00	0.00	1.91	0.03	NA	NA	0.00	0.00	0.31	0.04	0.44	0.02	NA	NA
2015.23	0.00	0.00	1.61	0.17	NA	NA	0.00	0.00	0.25	0.04	0.23	0.07	NA	NA
2015.25	0.00	0.00	3.55	0.14	NA	NA	0.00	0.00	0.94	0.05	NA	NA	2.96	0.13
2015.27	0.00	0.00	2.62	0.10	NA	NA	0.00	0.00	0.59	0.03	NA	NA	1.92	0.08

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.2 **Supplementary Table 2.** Acetone levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Acetone (deposited µg/mL)														
Year. Calendar week	3R4F (Air)		3R4F (0.32)		3R4F (0.51)		THS2.2 (Air)		THS2.2 (0.31)		THS2.2 (0.46)		THS2.2 (1.09)	
	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.03	0.00	1.07	0.15	NA	NA	0.05	0.00	0.19	0.03	NA	NA	NA	NA
2014.19	0.04	0.00	1.36	0.02	NA	NA	0.05	0.00	0.17	0.01	NA	NA	NA	NA
2014.25	0.03	0.00	1.21	0.09	NA	NA	0.02	0.01	0.13	0.00	0.17	0.00	NA	NA
2015.11	0.09	0.04	1.33	0.03	NA	NA	0.01	0.01	0.11	0.00	0.13	0.01	NA	NA
2015.12	0.00	0.00	1.36	0.12	NA	NA	0.00	0.00	0.17	0.02	0.19	0.02	NA	NA
2015.15	0.03	0.00	1.38	0.03	2.20	0.03	0.02	0.01	0.16	0.00	0.20	0.00	0.51	0.01
2015.16	0.00	0.00	1.26	0.01	NA	NA	0.01	0.01	0.11	0.00	0.13	0.01	NA	NA
2015.23	0.00	0.00	1.12	0.10	NA	NA	0.00	0.00	0.12	0.00	0.12	0.01	NA	NA
2015.25	0.19	0.12	1.79	0.01	NA	NA	0.08	0.00	0.23	0.01	NA	NA	0.56	0.02
2015.27	0.03	0.00	1.35	0.00	NA	NA	0.02	0.00	0.13	0.01	NA	NA	0.37	0.01

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.3 **Supplementary Table 3.** Acrolein Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Acrolein (deposited µg/mL)														
Year. Calendar week	3R4F (Air)		3R4F (0.32)		3R4F (0.51)		THS2.2 (Air)		THS2.2 (0.31)		THS2.2 (0.46)		THS2.2 (1.09)	
	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.10	0.00	NA	NA	0.00	0.00	0.03	0.01	NA	NA	NA	NA
2014.19	0.00	0.00	0.12	0.00	NA	NA	0.00	0.00	0.02	0.00	NA	NA	NA	NA
2014.25	0.00	0.00	0.15	0.01	NA	NA	0.00	0.00	0.02	0.00	0.03	0.00	NA	NA
2015.11	0.01	0.01	0.15	0.00	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.12	0.00	0.00	0.18	0.01	NA	NA	0.00	0.00	0.01	0.01	0.02	0.02	NA	NA
2015.15	0.00	0.00	0.17	0.00	0.29	0.01	0.00	0.00	0.03	0.00	0.03	0.00	0.08	0.00
2015.16	0.00	0.00	0.14	0.00	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.23	0.00	0.00	0.16	0.02	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.25	0.00	0.00	0.22	0.01	NA	NA	0.00	0.00	0.02	0.00	NA	NA	0.07	0.00
2015.27	0.00	0.00	0.11	0.00	NA	NA	0.00	0.00	0.02	0.00	NA	NA	0.04	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.4 **Supplementary Table 4.** Propionaldehyde Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Propionaldehyde (deposited µg/mL)														
Year. Calendar week	**3R4F (Air)**		**3R4F (0.32)**		**3R4F (0.51)**		**THS2.2 (Air)**		**THS2.2 (0.31)**		**THS2.2 (0.46)**		**THS2.2 (1.09)**	
	M	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**
2014.14	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.02	0.01	NA	NA	NA	NA
2014.19	0.00	0.00	0.11	0.00	NA	NA	0.00	0.00	0.03	0.00	NA	NA	NA	NA
2014.25	0.00	0.00	0.10	0.01	NA	NA	0.00	0.00	0.03	0.00	0.04	0.00	NA	NA
2015.11	0.01	0.01	0.13	0.00	NA	NA	0.00	0.00	0.03	0.00	0.04	0.00	NA	NA
2015.12	0.00	0.00	0.21	0.01	NA	NA	0.00	0.00	0.05	0.00	0.07	0.02	NA	NA
2015.15	0.00	0.00	0.18	0.01	0.29	0.01	0.00	0.00	0.06	0.00	0.07	0.00	0.18	0.01
2015.16	0.00	0.00	0.10	0.00	NA	NA	0.00	0.00	0.03	0.00	0.03	0.00	NA	NA
2015.23	0.00	0.00	0.10	0.01	NA	NA	0.00	0.00	0.03	0.00	0.03	0.00	NA	NA
2015.25	0.00	0.00	0.24	0.01	NA	NA	0.00	0.00	0.07	0.00	NA	NA	0.19	0.01
2015.27	0.00	0.00	0.14	0.00	NA	NA	0.00	0.00	0.04	0.00	NA	NA	0.10	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.5 **Supplementary Table 5.** Crotonaldehyde Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Crotonaldehyde (deposited µg/mL)														
Year. Calendar week	**3R4F (Air)**		**3R4F (0.32)**		**3R4F (0.51)**		**THS2.2 (Air)**		**THS2.2 (0.31)**		**THS2.2 (0.46)**		**THS2.2 (1.09)**	
	M	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**
2014.14	0.00	0.00	0.14	0.00	NA	NA	0.00	0.00	0.02	0.00	NA	NA	NA	NA
2014.19	0.00	0.00	0.12	0.00	NA	NA	0.00	0.00	0.01	0.00	NA	NA	NA	NA
2014.25	0.00	0.00	0.13	0.01	NA	NA	0.00	0.00	0.01	0.00	0.02	0.00	NA	NA
2015.11	0.01	0.00	0.12	0.00	NA	NA	0.00	0.00	0.01	0.00	0.01	0.00	NA	NA
2015.12	0.00	0.00	0.12	0.00	NA	NA	0.00	0.00	0.01	0.00	0.02	0.00	NA	NA
2015.15	0.00	0.00	0.13	0.00	0.21	0.01	0.00	0.00	0.01	0.00	0.02	0.00	0.04	0.00
2015.16	0.00	0.00	0.13	0.01	NA	NA	0.00	0.00	0.01	0.00	0.01	0.00	NA	NA
2015.23	0.00	0.00	0.10	0.01	NA	NA	0.00	0.00	0.01	0.00	0.01	0.00	NA	NA
2015.25	0.00	0.00	0.16	0.02	NA	NA	0.00	0.00	0.02	0.00	NA	NA	0.05	0.00
2015.27	0.00	0.00	0.11	0.01	NA	NA	0.00	0.00	0.01	0.00	NA	NA	0.04	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.6 **Supplementary Table 6.** Methyl Ethyl Ketone Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Methyl ethyl ketone (deposited µg/mL)														
Year. Calendar week	3R4F (Air)		3R4F (0.32)		3R4F (0.51)		THS2.2 (Air)		THS2.2 (0.31)		THS2.2 (0.46)		THS2.2 (1.09)	
	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.03	0.00	0.30	0.02	NA	NA	0.03	0.00	0.06	0.00	NA	NA	NA	NA
2014.19	0.03	0.00	0.35	0.00	NA	NA	0.04	0.00	0.06	0.00	NA	NA	NA	NA
2014.25	0.03	0.00	0.31	0.02	NA	NA	0.03	0.00	0.05	0.00	0.06	0.00	NA	NA
2015.11	0.05	0.02	0.32	0.00	NA	NA	0.01	0.01	0.03	0.00	0.03	0.00	NA	NA
2015.12	0.01	0.00	0.32	0.02	NA	NA	0.00	0.00	0.04	0.00	0.04	0.00	NA	NA
2015.15	0.02	0.00	0.35	0.00	0.55	0.01	0.02	0.00	0.04	0.00	0.05	0.00	0.11	0.01
2015.16	0.02	0.01	0.33	0.01	NA	NA	0.01	0.00	0.03	0.00	0.05	0.01	NA	NA
2015.23	0.01	0.00	0.29	0.03	NA	NA	0.01	0.00	0.03	0.00	0.03	0.00	NA	NA
2015.25	0.07	0.00	0.42	0.03	NA	NA	0.08	0.00	0.11	0.00	NA	NA	0.17	0.01
2015.27	0.02	0.00	0.35	0.00	NA	NA	0.02	0.00	0.04	0.00	NA	NA	0.09	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

14.1.1.7 **Supplementary Table 7.** Butyraldehyde Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Butyraldehyde (deposited µg/mL)														
Year. Calendar week	3R4F (Air)		3R4F (0.32)		3R4F (0.51)		THS2.2 (Air)		THS2.2 (0.31)		THS2.2 (0.46)		THS2.2 (1.09)	
	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM	M	SEM
2014.14	0.00	0.00	0.04	0.00	NA	NA	0.00	0.00	0.03	0.01	NA	NA	NA	NA
2014.19	0.00	0.00	0.01	0.00	NA	NA	0.00	0.00	0.00	0.00	NA	NA	NA	NA
2014.25	0.00	0.00	0.01	0.00	NA	NA	0.00	0.00	0.01	0.00	0.01	0.00	NA	NA
2015.11	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.12	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.03	0.00	0.03	0.00	NA	NA
2015.15	0.00	0.00	0.03	0.00	0.05	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.07	0.00
2015.16	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.23	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.02	0.00	0.02	0.00	NA	NA
2015.25	0.00	0.00	0.05	0.00	NA	NA	0.00	0.00	0.06	0.03	NA	NA	0.09	0.01
2015.27	0.00	0.00	0.04	0.00	NA	NA	0.00	0.00	0.02	0.00	NA	NA	0.06	0.00

The method to measure various carbonyls in PBS-exposed samples has not been validated. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

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.

14.1.1.8 **Supplementary Table 8.** Formaldehyde Levels deposited in the cultivation base module of the Vitrocell 24/48 exposure system

Descriptive Statistics: Formaldehyde (deposited µg/mL)														
Year. Calendar week	**3R4F (Air)**		**3R4F (0.32)**		**3R4F (0.51)**		**THS2.2 (Air)**		**THS2.2 (0.31)**		**THS2.2 (0.46)**		**THS2.2 (1.09)**	
	M	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**	**M**	**SEM**
2014.14	0.03	0.01	0.22	0.09	NA	NA	0.18	0.06	0.28	0.07	NA	NA	NA	NA
2014.19	0.00	0.00	0.03	0.00	NA	NA	0.00	0.00	0.00	0.00	NA	NA	NA	NA
2014.25	0.00	0.00	0.05	0.00	NA	NA	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
2015.11	0.02	0.00	0.10	0.01	NA	NA	0.01	0.00	0.03	0.01	0.03	0.01	NA	NA
2015.12	0.07	0.02	0.24	0.03	NA	NA	0.04	0.01	0.11	0.02	0.10	0.04	NA	NA
2015.15	0.05	0.01	0.19	0.01	0.26	0.01	0.04	0.02	0.08	0.01	0.07	0.00	0.12	0.01
2015.16	0.00	0.00	0.05	0.00	NA	NA	0.03	0.03	0.02	0.02	0.01	0.01	NA	NA
2015.23	0.00	0.00	0.00	0.00	NA	NA	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
2015.25	0.10	0.02	0.22	0.00	NA	NA	0.11	0.01	0.13	0.01	NA	NA	0.16	0.02
2015.27	0.05	0.01	0.12	0.01	NA	NA	0.04	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. For the indicated nicotine doses (in mg/L) of the reference smoke and test aerosols, as well as the corresponding air controls, the carbonyls were measured in phosphate buffered saline (PBS) following a 28-minute exposure (see Testing Procedure). Abbreviations: Cig, cigarette; M, mean; NA, not available (no measurement for the indicated calendar week); SEM, standard error mean.

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.

14.1.2 Descriptive Statistics: Morphological Evaluation

14.1.2.1 **Supplementary Table 9**. Descriptive Statistics: Cellular alteration

Collection Time		N	Mean	SEM	Score					
					0	1	2	3	4	5
Shipment*		12	3.17	0.27	.	.	3	5	3	1
Monday IC*		12	3.75	0.18	.	.	1	1	10	.
Wednesday IC*		9	2.78	0.22	.	.	3	5	1	.
Thursday IC*		9	0.78	0.22	3	5	1	.	.	.
Friday IC*		12	0.25	0.13	9	3	.	.	.	.
48 h PE	**3R4F (Air)**	12	2.17	0.21	.	2	6	4	.	.
	3R4F (0.32)	12	1.83	0.21	1	1	9	1	.	.
	3R4F (0.51)	12	2.42	0.23	.	1	6	4	1	.
	THS2.2 (Air)	12	2.50	0.26	.	1	6	3	2	.
	THS2.2 (0.31)	12	2.25	0.30	1	1	5	4	1	.
	THS2.2 (0.46)	12	2.25	0.28	1	.	7	3	1	.
	THS2.2 (1.09)	12	1.92	0.19	1	.	10	1	.	.
72 h PE	**3R4F (Air)**	12	1.08	0.26	4	3	5	.	.	.
	3R4F (0.32)	12	0.75	0.28	6	4	1	1	.	.
	3R4F (0.51)	12	0.75	0.22	5	5	2	.	.	.
	THS2.2 (Air)	12	1.17	0.21	2	6	4	.	.	.
	THS2.2 (0.31)	12	1.17	0.30	3	6	1	2	.	.
	THS2.2 (0.46)	12	0.92	0.23	4	5	3	.	.	.
	THS2.2 (1.09)	12	0.92	0.23	4	5	3	.	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.2 **Supplementary Table 10**. Descriptive Statistics: Epithelial Atrophy

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	4
Shipment*		12	2.00	0.30	.	6	.	6	.
Monday IC*		12	0.92	0.26	5	3	4	.	.
Wednesday IC*		9	0.00	0.00	9	.	.	.	.
Thursday IC*		9	0.00	0.00	9	.	.	.	.
Friday IC*		12	0.08	0.08	11	1	.	.	.
48 h PE	**3R4F (Air)**	12	0.25	0.18	10	1	1	.	.
	3R4F (0.32)	12	0.08	0.08	11	1	.	.	.
	3R4F (0.51)	12	0.08	0.08	11	1	.	.	.
	THS2.2 (Air)	12	0.08	0.08	11	1	.	.	.
	THS2.2 (0.31)	12	0.50	0.36	10	.	1	.	1
	THS2.2 (0.46)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.	.
72 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.	.
	3R4F (0.32)	12	0.42	0.26	9	2	.	1	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (0.31)	12	0.08	0.08	11	1	.	.	.
	THS2.2 (0.46)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.3 **Supplementary Table 11**. Descriptive Statistics: Cysts

Collection Time		N	Mean	SEM	Score		
					0	1	2
Shipment*		12	0.50	0.19	7	4	1
Monday IC*		12	0.50	0.19	7	4	1
Wednesday IC*		9	0.33	0.17	6	3	.
Thursday IC*		9	0.67	0.24	4	4	1
Friday IC*		12	0.50	0.23	8	2	2
48 h PE	**3R4F (Air)**	12	0.42	0.19	8	3	1
	3R4F (0.32)	12	0.67	0.19	5	6	1
	3R4F (0.51)	12	0.25	0.13	9	3	.
	THS2.2 (Air)	12	0.33	0.14	8	4	.
	THS2.2 (0.31)	12	0.50	0.15	6	6	.
	THS2.2 (0.46)	12	0.67	0.26	7	2	3
	THS2.2 (1.09)	12	0.33	0.14	8	4	.
72 h PE	**3R4F (Air)**	12	0.83	0.21	4	6	2
	3R4F (0.32)	12	0.25	0.13	9	3	.
	3R4F (0.51)	12	0.08	0.08	11	1	.
	THS2.2 (Air)	12	1.17	0.17	1	8	3
	THS2.2 (0.31)	12	1.08	0.19	2	7	3
	THS2.2 (0.46)	12	0.92	0.23	4	5	3
	THS2.2 (1.09)	12	0.83	0.24	5	4	3

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.4 **Supplementary Table 12**. Descriptive Statistics: Ectopic Keratinization

Collection Time		N	Mean	SEM	Score			
					0	1	2	3
Shipment*		12	0.17	0.11	10	2	.	.
Monday IC*		12	1.00	0.21	3	6	3	.
Wednesday IC*		9	0.67	0.17	3	6	.	.
Thursday IC*		9	1.11	0.20	1	6	2	.
Friday IC*		12	0.92	0.19	3	7	2	.
48 h PE	**3R4F (Air)**	12	1.08	0.19	2	7	3	.
	3R4F (0.32)	12	1.17	0.11	.	10	2	.
	3R4F (0.51)	12	1.17	0.21	1	9	1	1
	THS2.2 (Air)	12	1.25	0.13	.	9	3	.
	THS2.2 (0.31)	12	1.00	0.12	1	10	1	.
	THS2.2 (0.46)	12	1.08	0.15	1	9	2	.
	THS2.2 (1.09)	12	1.25	0.13	.	9	3	.
72 h PE	**3R4F (Air)**	12	0.92	0.15	2	9	1	.
	3R4F (0.32)	12	1.42	0.15	.	7	5	.
	3R4F (0.51)	12	1.33	0.22	1	7	3	1
	THS2.2 (Air)	12	0.92	0.08	1	11	.	.
	THS2.2 (0.31)	12	0.83	0.17	3	8	1	.
	THS2.2 (0.46)	12	0.75	0.18	4	7	1	.
	THS2.2 (1.09)	12	0.50	0.15	6	6	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.5 **Supplementary Table 13.** Descriptive Statistics: Apical Keratinization

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	4
Shipment*		12	0.58	0.15	5	7	.	.	.
Monday IC*		12	1.08	0.29	4	4	3	1	.
Wednesday IC*		9	0.89	0.26	3	4	2	.	.
Thursday IC*		9	1.00	0.24	2	5	2	.	.
Friday IC*		12	1.17	0.24	2	7	2	1	.
48 h PE	**3R4F (Air)**	12	1.50	0.26	.	9	.	3	.
	3R4F (0.32)	12	1.58	0.31	2	4	3	3	.
	3R4F (0.51)	12	2.25	0.25	.	3	3	6	.
	THS2.2 (Air)	12	1.33	0.19	1	6	5	.	.
	THS2.2 (0.31)	12	1.17	0.24	3	4	5	.	.
	THS2.2 (0.46)	12	1.58	0.29	1	6	2	3	.
	THS2.2 (1.09)	12	1.17	0.21	1	9	1	1	.
72 h PE	**3R4F (Air)**	12	0.67	0.19	5	6	1	.	.
	3R4F (0.32)	12	1.17	0.24	3	4	5	.	.
	3R4F (0.51)	12	2.17	0.34	1	2	5	2	2
	THS2.2 (Air)	12	1.00	0.17	2	8	2	.	.
	THS2.2 (0.31)	12	0.83	0.17	3	8	1	.	.
	THS2.2 (0.46)	12	1.00	0.17	2	8	2	.	.
	THS2.2 (1.09)	12	0.67	0.22	6	4	2	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.6 **Supplementary Table 14**. Descriptive Statistics: Detachment of Culture above Basal Cell Level

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	4
Shipment*		12	3.67	0.14	.	.	.	4	8
Monday IC*		12	0.50	0.23	8	2	2	.	.
Wednesday IC*		9	0.56	0.24	5	3	1	.	.
Thursday IC*		9	0.89	0.45	6	.	1	2	.
Friday IC*		12	0.83	0.39	8	1	.	3	.
48 h PE	**3R4F (Air)**	12	0.33	0.22	10	.	2	.	.
	3R4F (0.32)	12	1.08	0.31	5	2	4	1	.
	3R4F (0.51)	12	1.50	0.36	4	1	4	3	.
	THS2.2 (Air)	12	0.67	0.31	8	1	2	1	.
	THS2.2 (0.31)	12	0.83	0.32	6	4	.	2	.
	THS2.2 (0.46)	12	0.50	0.23	8	2	2	.	.
	THS2.2 (1.09)	12	0.75	0.30	7	2	2	1	.
72 h PE	**3R4F (Air)**	12	1.00	0.43	7	2	.	2	1
	3R4F (0.32)	12	1.75	0.41	4	1	1	6	.
	3R4F (0.51)	12	2.58	0.38	2	.	1	7	2
	THS2.2 (Air)	12	1.08	0.36	6	1	3	2	.
	THS2.2 (0.31)	12	1.33	0.33	4	2	4	2	.
	THS2.2 (0.46)	12	1.17	0.41	6	1	3	1	1
	THS2.2 (1.09)	12	1.33	0.38	4	3	3	1	1

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.7 **Supplementary Table 15**. Descriptive Statistics: Detachment of Culture below Basal Cell Level

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	5
Shipment*		12	0.00	0.00	12	.	.	.	.
Monday IC*		12	0.83	0.46	8	2	.	1	1
Wednesday IC*		9	0.11	0.11	8	1	.	.	.
Thursday IC*		9	0.11	0.11	8	1	.	.	.
Friday IC*		12	0.08	0.08	11	1	.	.	.
48 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.	.
	3R4F (0.32)	12	0.50	0.29	9	1	1	1	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (0.31)	12	0.17	0.17	11	.	1	.	.
	THS2.2 (0.46)	12	0.33	0.19	9	2	1	.	.
	THS2.2 (1.09)	12	0.25	0.18	10	1	1	.	.
72 h PE	**3R4F (Air)**	12	0.42	0.29	10	.	1	1	.
	3R4F (0.32)	12	0.67	0.33	8	2	.	2	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.	.
	THS2.2 (Air)	12	0.17	0.11	10	2	.	.	.
	THS2.2 (0.31)	12	0.25	0.25	11	.	.	1	.
	THS2.2 (0.46)	12	0.08	0.08	11	1	.	.	.
	THS2.2 (1.09)	12	0.25	0.18	10	1	1	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.8 **Supplementary Table 16**. Descriptive Statistics: Extracellular Granules

Collection Time		N	Mean	SEM	Score			
					0	1	2	3
Shipment*		12	1.75	0.30	1	5	2	4
Monday IC*		12	0.00	0.00	12	.	.	.
Wednesday IC*		9	0.00	0.00	9	.	.	.
Thursday IC*		9	0.00	0.00	9	.	.	.
Friday IC*		12	0.00	0.00	12	.	.	.
48 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.
	3R4F (0.32)	12	0.00	0.00	12	.	.	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.46)	12	0.00	0.00	12	.	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.
72 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.
	3R4F (0.32)	12	0.00	0.00	12	.	.	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.46)	12	0.00	0.00	12	.	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.9 **Supplementary Table 17**. Descriptive Statistics: Increased Cytoplasmic Vacuolation

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	4
Shipment*		12	1.50	0.31	3	2	5	2	.
Monday IC*		12	1.92	0.26	.	5	3	4	.
Wednesday IC*		9	1.00	0.24	2	5	2	.	.
Thursday IC*		9	0.22	0.15	7	2	.	.	.
Friday IC*		12	0.50	0.23	8	2	2	.	.
48 h PE	**3R4F (Air)**	12	1.00	0.28	4	5	2	1	.
	3R4F (0.32)	12	1.08	0.19	2	7	3	.	.
	3R4F (0.51)	12	1.25	0.22	2	5	5	.	.
	THS2.2 (Air)	12	1.00	0.21	3	6	3	.	.
	THS2.2 (0.31)	12	0.83	0.24	4	7	.	1	.
	THS2.2 (0.46)	12	1.33	0.33	2	7	1	1	1
	THS2.2 (1.09)	12	0.58	0.15	5	7	.	.	.
72 h PE	**3R4F (Air)**	12	0.75	0.28	6	4	1	1	.
	3R4F (0.32)	12	0.33	0.14	8	4	.	.	.
	3R4F (0.51)	12	0.33	0.14	8	4	.	.	.
	THS2.2 (Air)	12	0.58	0.19	6	5	1	.	.
	THS2.2 (0.31)	12	0.50	0.15	6	6	.	.	.
	THS2.2 (0.46)	12	0.58	0.29	8	2	1	1	.
	THS2.2 (1.09)	12	0.25	0.13	9	3	.	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.10 **Supplementary Table 18**. Descriptive Statistics: Increased Desquamation

Collection Time		N	Mean	SEM	Score			
					0	1	2	3
Shipment*		12	0.00	0.00	12	.	.	.
Monday IC*		12	0.08	0.08	11	1	.	.
Wednesday IC*		9	0.00	0.00	9	.	.	.
Thursday IC*		9	0.33	0.24	7	1	1	.
Friday IC*		12	0.50	0.34	10	.	.	2
48 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.
	3R4F (0.32)	12	0.33	0.26	10	1	.	1
	3R4F (0.51)	12	0.17	0.17	11	.	1	.
	THS2.2 (Air)	12	0.25	0.18	10	1	1	.
	THS2.2 (0.31)	12	0.25	0.18	10	1	1	.
	THS2.2 (0.46)	12	0.25	0.25	11	.	.	1
	THS2.2 (1.09)	12	0.08	0.08	11	1	.	.
72 h PE	**3R4F (Air)**	12	0.17	0.11	10	2	.	.
	3R4F (0.32)	12	0.83	0.30	6	3	2	1
	3R4F (0.51)	12	1.67	0.36	3	2	3	4
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.25	0.18	10	1	1	.
	THS2.2 (0.46)	12	0.67	0.33	8	2	.	2
	THS2.2 (1.09)	12	0.67	0.28	7	3	1	1

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.11 **Supplementary Table 19**. Descriptive Statistics: Intracellular Fine Granular Basophilic Material

Collection Time		N	Mean	SEM	Score		
					0	1	2
Shipment*		12	0.00	0.00	12	.	.
Monday IC*		12	0.00	0.00	12	.	.
Wednesday IC*		9	0.44	0.24	6	2	1
Thursday IC*		9	0.22	0.15	7	2	.
Friday IC*		12	0.00	0.00	12	.	.
48 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.
	3R4F (0.32)	12	0.25	0.13	9	3	.
	3R4F (0.51)	12	0.67	0.19	5	6	1
	THS2.2 (Air)	12	0.00	0.00	12	.	.
	THS2.2 (0.31)	12	0.00	0.00	12	.	.
	THS2.2 (0.46)	12	0.00	0.00	12	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.
72 h PE	**3R4F (Air)**	12	0.08	0.08	11	1	.
	3R4F (0.32)	12	0.58	0.19	6	5	1
	3R4F (0.51)	12	1.08	0.23	3	5	4
	THS2.2 (Air)	12	0.00	0.00	12	.	.
	THS2.2 (0.31)	12	0.00	0.00	12	.	.
	THS2.2 (0.46)	12	0.00	0.00	12	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.12 **Supplementary Table 19**. Descriptive Statistics: Longitudinal Cleft Formation

Collection Time		N	Mean	SEM	Score			
					0	1	2	3
Shipment*		12	0.00	0.00	12	.	.	.
Monday IC*		12	0.00	0.00	12	.	.	.
Wednesday IC*		9	0.22	0.22	8	.	1	.
Thursday IC*		9	0.33	0.33	8	.	.	1
Friday IC*		12	0.25	0.13	9	3	.	.
48 h PE	**3R4F (Air)**	12	0.00	0.00	12	.	.	.
	3R4F (0.32)	12	0.17	0.17	11	.	1	.
	3R4F (0.51)	12	0.25	0.25	11	.	.	1
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.17	0.17	11	.	1	.
	THS2.2 (0.46)	12	0.17	0.17	11	.	1	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.
72 h PE	**3R4F (Air)**	12	0.08	0.08	11	1	.	.
	3R4F (0.32)	12	0.58	0.23	7	3	2	.
	3R4F (0.51)	12	0.50	0.29	9	1	1	1
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.17	0.11	10	2	.	.
	THS2.2 (0.46)	12	0.25	0.18	10	1	1	.
	THS2.2 (1.09)	12	0.25	0.18	10	1	1	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.13 **Supplementary Table 20**. Descriptive Statistics: Single-cell Necrosis/Apoptosis

Collection Time		N	Mean	SEM	Score				
					0	1	2	3	4
Shipment*		12	1.92	0.19	.	3	7	2	.
Monday IC*		12	1.50	0.23	1	5	5	1	.
Wednesday IC*		9	1.22	0.22	1	5	3	.	.
Thursday IC*		9	1.00	0.24	2	5	2	.	.
Friday IC*		12	0.92	0.15	2	9	1	.	.
48 h PE	**3R4F (Air)**	12	1.25	0.25	2	6	3	1	.
	3R4F (0.32)	12	1.25	0.18	1	7	4	.	.
	3R4F (0.51)	12	1.08	0.15	1	9	2	.	.
	THS2.2 (Air)	12	1.67	0.19	.	5	6	1	.
	THS2.2 (0.31)	12	1.67	0.26	.	6	5	.	1
	THS2.2 (0.46)	12	1.33	0.14	.	8	4	.	.
	THS2.2 (1.09)	12	1.50	0.19	.	7	4	1	.
72 h PE	**3R4F (Air)**	12	1.25	0.22	2	5	5	.	.
	3R4F (0.32)	12	1.17	0.17	1	8	3	.	.
	3R4F (0.51)	12	1.00	0.17	2	8	2	.	.
	THS2.2 (Air)	12	1.67	0.19	.	5	6	1	.
	THS2.2 (0.31)	12	1.83	0.24	1	2	7	2	.
	THS2.2 (0.46)	12	1.67	0.26	1	4	5	2	.
	THS2.2 (1.09)	12	1.17	0.21	2	6	4	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.14 **Supplementary Table 21**. Descriptive Statistics: Culture Thickness Inhomogeneity

Collection Time		N	Mean	SEM	Score			
					0	1	2	3
Shipment*		12	0.00	0.00	12	.	.	.
Monday IC*		12	0.17	0.17	11	.	1	.
Wednesday IC*		9	0.22	0.15	7	2	.	.
Thursday IC*		9	0.00	0.00	9	.	.	.
Friday IC*		12	0.17	0.17	11	.	1	.
48 h PE	**3R4F (Air)**	12	0.42	0.19	8	3	1	.
	3R4F (0.32)	12	0.08	0.08	11	1	.	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.
	THS2.2 (Air)	12	0.25	0.18	10	1	1	.
	THS2.2 (0.31)	12	0.25	0.25	11	.	.	1
	THS2.2 (0.46)	12	0.08	0.08	11	1	.	.
	THS2.2 (1.09)	12	0.33	0.22	10	.	2	.
72 h PE	**3R4F (Air)**	12	0.08	0.08	11	1	.	.
	3R4F (0.32)	12	0.00	0.00	12	.	.	.
	3R4F (0.51)	12	0.00	0.00	12	.	.	.
	THS2.2 (Air)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.31)	12	0.00	0.00	12	.	.	.
	THS2.2 (0.46)	12	0.08	0.08	11	1	.	.
	THS2.2 (1.09)	12	0.00	0.00	12	.	.	.

The mean is calculated from the number of observations multiplied by the score grade, and divided by the total number of replicates. Scores are for the cellular alteration of the cultures. *Incubator control (IC) samples collected on shipment day or on the indicated day throughout the exposure week. Abbreviations: IC, incubator control; PE, post-exposure; SEM, standard error of the mean.

14.1.2.15 **Supplementary Table 22**. Inferential Statistics for 3R4F *vs*. THS2.2 Comparison

Features		3R4F (0.32) vs. THS2.2 (0.31)		3R4F (0.32) vs. THS2.2 (0.46)		3R4F (0.32) vs. THS2.2 (1.09)		3R4F (0.51) vs. THS2.2 (0.46)		3R4F (0.51) vs. THS2.2 (1.09)	
		48:00	**72:00**	**48:00**	**72:00**	**48:00**	**72:00**	**48:00**	**72:00**	**48:00**	**72:00**
Apical keratinization	DOS	0.25	0.57	−0.17	0.50	0.25	0.70	0.50	1.50	0.92	1.70
Areas of cellular alteration (epithelial dysplasia)	DOS	−0.08	−0.20	−0.08	−0.20	0.25	−0.07	0.50	−0.20	0.83	−0.07
Atrophy	DOS	−0.58	0.35	−0.08	0.42	−0.08	0.42	−0.08	0.00	−0.08	0.00
Cyst(s)	DOS	0.08	−0.52	−0.08	−0.45	0.25	−0.38	−0.50	−0.62	−0.17	−0.55
Ectopic keratinization	DOS	0.33	0.57	0.25	0.70	0.08	0.83	0.25	0.62	0.08	0.75
Epithelial detachment above basal cell level	DOS	0.58	0.68	0.92	0.82	0.67	0.68	1.33	1.65	1.08	1.52
Epithelial detachment below basal cell level	DOS	0.33	0.12	0.17	0.25	0.25	0.18	−0.33	−0.42	−0.25	−0.48
Extracellular bacteria	DOS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Foreign body/bodies	Bin.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Increased cytoplasmic vacuolation	DOS	0.25	−0.42	−0.25	−0.42	0.50	−0.15	−0.08	−0.42	0.67	−0.15
Increased desquamation	DOS	0.33	0.40	0.33	0.13	0.50	0.13	0.17	0.97	0.33	0.97
Intracellular fine granular basophilic material	DOS	0.25	0.50	0.25	0.50	0.25	0.50	0.67	1.00	0.67	1.00
Longitudinal cleft	DOS	0.00	0.50	0.00	0.23	0.17	0.43	0.08	0.15	0.25	0.35
Mineralization	Bin.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Single-cell necrosis/apoptosis	DOS	0.00	−0.15	0.33	0.05	0.17	0.38	0.17	−0.12	0.00	0.22
Culture thickness inhomogeneity	DOS	−0.33	−0.15	−0.17	−0.15	−0.42	−0.08	−0.25	−0.15	−0.50	−0.08

Values indicate the absolute difference between the score means for a given feature for each exposure condition compared with the corresponding air control. Negative differences are indicated with the "−" sign. Differences between the score means that are below the raw *p*-value of 0.05 are highlighted in black. Abbreviations: DOS, Discrete Ordered Scale; bin, binary.

14.1.3 **Supplementary Table 23**. Descriptive Statistics: Cytotoxicity (RLU)

		Descriptive Statistics							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
NA	Untreated	4	1170	377	555	1045	1786	-29	2370
	TX-100	4	147383	15033	125881	146231	168885	99541	195225
4:00	3R4F (Air)	13	2319	751	720	1271	2482	665	3972
	3R4F (0.32)	12	2368	836	767	1041	2481	527	4209
	3R4F (0.51)	12	2554	905	640	1017	3946	563	4545
	THS2.2 (Air)	11	2126	600	643	1537	2656	805	3447
	THS2.2 (0.31)	12	2711	929	729	1284	3079	666	4756
	THS2.2 (0.46)	12	2468	782	705	1336	2801	747	4188
	THS2.2 (1.09)	12	2465	797	649	1406	2578	710	4219
24:00	3R4F (Air)	12	3506	991	1242	2353	3517	1324	5688
	3R4F (0.32)	12	4368	1353	1304	2336	5530	1390	7345
	3R4F (0.51)	12	5844	1962	1553	2416	9955	1527	10161
	THS2.2 (Air)	12	2841	942	1001	1774	3242	767	4915
	THS2.2 (0.31)	12	3501	1284	1154	1708	2827	675	6328
	THS2.2 (0.46)	12	5660	2285	1033	2252	7030	631	10689
	THS2.2 (1.09)	12	3854	1350	930	1900	3851	883	6824
48:00	3R4F (Air)	12	5005	1750	1607	2523	4611	1152	8857
	3R4F (0.32)	12	6792	2211	2253	3155	7097	1925	11660
	3R4F (0.51)	12	9707	2949	2556	5274	14946	3216	16198
	THS2.2 (Air)	12	4062	1441	1562	2367	4364	890	7234
	THS2.2 (0.31)	12	5659	1930	1482	2491	6109	1411	9906
	THS2.2 (0.46)	12	25844	20315	1551	2529	12567	-18870	70558
	THS2.2 (1.09)	12	5670	1984	1389	2145	6329	1303	10037
72:00	3R4F (Air)	12	6668	2100	2011	3375	8207	2047	11290
	3R4F (0.32)	12	8584	2466	3306	4639	9521	3156	14012
	3R4F (0.51)	12	11155	3535	3161	5143	17580	3374	18936
	THS2.2 (Air)	12	5439	1796	1931	2677	6584	1486	9392
	THS2.2 (0.31)	12	6785	2142	1696	3748	8102	2071	11500
	THS2.2 (0.46)	12	22393	15623	1899	4626	14381	-11993	56779
	THS2.2 (1.09)	12	6888	2193	1622	3586	9833	2061	11715

Abbreviations: h, hour; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; NA, not available; PE, post-exposure; RLU, relative luminescence units; SEM, standard error of the mean; TX-100, Triton X-100.

14.1.4 **Supplementary Table 24**. Descriptive Statistics: CYP1A1/1B1 Activity (RLU)

		Descriptive Statistics							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
NA	Untr	1	87	NA	87	87	87	NA	NA
	Luciferin	3	286	71	180	255	422	-22	593
	TCDD	4	8038	2595	4805	7377	11270	-220	16296
24:00	3R4F (Air)	12	353	74	193	303	405	189	516
	3R4F (0.32)	12	258	37	146	268	336	177	339
	3R4F (0.51)	12	177	26	112	157	247	120	234
	THS2.2 (Air)	12	289	37	185	316	351	207	370
	THS2.2 (0.31)	12	557	149	366	468	520	229	885
	THS2.2 (0.46)	12	696	135	430	602	759	399	994
	THS2.2 (1.09)	12	931	123	604	907	1210	660	1203
48:00	3R4F (Air)	12	465	94	217	396	631	257	673
	3R4F (0.32)	12	581	121	287	468	750	314	847
	3R4F (0.51)	12	298	49	169	294	382	190	406
	THS2.2 (Air)	12	360	46	216	377	466	259	460
	THS2.2 (0.31)	12	609	142	422	506	641	296	922
	THS2.2 (0.46)	12	722	136	501	621	777	424	1021
	THS2.2 (1.09)	12	1021	133	660	998	1337	728	1314

Abbreviations: h, hour; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PE, post-exposure; SEM, standard error of the mean; TCDD, 2,3,7,8-Tetrachlorodibenzodioxin; Untr, untreated.

14.1.5 Descriptive Statistics: Concentration of Pro-Inflammatory Mediators in the Basolateral Media

14.1.5.1 **Supplementary Table 25**. Descriptive Statistics: CSF3 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	110.42	62.25	37.06	68.44	183.77	−87.68	308.51
	TNFA+IL1B	4	2141.77	407.95	1591.25	2332.90	2692.29	843.48	3440.06
24:00	3R4F (Air)	12	103.51	15.91	62.34	107.18	128.72	68.48	138.54
	3R4F (0.32)	12	293.07	189.03	57.52	112.07	181.74	−122.97	709.12
	3R4F (0.51)	12	146.62	75.41	45.41	80.14	94.79	−19.36	312.60
	THS2.2 (Air)	12	115.65	23.69	59.02	108.07	135.36	63.51	167.79
	THS2.2 (0.31)	12	112.94	15.73	69.49	107.44	149.79	78.32	147.56
	THS2.2 (0.46)	12	469.72	345.22	42.72	148.59	188.87	−290.11	1229.55
	THS2.2 (1.09)	12	547.03	397.35	68.06	159.05	212.24	−327.55	1421.60
48:00	3R4F (Air)	12	587.51	419.27	73.86	154.02	194.09	−335.30	1510.32
	3R4F (0.32)	12	202.40	35.04	96.11	194.47	282.63	125.28	279.53
	3R4F (0.51)	12	188.92	30.06	89.84	217.47	271.45	122.77	255.08
	THS2.2 (Air)	12	168.35	32.36	76.86	161.93	218.56	97.13	239.57
	THS2.2 (0.31)	12	351.24	109.16	166.41	273.86	365.05	110.97	591.50
	THS2.2 (0.46)	12	447.85	124.72	164.99	280.67	633.80	173.35	722.35
	THS2.2 (1.09)	12	698.85	364.11	147.73	242.63	394.72	−102.56	1500.26
72:00	3R4F (Air)	12	661.45	419.28	77.46	188.50	324.66	−261.38	1584.28
	3R4F (0.32)	12	843.64	341.68	148.82	335.34	903.21	91.60	1595.67
	3R4F (0.51)	12	283.26	57.59	117.34	249.32	456.20	156.51	410.01
	THS2.2 (Air)	12	305.74	89.15	106.50	193.81	359.21	109.51	501.96
	THS2.2 (0.31)	12	349.17	89.18	150.42	256.32	438.68	152.89	545.45
	THS2.2 (0.46)	12	360.83	67.83	141.23	357.24	530.97	211.53	510.12
	THS2.2 (1.09)	12	630.88	234.76	133.49	320.11	701.05	114.19	1147.58

Abbreviations: CSF3, colony-stimulating factor 3; IL, interleukin; h, hour; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.2 **Supplementary Table 26**. Descriptive Statistics: CSF2 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	12.17	3.20	7.22	13.61	17.12	1.99	22.34
	TNFA+IL1B	4	61.88	9.99	48.86	69.71	74.90	30.10	93.66
24:00	3R4F (Air)	12	18.99	2.20	13.78	17.58	20.67	14.15	23.84
	3R4F (0.32)	12	17.89	5.90	10.92	13.30	15.23	4.90	30.88
	3R4F (0.51)	12	12.14	2.02	7.40	11.47	13.48	7.70	16.58
	THS2.2 (Air)	12	18.18	0.98	15.90	18.69	21.02	16.02	20.34
	THS2.2 (0.31)	12	19.54	1.75	14.57	19.04	23.85	15.69	23.39
	THS2.2 (0.46)	12	27.49	6.31	17.93	21.98	24.97	13.60	41.37
	THS2.2 (1.09)	12	28.25	11.33	12.73	18.40	23.51	3.32	53.19
48:00	3R4F (Air)	12	38.03	9.31	22.13	31.81	40.55	17.55	58.51
	3R4F (0.32)	12	24.55	2.21	18.81	26.36	27.97	19.68	29.42
	3R4F (0.51)	12	24.60	2.53	18.22	25.73	31.34	19.03	30.17
	THS2.2 (Air)	12	39.92	6.19	22.58	34.61	49.15	26.30	53.54
	THS2.2 (0.31)	12	43.83	4.19	35.37	42.47	55.91	34.60	53.05
	THS2.2 (0.46)	12	47.25	5.69	29.69	47.86	59.31	34.73	59.77
	THS2.2 (1.09)	12	43.12	7.08	31.17	34.57	52.84	27.55	58.69
72:00	3R4F (Air)	12	48.67	5.88	40.83	44.15	47.34	35.73	61.60
	3R4F (0.32)	12	45.31	7.03	29.73	39.06	50.79	29.84	60.78
	3R4F (0.51)	12	32.49	4.64	20.58	29.85	37.44	22.28	42.70
	THS2.2 (Air)	12	48.23	6.15	36.44	42.29	65.60	34.69	61.77
	THS2.2 (0.31)	12	54.22	5.98	36.23	55.19	71.28	41.06	67.38
	THS2.2 (0.46)	12	56.10	6.25	36.52	59.76	71.42	42.34	69.86
	THS2.2 (1.09)	12	49.90	6.20	33.92	49.94	56.13	36.25	63.55

Abbreviations: CSF2, colony-stimulating factor 2; IL, interleukin; h, hour; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha..

14.1.5.3 **Supplementary Table 27**. Descriptive Statistics: IL1A Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	18.37	6.56	10.27	16.26	26.48	−2.50	39.25
	TNFA+IL1B	4	27.00	8.00	15.41	32.16	38.59	1.56	52.44
24:00	3R4F (Air)	12	34.04	5.57	23.62	28.16	41.09	21.79	46.29
	3R4F (0.32)	12	44.59	8.97	25.80	35.97	56.76	24.85	64.33
	3R4F (0.51)	12	52.96	14.95	21.60	31.23	75.53	20.05	85.87
	THS2.2 (Air)	12	34.95	5.07	19.78	27.05	53.47	23.78	46.12
	THS2.2 (0.31)	12	31.61	4.78	20.30	26.28	40.03	21.10	42.12
	THS2.2 (0.46)	12	49.10	9.94	30.65	32.67	60.49	27.21	70.98
	THS2.2 (1.09)	12	51.51	13.71	27.18	34.65	64.84	21.33	81.69
48:00	3R4F (Air)	12	62.59	17.06	26.75	44.38	69.91	25.03	100.15
	3R4F (0.32)	12	46.93	5.35	32.38	49.15	59.34	35.15	58.70
	3R4F (0.51)	12	72.32	17.23	36.74	52.73	88.22	34.41	110.24
	THS2.2 (Air)	12	48.16	5.96	40.19	46.13	56.59	35.04	61.27
	THS2.2 (0.31)	12	66.98	13.69	35.41	63.45	70.64	36.86	97.10
	THS2.2 (0.46)	12	70.73	13.82	35.90	59.96	87.40	40.33	101.14
	THS2.2 (1.09)	12	73.79	14.58	42.53	57.60	94.80	41.69	105.89
72:00	3R4F (Air)	12	84.10	13.45	49.68	65.78	116.44	54.50	113.70
	3R4F (0.32)	12	87.87	19.02	43.59	71.24	109.84	46.02	129.73
	3R4F (0.51)	12	79.17	19.21	30.53	71.27	90.71	36.90	121.45
	THS2.2 (Air)	12	78.85	11.47	48.49	70.57	101.26	53.60	104.11
	THS2.2 (0.31)	12	81.43	11.57	48.82	82.69	88.04	55.96	106.90
	THS2.2 (0.46)	12	88.10	11.61	60.34	80.31	109.87	62.54	113.65
	THS2.2 (1.09)	12	104.36	22.64	44.01	95.21	144.37	54.53	154.20

Abbreviations: h, hour; LCLM, lower confidence limit of the mean; IL, interleukin; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.4 **Supplementary Table 28.** Descriptive Statistics: IL1B Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	5.34	2.26	2.67	4.19	8.00	−1.85	12.52
	TNFA+IL1B	4	8273.42	62.39	8181.26	8254.22	8365.59	8074.86	8471.98
24:00	3R4F (Air)	12	3.08	0.51	1.78	2.02	4.86	1.97	4.20
	3R4F (0.32)	12	28.99	8.68	10.43	23.02	29.24	9.87	48.10
	3R4F (0.51)	12	53.34	13.80	29.90	46.18	52.49	22.96	83.72
	THS2.2 (Air)	12	4.70	2.01	1.28	2.74	4.77	0.28	9.13
	THS2.2 (0.31)	12	3.24	0.66	2.36	2.73	3.79	1.80	4.68
	THS2.2 (0.46)	12	7.90	2.82	1.19	6.26	7.98	1.71	14.10
	THS2.2 (1.09)	12	14.61	4.84	4.26	10.40	14.82	3.97	25.26
48:00	3R4F (Air)	12	12.59	7.38	1.39	3.19	9.69	−3.65	28.83
	3R4F (0.32)	12	36.13	6.39	17.19	29.22	53.59	22.06	50.20
	3R4F (0.51)	12	55.08	10.82	29.70	36.88	72.93	31.27	78.88
	THS2.2 (Air)	12	4.97	1.63	1.32	3.15	5.53	1.38	8.56
	THS2.2 (0.31)	12	10.44	3.81	2.65	5.44	13.09	2.05	18.83
	THS2.2 (0.46)	12	13.62	6.08	1.53	2.86	20.48	0.24	27.00
	THS2.2 (1.09)	12	21.23	10.80	4.26	8.02	10.55	−2.53	45.00
72:00	3R4F (Air)	12	16.94	8.13	2.50	4.79	10.52	−0.96	34.85
	3R4F (0.32)	12	52.98	14.17	20.22	39.77	63.85	21.79	84.16
	3R4F (0.51)	12	67.88	10.72	40.09	64.71	87.45	44.30	91.47
	THS2.2 (Air)	12	13.46	5.06	1.48	4.75	25.09	2.32	24.60
	THS2.2 (0.31)	12	10.94	4.23	2.75	6.83	9.72	1.63	20.25
	THS2.2 (0.46)	12	9.28	2.65	2.53	5.64	12.51	3.46	15.10
	THS2.2 (1.09)	12	25.95	10.33	3.48	10.03	35.48	3.22	48.69

Abbreviations: h, hour; LCLM, lower confidence limit of the mean; IL, interleukin; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.5 **Supplementary Table 29.** Descriptive Statistics: IL6 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	16.46	5.57	8.69	19.65	24.23	−1.26	34.18
	TNFA+IL1B	4	91.22	22.53	66.11	110.43	116.32	19.51	162.92
24:00	3R4F (Air)	12	18.63	4.08	5.82	19.46	30.40	9.64	27.62
	3R4F (0.32)	12	14.32	3.75	5.02	12.00	20.05	6.06	22.57
	3R4F (0.51)	12	8.29	2.10	2.35	5.53	14.60	3.68	12.91
	THS2.2 (Air)	12	16.75	3.29	6.86	16.67	25.77	9.51	24.00
	THS2.2 (0.31)	12	16.25	3.24	7.14	15.20	26.17	9.11	23.39
	THS2.2 (0.46)	12	27.25	12.09	2.87	14.25	30.84	0.65	53.85
	THS2.2 (1.09)	12	30.94	14.86	5.18	18.61	27.02	−1.78	63.66
48:00	3R4F (Air)	12	30.91	7.45	11.50	27.34	42.06	14.51	47.30
	3R4F (0.32)	12	10.09	3.02	3.11	7.08	12.63	3.46	16.73
	3R4F (0.51)	12	9.89	2.98	1.12	4.55	18.36	3.32	16.45
	THS2.2 (Air)	12	26.63	5.18	8.88	32.64	38.38	15.23	38.03
	THS2.2 (0.31)	12	28.77	5.48	11.23	35.12	44.10	16.70	40.84
	THS2.2 (0.46)	12	28.64	6.35	7.33	31.80	41.22	14.66	42.62
	THS2.2 (1.09)	12	25.37	6.16	13.86	22.67	27.96	11.82	38.92
72:00	3R4F (Air)	12	40.93	9.44	14.19	42.06	47.44	20.15	61.71
	3R4F (0.32)	12	19.20	4.93	4.74	13.40	32.39	8.35	30.05
	3R4F (0.51)	12	7.34	1.52	3.15	7.13	10.21	3.98	10.69
	THS2.2 (Air)	12	39.46	8.96	12.39	36.26	64.25	19.74	59.19
	THS2.2 (0.31)	12	33.29	6.40	15.64	36.15	45.29	19.19	47.38
	THS2.2 (0.46)	12	36.94	7.07	13.96	41.78	59.23	21.38	52.51
	THS2.2 (1.09)	12	23.96	5.53	6.08	22.44	40.92	11.80	36.13

Abbreviations: h, hour; LCLM, lower confidence limit of the mean; IL, interleukin; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.6 **Supplementary Table 30**. Descriptive Statistics: CXCL8 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	273.54	84.76	162.35	276.09	384.73	3.81	543.28
	TNFA+IL1B	4	3750.64	540.93	3112.51	4175.67	4388.76	2029.15	5472.13
24:00	3R4F (Air)	12	408.04	59.39	244.41	440.37	509.95	277.33	538.76
	3R4F (0.32)	12	465.52	141.92	238.79	399.08	437.64	153.15	777.89
	3R4F (0.51)	12	360.77	59.55	218.77	377.77	424.89	229.69	491.84
	THS2.2 (Air)	12	338.41	47.68	213.91	321.44	417.34	233.48	443.34
	THS2.2 (0.31)	12	312.13	32.94	229.48	291.53	437.41	239.64	384.62
	THS2.2 (0.46)	12	541.97	185.14	242.00	390.23	436.43	134.47	949.47
	THS2.2 (1.09)	12	558.61	237.89	169.16	330.93	490.46	35.02	1082.20
48:00	3R4F (Air)	12	1006.37	424.05	424.82	632.98	782.54	73.05	1939.70
	3R4F (0.32)	12	531.54	58.44	360.51	563.01	679.81	402.92	660.16
	3R4F (0.51)	12	569.54	67.00	436.46	607.06	694.97	422.07	717.02
	THS2.2 (Air)	12	664.75	102.64	357.92	701.50	846.82	438.85	890.66
	THS2.2 (0.31)	12	768.43	113.18	564.91	807.58	830.74	519.32	1017.53
	THS2.2 (0.46)	12	910.73	170.97	464.41	802.43	1228.02	534.43	1287.02
	THS2.2 (1.09)	12	909.67	223.43	486.59	754.83	880.94	417.91	1401.43
72:00	3R4F (Air)	12	1009.56	214.66	548.16	881.08	1010.65	537.10	1482.01
	3R4F (0.32)	12	820.92	142.18	476.81	747.20	954.34	507.98	1133.87
	3R4F (0.51)	12	601.37	70.24	430.10	668.13	717.66	446.78	755.96
	THS2.2 (Air)	12	887.93	145.41	455.18	826.58	1166.29	567.88	1207.98
	THS2.2 (0.31)	12	882.16	121.51	584.70	897.04	1133.30	614.71	1149.60
	THS2.2 (0.46)	12	1035.54	162.32	529.76	1118.70	1282.99	678.28	1392.79
	THS2.2 (1.09)	12	998.66	173.60	390.02	1034.61	1268.86	616.57	1380.76

Abbreviations: CXCL, chemokine (C-X-C motif) ligand; h, hour; IL, interleukin; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.7 **Supplementary Table 31**. Descriptive Statistics: CXCL10 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	82.93	23.58	44.92	86.62	120.93	7.87	157.98
	TNFA+IL1B	4	1347.76	273.23	945.70	1199.28	1749.82	478.22	2217.31
24:00	3R4F (Air)	12	95.96	15.14	57.04	80.21	121.02	62.64	129.28
	3R4F (0.32)	12	55.17	3.98	48.01	56.92	61.91	46.42	63.92
	3R4F (0.51)	12	56.63	4.28	47.42	54.39	66.57	47.22	66.04
	THS2.2 (Air)	12	103.89	15.61	60.22	97.74	122.18	69.54	138.24
	THS2.2 (0.31)	12	100.74	11.90	60.68	108.02	126.43	74.56	126.93
	THS2.2 (0.46)	12	101.48	11.43	72.83	101.71	120.90	76.33	126.63
	THS2.2 (1.09)	12	83.69	11.79	48.26	91.99	115.85	57.73	109.64
48:00	3R4F (Air)	12	218.47	34.23	152.91	172.85	318.86	143.12	293.81
	3R4F (0.32)	12	105.54	14.27	53.98	105.88	146.92	74.14	136.95
	3R4F (0.51)	12	85.87	7.95	68.15	73.40	105.96	68.37	103.37
	THS2.2 (Air)	12	309.68	79.87	171.80	236.57	353.81	133.88	485.48
	THS2.2 (0.31)	12	192.31	21.98	161.17	186.32	235.48	143.93	240.68
	THS2.2 (0.46)	12	157.82	19.87	104.31	163.73	215.77	114.09	201.54
	THS2.2 (1.09)	12	135.38	11.44	110.32	128.20	169.13	110.21	160.55
72:00	3R4F (Air)	12	322.72	39.66	228.59	314.75	379.94	235.44	410.00
	3R4F (0.32)	12	140.50	18.51	96.08	127.71	187.73	99.76	181.25
	3R4F (0.51)	12	133.05	19.06	89.71	108.64	167.58	91.10	175.01
	THS2.2 (Air)	12	426.34	90.02	217.07	341.07	418.20	228.21	624.47
	THS2.2 (0.31)	12	329.12	55.85	192.46	290.36	404.42	206.21	452.04
	THS2.2 (0.46)	12	257.81	34.83	189.76	264.01	344.82	181.17	334.46
	THS2.2 (1.09)	12	206.34	23.19	149.22	185.96	245.56	155.30	257.39

Abbreviations: CXCL, chemokine (C-X-C motif) ligand; h, hour; IL, interleukin; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.8 **Supplementary Table 32**. Descriptive Statistics: MMP-1 Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	8844.69	1908.74	5611.55	8889.08	12077.84	2770.24	14,919.15
	TNFA+IL1B	4	11,371.50	3497.19	5572.03	10,662.18	17,170.97	241.89	22,501.12
24:00	3R4F (Air)	12	11,723.97	977.48	9874.11	11,722.98	13,441.23	9572.54	13,875.40
	3R4F (0.32)	12	26,138.69	3139.89	19,834.18	26,040.12	28,650.13	19,227.85	33,049.53
	3R4F (0.51)	12	35,420.81	3356.04	29,395.46	35,607.21	44,070.41	28,034.22	42,807.40
	THS2.2 (Air)	12	9089.93	1089.51	5682.61	10,903.90	11,961.63	6691.95	11,487.92
	THS2.2 (0.31)	12	11,057.61	1226.56	7756.58	11,747.93	13,655.90	8357.97	13,757.25
	THS2.2 (0.46)	12	15,769.10	2970.69	9294.77	12,138.43	20,200.72	9230.65	22,307.54
	THS2.2 (1.09)	12	18,189.40	4389.94	9214.34	14,980.94	21,324.46	8527.20	27,851.60
48:00	3R4F (Air)	12	15,054.54	3090.02	8851.68	13,908.85	16,293.41	8253.46	21,855.63
	3R4F (0.32)	12	31,454.88	3781.17	19,865.57	30,966.69	37,684.79	23,132.59	39,777.18
	3R4F (0.51)	12	34,956.42	4019.61	23,975.65	38,379.64	42,645.85	26,109.31	43,803.52
	THS2.2 (Air)	12	11,239.09	934.47	9708.74	11,183.82	14,066.35	9182.33	13,295.86
	THS2.2 (0.31)	12	14,608.41	1648.93	11,317.14	13,137.60	17,432.47	10,979.14	18,237.69
	THS2.2 (0.46)	12	15,881.70	2332.83	10,483.01	12,746.04	21,585.65	10,747.17	21,016.22
	THS2.2 (1.09)	12	23,469.31	5307.81	12,282.01	15,518.13	31,350.55	11,786.89	35,151.72
72:00	3R4F (Air)	12	24,636.75	4533.36	12,015.26	20,939.36	29,086.74	14,658.89	34,614.60
	3R4F (0.32)	12	85,478.85	17,883.61	42,773.17	60,330.14	96,500.95	46,117.29	12,4840.4
	3R4F (0.51)	12	81,057.57	20,396.44	41,850.26	63,282.78	88,094.82	36,165.32	12,5949.8
	THS2.2 (Air)	12	13,374.66	1919.22	9054.23	13,129.90	18,658.19	9150.49	17,598.83
	THS2.2 (0.31)	12	15,323.19	1807.86	11,252.26	13,294.08	19,455.17	11,344.12	19,302.26
	THS2.2 (0.46)	12	16,848.84	2658.67	10,450.78	14,348.34	20,329.08	10,997.15	22,700.53
	THS2.2 (1.09)	12	24,539.87	5015.28	11,513.93	22,347.09	28,427.59	13,501.31	35,578.42

Abbreviations: h, hour; IL, interleukin; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; MMP-1, matrix metalloproteinase-1; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha.

14.1.5.9 **Supplementary Table 33**. Descriptive Statistics: TGFA Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	5.90	1.83	3.13	4.97	8.67	0.07	11.74
	TNFA+IL1B	4	11.46	1.96	9.07	12.57	13.85	5.23	17.68
24:00	3R4F (Air)	12	7.08	0.83	5.51	6.40	8.69	5.26	8.90
	3R4F (0.32)	12	13.93	4.13	6.61	10.75	13.78	4.85	23.01
	3R4F (0.51)	12	15.45	2.08	10.19	14.36	18.55	10.89	20.02
	THS2.2 (Air)	12	7.98	1.50	4.10	7.37	11.35	4.67	11.29
	THS2.2 (0.31)	12	6.86	0.90	4.08	6.48	10.11	4.88	8.84
	THS2.2 (0.46)	12	13.16	3.68	5.02	11.53	14.08	5.05	21.27
	THS2.2 (1.09)	12	14.25	4.51	5.13	10.36	14.44	4.32	24.17
48:00	3R4F (Air)	12	16.41	6.69	6.56	9.90	12.78	1.68	31.15
	3R4F (0.32)	12	17.72	1.93	12.41	16.80	23.36	13.47	21.97
	3R4F (0.51)	12	24.19	1.97	18.58	25.41	28.84	19.87	28.52
	THS2.2 (Air)	12	8.71	0.84	6.18	8.51	11.74	6.85	10.56
	THS2.2 (0.31)	12	12.16	1.96	7.50	10.86	14.06	7.85	16.48
	THS2.2 (0.46)	12	13.11	2.54	7.02	9.88	18.80	7.52	18.69
	THS2.2 (1.09)	12	16.65	4.33	8.55	11.57	15.25	7.11	26.18
72:00	3R4F (Air)	12	15.50	3.40	7.04	9.49	21.78	8.01	22.98
	3R4F (0.32)	12	39.00	7.09	20.08	36.88	42.91	23.39	54.61
	3R4F (0.51)	12	53.56	7.87	34.77	46.65	65.83	36.25	70.88
	THS2.2 (Air)	12	12.83	1.94	5.98	12.30	18.06	8.57	17.09
	THS2.2 (0.31)	12	12.09	1.74	7.35	9.80	16.26	8.25	15.93
	THS2.2 (0.46)	12	10.58	1.57	5.72	9.74	16.16	7.11	14.04
	THS2.2 (1.09)	12	16.85	3.42	7.28	14.57	20.12	9.33	24.37

Abbreviations: h, hour; IL, interleukin; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TGFA, transforming growth factor alpha; TNFA, tumor necrosis factor alpha.

14.1.5.10 **Supplementary Table 34**. Descriptive Statistics: VEGFA Protein in the Basolateral Media

		Descriptive Statistics (pg/mL)							
PE (h)	**Exposure**	**N**	**M**	**SEM**	**Q1**	**Median**	**Q3**	**LCLM**	**UCLM**
0:00	PBS	4	227.37	13.61	210.79	233.09	243.96	184.05	270.69
	TNFA+IL1B	4	368.29	39.08	305.31	353.37	431.26	243.92	492.65
24:00	3R4F (Air)	12	239.43	15.43	208.30	229.75	282.33	205.46	273.40
	3R4F (0.32)	12	378.81	15.01	340.29	378.51	413.47	345.78	411.84
	3R4F (0.51)	12	423.75	34.44	340.72	381.78	480.87	347.95	499.56
	THS2.2 (Air)	12	219.30	26.24	160.87	189.91	271.05	161.54	277.06
	THS2.2 (0.31)	12	233.96	25.25	161.28	227.63	282.57	178.37	289.54
	THS2.2 (0.46)	12	340.14	70.64	189.34	262.45	418.72	184.66	495.63
	THS2.2 (1.09)	12	318.82	47.02	199.12	243.27	443.62	215.33	422.31
48:00	3R4F (Air)	12	444.79	33.44	370.65	389.17	500.62	371.19	518.40
	3R4F (0.32)	12	605.71	20.45	544.68	599.36	674.04	560.70	650.72
	3R4F (0.51)	12	623.27	26.52	551.22	628.68	688.90	564.91	681.63
	THS2.2 (Air)	12	537.49	54.33	420.13	506.21	539.34	417.91	657.06
	THS2.2 (0.31)	12	520.28	44.92	412.97	479.66	613.74	421.42	619.14
	THS2.2 (0.46)	12	491.01	37.00	397.56	461.08	590.05	409.57	572.44
	THS2.2 (1.09)	12	643.39	55.50	466.53	632.90	748.57	521.23	765.55
72:00	3R4F (Air)	12	767.26	52.50	623.30	737.12	892.89	651.71	882.80
	3R4F (0.32)	12	845.67	47.90	733.41	812.52	968.77	740.25	951.10
	3R4F (0.51)	12	806.92	43.98	664.31	771.80	927.00	710.12	903.73
	THS2.2 (Air)	12	781.06	78.70	591.09	629.71	1039.42	607.86	954.27
	THS2.2 (0.31)	12	750.38	58.56	580.06	685.03	928.99	621.50	879.26
	THS2.2 (0.46)	12	727.37	60.01	545.95	663.85	900.23	595.28	859.45
	THS2.2 (1.09)	12	880.75	87.33	644.97	796.50	1002.51	688.53	1072.97

Abbreviations: h, hour; IL, interleukin; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; PBS, phosphate-buffered saline; PE, post-exposure; SEM, standard error of the mean; TNFA, tumor necrosis factor alpha; VEGFA, vascular epithelial growth factor A.

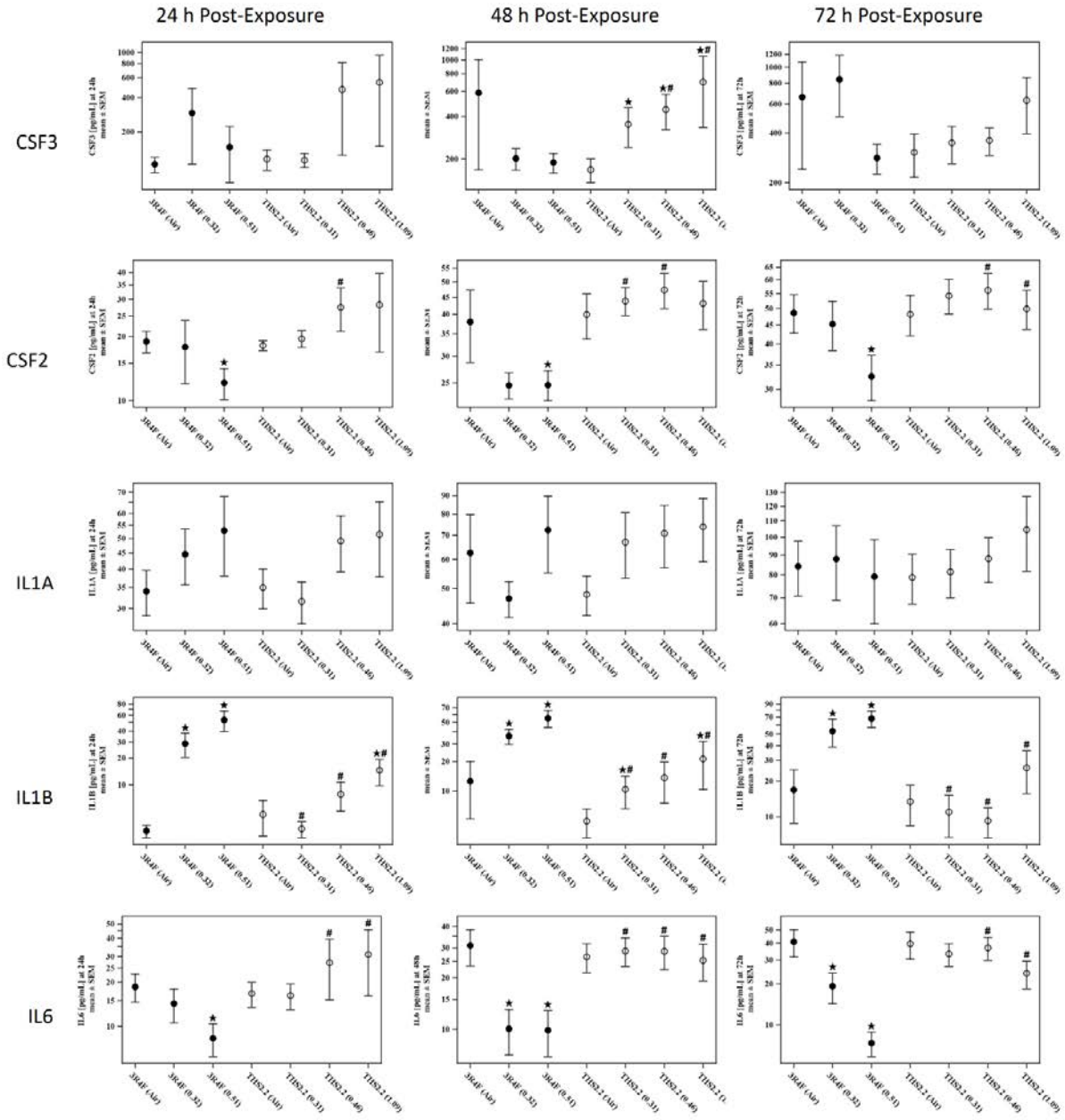
14.1.6 **Supplementary Table 35**. Descriptive Statistics: Cytotoxicity (%) in Dose Range Assessment of THS2.2

PE (h)	Exposure	N	M	SEM	Q1	Median	Q3	LCLM	UCLM
72:00	THS2.2 (Air)	3	0.74	0.14	0.57	0.62	1.02	0.12	1.35
	THS2.2 (0.34)	3	0.70	0.07	0.62	0.65	0.84	0.40	1.01
	THS2.2 (0.45)	3	0.72	0.06	0.61	0.75	0.81	0.47	0.98
	THS2.2 (1.02)	3	0.84	0.07	0.72	0.84	0.95	0.54	1.13
	THS2.2 (1.79)	3	0.91	0.08	0.79	0.86	1.07	0.55	1.27

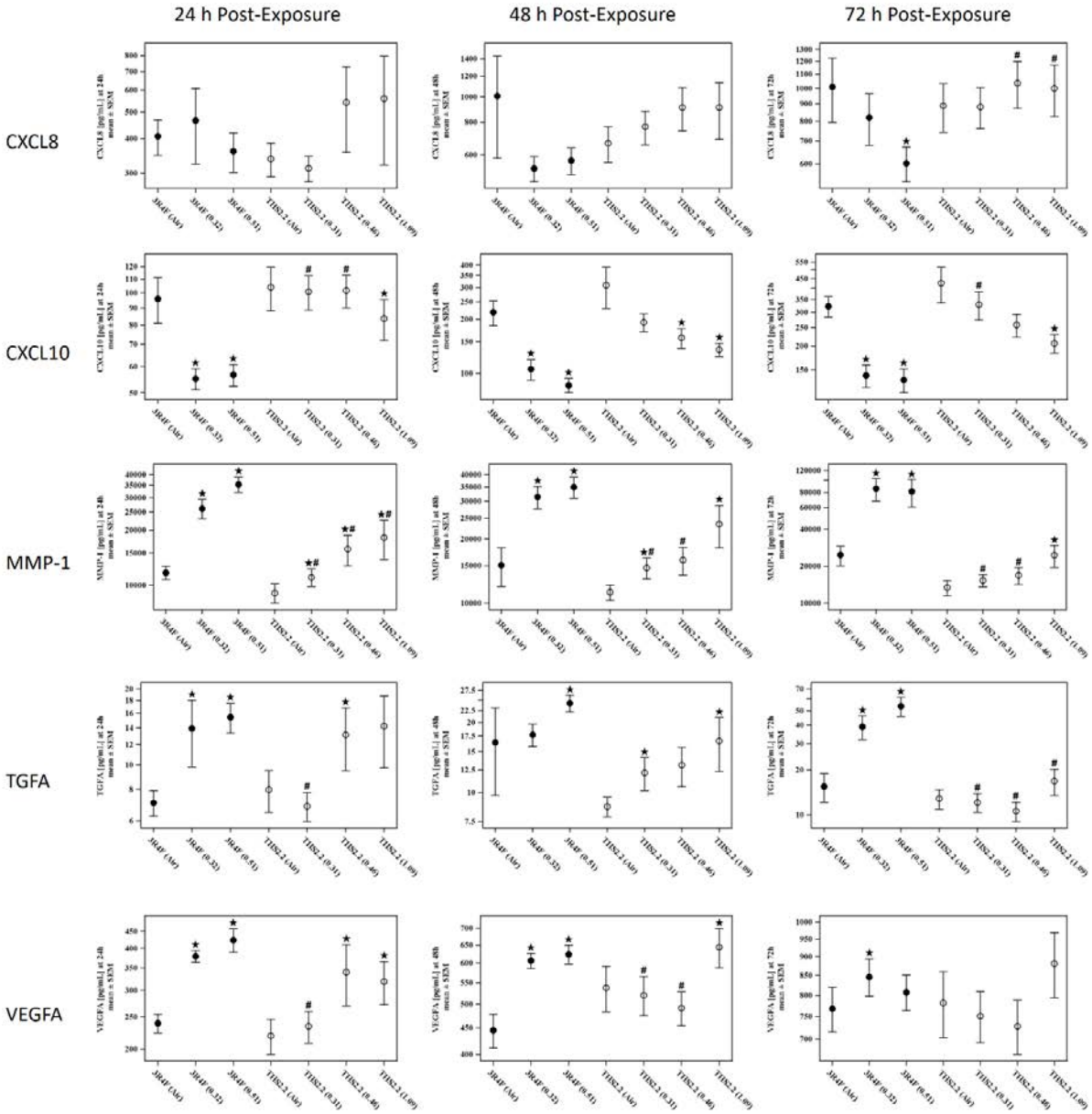
Abbreviations: h, hour; LCLM, lower confidence limit of the mean; M, mean; N, number of replicates; Q1, first quartile; Q3, third quartile; UCLM, upper confidence limit of the mean; SEM, standard error of the mean.

14.2 Supplementary Figures

14.2.1 **Supplementary Figure 1 (figure continues)**. Individual Plots of Pro-Inflammatory Mediators Secreted in Basolateral Media



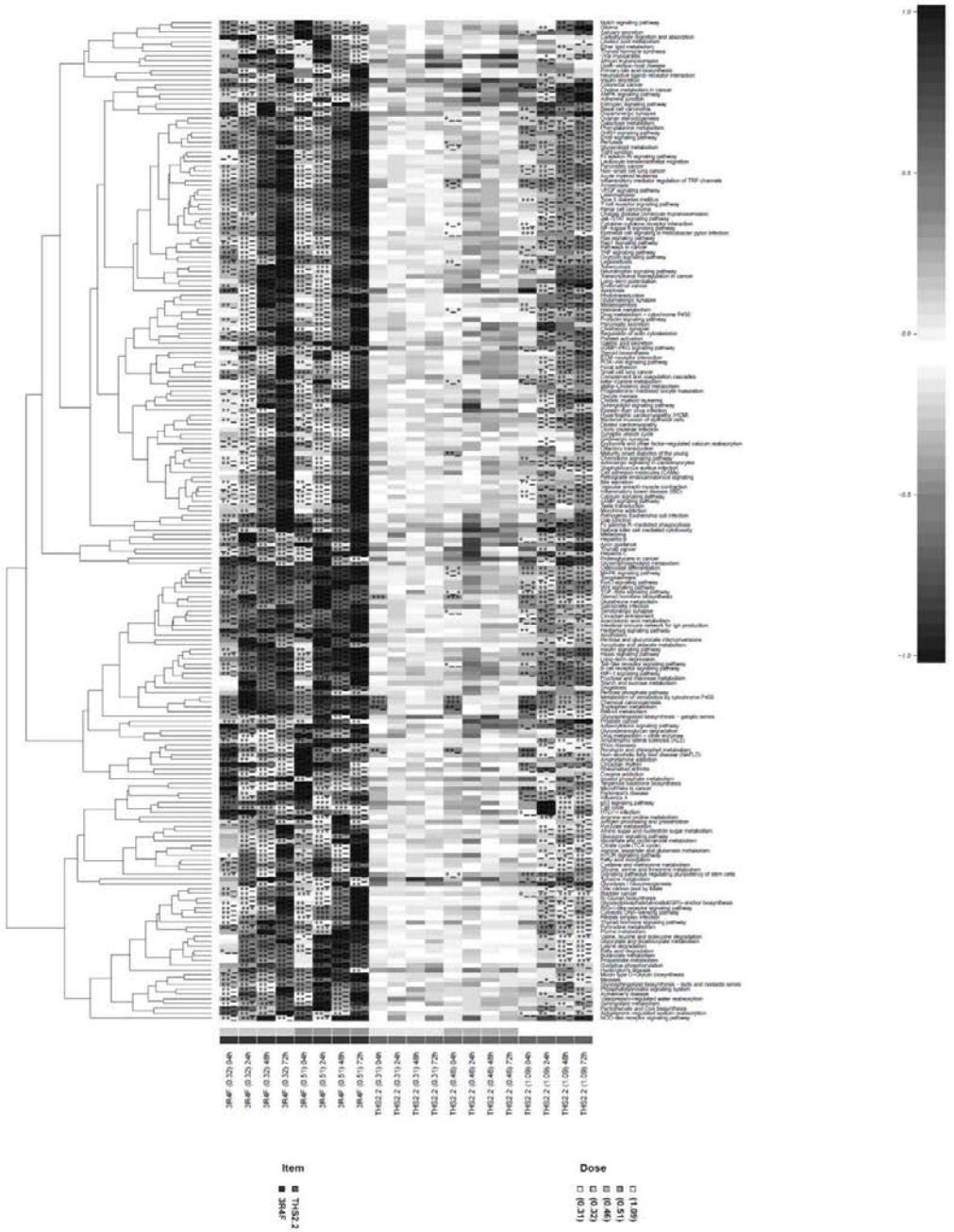
14.2.1 **Supplementary Figure 1 (end of the figure)**.



Supplementary Figure 1. Individual Plots of Inflammatory Mediators Secreted in Basolateral Media.

The mean concentration of the indicated mediator and SEM (N = 11–12, from four experimental repetitions with three exposure runs/repetition) are shown. ⋆ indicates a significant difference compared with the corresponding air controls ($p < 0.05$). # indicates a significant difference compared with 3R4F at the comparable nicotine concentration ($p < 0.05$). Abbreviations: CSF, colony-stimulating factor; CXCL, chemokine (C-X-C motif) ligand; h, hour; IL, interleukin; MMP, matrix metalloproteinase; SEM, standard error of the mean; TGFA, transforming growth factor alpha, VEGFA, vascular endothelial growth factor alpha.

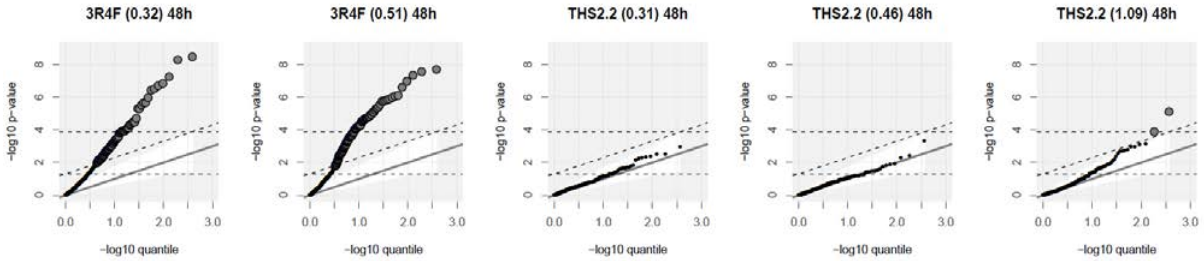
14.2.2 **Supplementary Figure 2.** Gene-set Analysis of Differentially Expressed Genes



Supplementary Figure 2. Gene-set Analysis of Differentially Expressed Genes.

Gene-set enrichment was assessed using over-representation analysis and gene-set analysis. Three types of significance (*) are marked in each cell for each annotation: The first character indicates the adjusted Fisher *p*-value, the second character indicates the GSA Q2 test, and the third character indicates the over-representation analysis test. Non-significant statistics are indicated with the "-" symbol.

14.2.3 **Supplementary Figure 3**. Quantile–Quantile (QQ) Plots of Differentially Expressed miRNA in All Experimental Groups



Supplementary Figure 3. Quantile–quantile (QQ) plots representing the significance of miRNA differential expression profiles.
Each dot represents a miRNA generated from four experimental repetitions, N = 11–12. Statistical significance (y-axis) is proportionally expressed as $-\log_{10}(raw\ p-value)$. The corresponding quantiles are plotted on the x-axis. A miRNA is considered to be significantly differentially expressed if it falls above the oblique gray line.

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