STUDY PLAN

Field for Barcode

TESTING OF REPEATED EXPOSURE OF 3R4F CIGARETTE SMOKE AND THS2.2 AEROSOL ON PBS-SUBMERSED GINGIVAL ORGANOTYPIC TISSUE CULTURES (STUDY NUMBER 179800)

Program Name	System Toxicology
Project Name	Cellular System Biology
Work Package Name	Organotypic investigations
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5. INTRODUCTION

This is an exploratory study to assess the impact of sub-chronic exposure to 3R4F cigarette smoke (CS) and Tobacco Heating System 2.2 (THS2.2) aerosol on human organotypic gingival epithelial tissue cultures covered by a thin layer of phosphate buffered saline (PBS).
The impact of the aerosol will be compared with the impact of smoke generated from the combustion of the reference cigarette 3R4F. The gingival organotypic culture model has been investigated before in the study by Schlage (Schlage 2014), where the effect of repeated exposure to whole cigarette smoke was investigated. The former tissue culture was the full thickness gingival epithelium model of MatTek and was co-cultured with fibroblasts (GIN-300-FT-1, MatTek, Ashland, MA, USA).
In a recent study (S178600), we measured the response of a different gingival model, the EpiGingival™ GIN-100 (MatTek), to different concentrations of THS2.2 aerosol and 3R4F CS at the air liquid interface (ALI). We gained informations about the histology, which was resembling the pictures provided by MatTek, with the exception of the upper cornified layers, which look more developed and fragile in our hands (Figure 1A). Cytotoxicity was also measured (Adenylate Kinase assay). Moreover, we tested the induction of cytochrome P450 (CYP) 1A1/1B1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), with positive results (Figure 1B).

In the present study, we will test the same gingival monoculture of human keratinocytes used in the last study (GIN-100, MatTek) which will address specifically the response of a single cell type (keratinocytes) to the aerosol/smoke. Different from our previous studies, the apical exposure to PBS will mimic the *in-vivo* situation, where saliva is constantly moistening the gingival epithelium.
To robustly assess the impact of exposure to inhaled aerosols (e.g., CS and nanoparticles), the chosen *in vitro* test system should display several key features, which the human gingival organotypic model fulfils.

First, the *in vitro* systems used to investigate the effects of exposure on respiratory toxicity should resemble the *in vivo* situation (Baxter 2015). Many studies in the last years use organotypic gingival tissue cultures with comparable results to the *in vivo* paradigm. Hai and colleagues demonstrated that organotypic EpiGingival™ tissues resemble the *in vivo* paradigm under cytomegalovirus infection (Hai 2006); EpiGingival™ cultures were utilized for carcinogenic studies with good reproducibility of the *in vivo* situation (Agrawal 2013, Mitchell 2012); finally, the EpiGingival™ model has been proved to be suitable for oral care product testing (Yang 2011). In addition, the air-liquid interface to which this 3D cultures are cultivated, allows the direct exposure to cigarette smoke or THS2.2 aerosol which better mimic the *in vivo* exposure situation (Schlage 2014).

Second, the test systems should retain normal metabolic and molecular activities (Huh 2011, Nichols 2014). Interestingly, the organotypic cultured cells have been shown to retain their ability to release pro-inflammatory markers (e.g., cytokines, chemokines, and growth factors) and reactive oxygen species, allowing researchers to investigate the potential mechanisms underlying the local and potentially systemic effects of the exposure (Huh 2011, Nichols 2014). In particular, Schlage and colleagues (2014) showed that following CS exposure, full thickness gingival organotypic cultures co-cultured with fibroblasts released high levels of MMP-1 and VEGF and decreased IP-10, a common feature of *in vivo* oral inflammations (Gemmell 2001, Rubini 2011). Reactive metabolites are formed by the metabolism of pro-toxicants (e.g., pollutants, CS, and nanoparticles) in cells, and these reactive metabolites may be more damaging than the original molecule of exposure (Baxter 2015, Schlage 2014). Gingival cells

express several members of the cytochrome P450 system, such as CYP1A1 and CYP1B1, which are involved in the xenobiotic metabolism of environmental toxicants, and the changes in expression and activity of these enzymes can be monitored following exposure to CS or other toxicants (Schlage 2014).

Finally, the use of tissues isolated from the upper respiratory tract, like gingival tissues, makes the collection from the donor less invasive. In regard to smoking, the oral tissues are more intensely and proportionally exposed to CS as compared to other respiratory tissues (i.e. nasal, bronchial), therefore resulting in a more consistent response (Schlage 2014).

The MatTek gingival tissue model (EpiGingival™) contains normal human gingival keratinocytes cultured in serum-free medium to form three-dimensional differentiated tissues. Hematoxylin and eosin staining of tissue sections shows that the architecture of the tissue is very similar to human gingival mucosa *in vivo* (Hai 2006). The cultured tissue is 9-13 cell layers thick and consists of partially cornified apical surface and a non cornified basal region (Figure 1A). The thickness and morphology of the apical stratum corneum and the basal cell layers are similar to those in the gingival tissues *in vivo*. Indeed, as happens *in vivo*, cells at the basal region of the cultured tissue continue to divide and differentiate, and apical surface cells continue to cornify to form the stratum corneum. Furthermore, the distributions of cytokeratins (e.g. K13 and K14), as assessed by immunohistochemical staining, are like those found *in vivo* (Hai 2006, Oda 1990). In summary, the cultured tissue exhibits characteristics in structure (thickness, morphology, and organization), cell type and differentiation, and protein expression and composition as observed *in vivo*, and can be therefore representative of the gingival tissue.

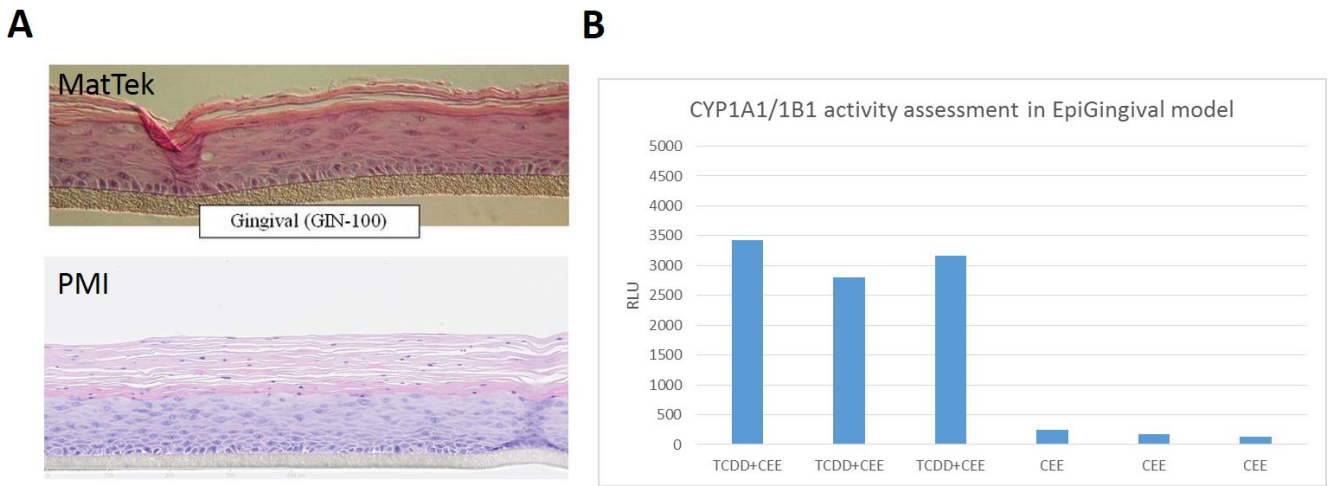

Figure 1. MatTek EpiGingival™ model.
(**A**) Comparison between a picture of the histology of GIN-100 tissues provided by MatTek and a picture of a GIN-100 in house. (**B**) CYP1A1/1B1 activity in GIN-100 tissues after stimulus with TCDD (TCDD+CEE) or negative control (CEE). Abbreviations: CYP, cytochrome P450; CEE, Luciferin; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin. RLU, Relative Light Units.

6. OBJECTIVES

6.1 Primary Objectives

- To determine if and after how long we may observe alterations (cytotoxicity, osmotic stress, release of pro-inflammatory mediators) due to PBS apical exposure in organotypic gingival tissue cultures.
- To evaluate at which concentrations of 3R4F CS and THS2.2 aerosol we may start to observe toxicity or morphological changes in PBS-submersed human gingival epithelial tissue cultures after repeated exposure of 28 min. Various doses (up to 50% 3R4F CS and 100% THS2.2) will be tested and a complete histological assessment, of the exposed inserts will be performed. In parallel, cytotoxicity will be measured using the Adenylate Kinase (AK) assay, CYP activity and gene expression.
- To compare the impact between THS2.2 aerosol and combustible 3R4F CS sub-chronic exposure at a comparable nicotine concentration on PBS-submersed human organotypic gingival tissue cultures by assessing tissue histology, cytotoxicity, secretion of pro-inflammatory mediators, and cytochrome P450 (CYP) activity, as well as transcriptomic (mRNA, miRNA) and metabolomic profiles.

6.2 Secondary Objectives

- To explore the toxicity and molecular profiles of PBS submersed gingival tissue cultures at a single time point after repeated daily exposure of 28 minutes to THS2.2 aerosol or 3R4F CS.
- To explore the alterations that are linked to THS2.2 exposure in particular.

7. EXPERIMENTAL DESIGN

The design of the current study will consist in a first phase, aimed at testing the impact of prolonged apical exposure (up to 96 hours) to PBS of the organotypic gingival tissue cultures. Different endpoints will be analyzed every 24 hours, including the measurement of AK and pro-inflammatory mediators release, Methyl Thiazoyl Tetrazolium (MTT) coloration and expression changes for genes of interest, mainly involved in osmotic stress (by qPCR). We will select the latest timepoint at which we will not observe alterations in the physiology of the tissues due to PBS apical exposure.
The second phase will consist in a Dose Range Assessment (DRA) to evaluate at which concentrations of 3R4F CS and THS2.2 aerosol we may start to observe toxicity or morphological changes in PBS-submersed human gingival epithelial tissue cultures after a repeated exposure of 28 min every 24 h. The endpoints (AK, CYP activity, tissue histology and gene expression) will be tested at the unique time point selected during Phase I.

The highest concentration of 3R4F and THS2.2 aerosol that will be chosen will not exceed the limit (based on the DRA results) after which tissue damage will be observed without obvious changes on AK-based cytotoxicity at the selected time point. This in order to allow the assessment of toxicity-specific mechanisms through the study in tissue cultures that exhibit minimal tissue damage; indeed, molecular changes in a tissue that is already severely damaged cannot be used to infer the mechanistic changes associated to the exposure but only reflect the damage that already occurred (Davis 2013).

Example: if we observe that PBS is altering the cultures physiology at 96 h, we will choose 72 h as time point for analysis; in the second phase we will expose the tissues to 3R4F CS or THS2.2 aerosol at 0, 24 h and 48 h and we will measure the endpoints at the sole 72 h timepoint.

Nicotine concentrations will be measured from PBS that will be placed in empty inserts. This will be necessary to compare the concentrations of smoke/aerosol from 3R4F and THS2.2 and to compare our results to the *in vivo* situation, where nicotine in the saliva of smokers is set in the range between 1 to 5 µM. These measurements will be performed in the same week as Phase I and repeated on each subsequent phase (DRA + MPs).

After the setting of the experimental conditions, three main phases (MPs) will be performed. During the MPs we will focus on the measurement of the repeated daily impact of 28 min exposure of the selected concentrations of 3R4F CS (2 concentrations) and THS2.2 aerosol (3 concentrations, 2 matching with 3R4F and 1 higher) (determined in Phase II) on the PBS-submersed gingival tissue cultures by analyzing different biological endpoints (AK, MAP, CYP activity, tissue histology) and the perturbation of the molecular network at a unique time point, determined after the Phase I of the study.

In addition, tissues will be dedicated specifically to Metabolomic analysis (see 7.5.14 for details).

Note that the design of the MPs will be decided after the analysis of the DRA results. Specific endpoints, controls, test and reference item concentrations will be reported for each MP in the corresponding exposure plan and metadatasheet saved in the following folder:

Study Documents are available in the corresponding Data storage folder.

Therefore, the present document will cover the detailed description of the PBS apical exposure assessment (Phase I) and of the DRA (Phase II) and will describe the endpoints and relative methods we will analyze in the following MPs of the study.

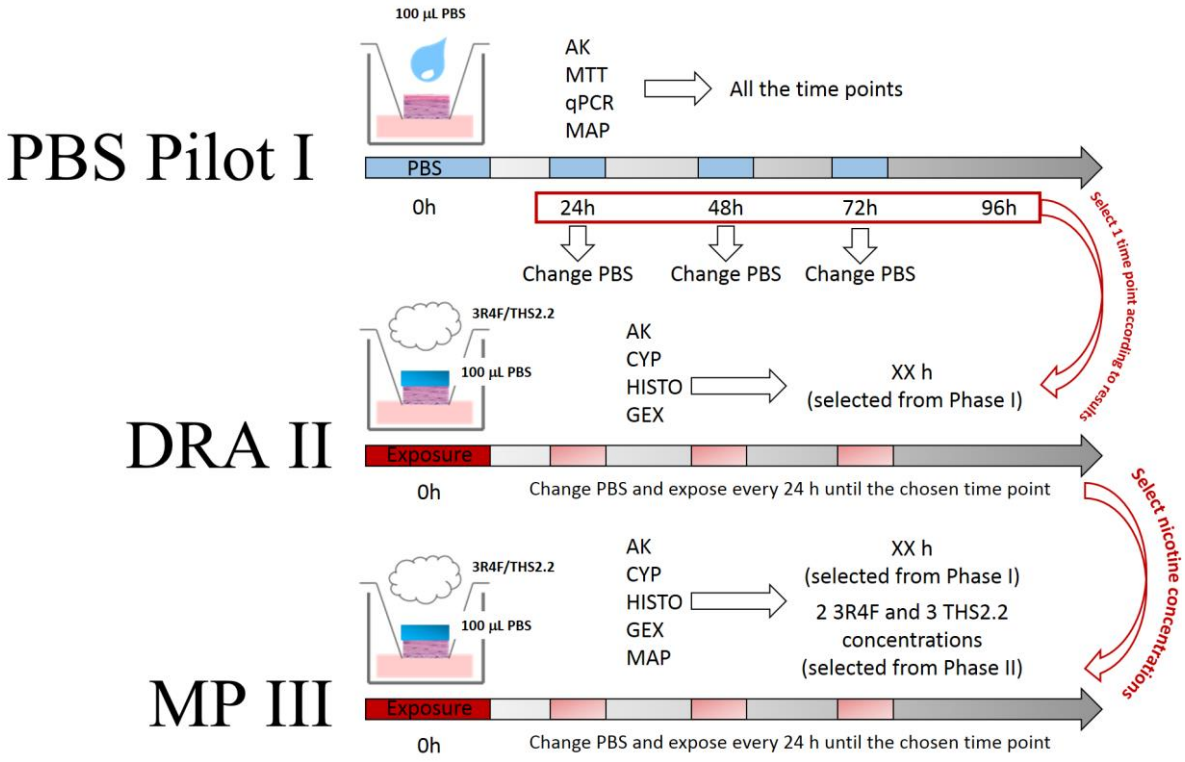

Figure 2. Summary of the experimental design for Phase I, II and III.

7.1 **Exposure Items**

7.1.1 Exposure Items Phase I: Apical exposure to PBS

Gingival tissues will be treated apically with 100 µL of sterile PBS (Sigma-Aldrich, D8662 with calcium and magnesium). PBS will be replaced every 24 h until the end of the exposure duration (96 h). Control tissues will be left untreated.

7.1.2 Phase II: DRA of 3R4F CS and THS2.2 aerosol

All exposure will be conducted according to Health Canada (HC) smoking regimen adapted to meet the special conditions for the Vitrocell® system specified in section 4.1.5. Exposures will be repeated every 24 h, accordingly to Phase I.

7.1.3 Phase III: Main Phase

As for the DRA phase, all exposure will be conducted according to Health Canada (HC) smoking regimen adapted to meet the special conditions for the Vitrocell® system specified in section 4.1.5. Exposures will be repeated every 24 h, accordingly to Phase I.

7.1.4 Test and reference items

Batch of THS2.2 regular test item have been produced by Philip Morris International, Neuchâtel, Switzerland, with a final production date: 12th of October 2015.

The expiration date for this batch is 12th of December 2016.

Packages of the sensorial media already opened are stored under controlled conditions. All test items will be conditioned between 7 and 21 days under controlled conditions from 22 ± 1ºC and a relative humidity of 60 ± 3% according to ISO guidelines.

3R4F cigarettes were purchased from the University of Kentucky (Lexington, KY). The receiving date is December 2014, the expiration date for this batch is May 2017.

Mass-produced test cigarettes are generally homogeneous and remain stable when stored under our standardized storage conditions (according to the results of INBIFO study P 0500/5312, 1998). However, all constituents derived from natural products resulting in a final product which is intrinsically variable. Determination of the test article characteristics including composition will be given in the batch release.

Table 1. Test and reference Items

Type	Short Name	Description	Pack Code	Stick Batch Number	Note
Test item	THS2.2	ZRH/DDA1/C3/CAST LEAF–CL/Flavor/Reynaldo	PCK.00497.RD	B-23862	Batch release certificate for THS2.2
Reference item	3R4F	Conventional Cigarettes	V350Y61B5	V350461B5	Expiry date: May 2017

Table 2. Device informations

Device information on THS2.2					
Product Code	B-178731	B-178731	B-178731	B-178731	B-178731
Receiving Date	27-02-2015	27-02-2015	27-02-2015	27-02-2015	27-02-2015
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.				

7.1.4.1 Exposure doses

The different dilutions tested in the DRA will allow the selection of the dose to test in the main phase where less than 20% cytotoxicity is observed and where no obvious signs of tissue damage (e.g. apoptotic cells, cleft formation, apical keratinization) is noted.

7.1.5 Smoking machine and exposure system

Smoke from the reference item (3R4F) will be generated using 30-port- carousel smoking machine SM 2000. Aerosol from the test item (THS2.2) will be generated using another 30-port carousel smoking machine (MM 2000 P1). Each of the smoking machine will be connected to a Vitrocell® exposure system (Vitrocell® System Gmbh, Waldkirch, Germany), where the tissue inserts will be exposed. The Vitrocell® exposure system is equipped with a dilution system. To achieve the desired target concentrations of nicotine in the smoke/aerosol, dilutions of the smoke/aerosol will be applied (diluted in fresh air). During the exposure experiment, the tissues will be exposed to the smoke/aerosol of the reference/test items at the specific target nicotine concentrations in the smoke/aerosol (Table 3) and various endpoints will be generated (Table 4, Table 5, and Table 6).

Table 3. Concentrations of 3R4F and THS2.2 aerosol.

Concentration	**5**	**4**	**3**	**2**	**1**
Aerosol (%)	10	20	35	75	100
Flow rate aerosol (L/min)	1.997	0.897	0.437	0.87	0
Smoke (%)	5	10	20	40	50
Flow rate smoke (L/min)	3.99	1.99	0.89	0.34	0.22

7.1.5.1 Quality check of the smoking run

The quality control (QC) will generate 3 TPM filters per day and 3 smoke bubbled PBS (sbPBS) for 3 consecutive days. The following actions will be taken for the QC of the aerosol generation, approximately starting 2-3 weeks before the actual exposure experiment:

- Installation and qualification of the smoking machine (IQ,OQ, PQ)
- Determination of the chemical characterization of the undiluted aerosol generated under the adapted HC protocol
- Any leakage of the exposure chamber will be controlled by checking valves and Borgwaldt leakage tester.
- The results of the IQ, OQ, PQ are collected and reported in the protocol stored in the study folder.

7.1.5.2 Characterization of the 3R4F smoke and THS2.2 aerosol

During each smoking run, TPM deposition using the microbalance will be measured for each dilution of 3R4F and THS2.2.

Carbonyls will be measured as part of a routine QC covering the percentage of smoke used in the experimental phases of the organotypic studies. The carbonyls data will be stored in the Carbonyl storage folder.

Nicotine content in PBS will also be measured during each smoking run of each phase as control. The data will be stored in the Nicotine storage folder.

7.2 **Test System**

7.2.1 Human EpiGingival™ tissue culture (without fibroblasts)

EpiGingival™ inserts will be provided by MatTek from the donor (G26, same as used in the oral study S174300) with the following characteristics:

- Age: 46 years old
- Gender: Male
- Non smoker
- No pathology reported
- Caucasian

The tissues belonging this donor will be used throughout the study. Different batches of EpiGingival™ will be used in each phase of the study. The dates of delivery are recorded in the Delivery date of inserts document.

Specific features of this organotypic culture:

1. Primary human cells
2. Human EpiGingival™ tissue cultures are reconstituted in separable Transwells of 6.5 mm of diameter on the polyester membrane with pore size of 0.4 µm (Greiner, cat. nr. 662640). These inserts are transparent, so the tissues can be visualized under the light microscope.
3. The gingival tissue cultures will arrive undifferentiated at PMI and will be cultured over the week-end in the manufacturer differentiation culture medium. On the following Monday, the inserts will be switch to maintenance medium for the whole duration of the study and will be then considered as differentiated epithelial tissue culture.

7.3 **Experimental Groups and Dosing**

7.3.1 Experimental Groups and dosing Phase I: Apical exposure to PBS

Note: No historical data about apical PBS exposure of gingival tissue cultures is available. 100 µL of sterile PBS with calcium and magnesium will be applied on the apical side of the EpiGingival™ cultures at the starting of the exposure phase. It will be replaced every 24 h with fresh PBS until 96 h.

Table 4. Pilot Phase I: list of endpoints per post-exposure time points.
Each endpoint will be measured for the two exposure conditions (PBS or no treatment control).

Experimental Phase	**Phase I: Apical Exposure to PBS**		
Experimental Week	**08-02-2016**		
Biological Endpoints	**Duration of Exposure (hours)**	**Post-Exposure (hours)**	**Tissue Inserts per Endpoint**
Cytotoxicity (AK)*#	24-48-72-96	0	5
Gene Expression (qPCR)*	24-48-72-96	0	5
Inflammatory mediators release (MAP)*	24-48-72-96	0	3 + 2[s]
Metabolic Activity (MTT)#	24-48-72-96	0	3
Exposure Characterization			
Nicotine (PBS)	✓		

[s] The basolateral medium will be collected for all the inserts (5) but analyzed for 3 only. In case of statistical inconsistency the other 2 inserts will be further added to the analysis. *# Common endpoints.

7.3.2 Experimental Groups and dosing Phase II: DRA of 3R4F CS and THS2.2 aerosol

Note: Previously generated data about the DRA of EpiGingival™ tissues are available from the study S178600. However, due to the different conditions of exposure (i.e. PBS on the apical side of the inserts and repeated CS/aerosol exposures) a new DRA needs to be run to correlate aerosol and CS dilutions within the Vitrocell® system to the extrapolated target nicotine concentrations and the effects of the different concentrations to the exposed tissues. The nicotine concentrations will be measured and confirmed (or adjusted when appropriate) starting from the DRA phase and following future measurements of nicotine concentrations throughout the study phases.

Table 5. DRA list of endpoints per post-exposure time points.
Each endpoint will be measured for all exposure conditions (Air, 3R4F, THS2.2) for all doses tested.

Experimental Phase	**DRA**		
Experimental Week	**22-02-2016**		
Biological Endpoints	**Duration of Exposure (hours)**	**Post Exposure (hours)**	**Tissue Inserts per Endpoint**
Cytotoxicity (AK)* #	according to Phase I	4-24	3
Histology (HE)*		24	3
CYP1A1/1B1 Activity*		24	3
Gene Expression (mRNA)#		4	3
Number of Runs	3 repetition every 24 h according to phase I		
Number of Tissue Insert/Run/Endpoint	1		
Exposure Characterization			
Nicotine (PBS)	✓		
Carbonyls	Will be part of QC assessment of the smoke/aerosol generation, samples will be generated throughout the study phases		

*# Common endpoints

Table 6. MP list of endpoints per post-exposure time point.

Each endpoint will be measured for all exposure conditions (Air, 3R4F, THS2.2) for all doses tested.

Experimental Phase	**Main Phase 1-2-3**		
Experimental Week	**07-03-2016 / 04-04-2016 / 18-04-2016**		
Biological Endpoints	**Duration of Exposure (hours)**	**Post Exposure (hours)**	**Tissue Inserts per Endpoint**
Cytotoxicity (AK)* #	according to Phase I	24	3
Gene Expression (mRNA/miRNA) #		4	3
Pro-inflammatory Mediators (MAP)#		24	3
CYP1A1/1B1 Activity*		24	3
Histology*		24	3
Metabolomic[$]		24	5
Nicotine Trapped in PBS	-	-	3
Number of Runs	3 repeated every 24 h according to phase I		
Number of Tissue Insert/Run	1		
Exposure Characterization			
Nicotine (PBS)	✓		
Carbonyls	Will be part of QC assessment of the smoke/aerosol generation, samples will be generated throughout the study phases		

The above table do not exclude the possibility that additional endpoints would be generated, the variations will be reported in the appropriate exposure plan. *# Common endpoints. [$] Only for one phase. Abbreviations: CYP, cytochrome.

7.4 Exposure Plan

7.4.1 Exposure Plan for Phase II DRA

Run A1			A	B	C	D	E	F	
3R4F		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	50.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	40.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	5.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

Run B1			A	B	C	D	E	F	
3R4F		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	50.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	40.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	5.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

Run C1			A	B	C	D	E	F	
3R4F		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	50.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	40.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	5.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

Run A2			A	B	C	D	E	F	
THS2.2		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	100.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	75.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	35.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

Run B2			A	B	C	D	E	F	
THS2.2		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	100.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	75.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	35.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

Run C2			A	B	C	D	E	F	
THS2.2		8	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	Repeated exposures - dates according to Phase I
	100.0%	7	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	75.0%	6	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	35.0%	5	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	20.0%	4	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
	10.0%	3	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity	SHAM	2	Nicotine Assessment	AK/GEX	Nicotine Assessment	AK/CYP/Histo	Nicotine Assessment	EMPTY	
AIR 60% Humidity		1	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	

7.4.2 Exposure Plan for the Pilot PBS and DRA

The exposure plan for the pilot PBS and DRA can be found in the Exposure plan PBS pilot and DRA file.

7.4.3 Exposure Plan for the MP

The exposure plan for the Main phases 1-3 can be found in the Study documents folder.

7.5 Procedures

All samples and collection dates are listed and recorded in the metadata files in the following folder:

- Metadata PBS pilot
- Metadata DRA phase

Metadata for each MP will be included in the following folders:

- Metadata main phase 1
- Metadata main phase 2
- Metadata main phase 3

The following sections includes the Methods that will be used in the PBS testing, in the DRA and in the following MPs.

7.5.1 Apical exposure of EpiGingivalTM tissues to PBS

EpiGingival™ tissue cultures will be exposed to 100 µL of Phosphate Buffer Saline (Sigma-Aldrich, D8662 with calcium and magnesium). PBS will be replaced with fresh one every 24 h until 96 h for Phase I, and until the selected time point for Phase II. Note that during Phase II (DRA) and III (MP), PBS will be kept on the apical surface of the tissue culture after exposure and changed before the following one, after 24 h.

7.5.2 Smoke and Aerosol Generation

Reference smoke will be generated from 3R4F reference cigarettes (Kentucky Tobacco Research & Development Center) using a 30-port carousel smoking machine type SM2000 (SM2000; Philip Morris, International) with a Programmable Dual Syringe Pump (PDSP) connected to the Vitrocell® 24/48 (Vitrocell® Systems GmbH, Waldkirch, Germany) (Vitrocell® 24/48 for 24 well sized inserts). Another 30-port carousel smoking machine type with a PDSP is used to generate the test aerosol from the modified risk tobacco product (THS2.2). The reference smoke and the test aerosol will be generated. The 3R4F cigarettes and THS2.2 sticks will be conditioned between 7 and 21 days at 22 ± 1°C with a relative humidity of 58 ± 3% to comply with the ISO standard 3402 (International Organization for Standardization 1999). The reference smoke and test aerosol will be generated according to the Intensive Health Canada protocol with a puff volume of 55 ml, puff duration of 2 sec. and a puff frequency of 2 times a minute. Supplementary, the ventilation holes of the reference cigarette will be blocked by installing special HC mouth pieces into the carousel of the SM 2000. As a special requirement for the Vitrocell® 24/48 the exhaust time from the PDSP is set to 8 seconds.

For the exposure experiments, each 3R4F cigarette will be smoked to a mean butt length of 35 mm ± 1 mm and each THS2.2 will be aerosolized with a pre-defined puff count of 12 puffs per THS2.2 stick. Before starting the 28 minutes exposure experiment a pre-smoke/aerosol phase will be implemented to get a steady state of both SM's. During the exposure experiment itself approximately ten 3R4F cigarettes will be smoked and approximately ten THS2.2 sticks will be aerosolized using the dedicated smoking machine.

7.5.3 Nicotine Determination in PBS exposed to Whole Smoke/ Aerosol

Deposition of nicotine will be measure in PBS-exposed samples. Briefly, 100 µl of PBS will be placed into steel inserts and exposed to cigarette smoke and P1 aerosols using the Vitrocell system. The nicotine concentrations will be measured using a LC-HRAM-MS (Q Exactive™, Thermo Fisher). A LC-HRAM-MS is a multi-analyte method in full scan positive electrospray ionization mode, using 2 sequential analytical columns (strong anion exchange/polymer backbone reversed phase). Quantification is performed by calibration curves of the analytes with stable isotope labelled internal standards for each analyte or analyte group. d3-Nicotine is used as internal standard for the determination of nicotine.

7.5.4 Carbonyls Determination in Whole Smoke/ Aerosol

Carbonyls will be measured in PBS following a 5 minutes exposure to whole smoke or test aerosol (total two sticks per test item, each of them will be smoked/aerosolized by applying a modified Heath Canada Intense (HCI) puffing protocol of 55 mL puff over 2 seconds, twice per minute with an 8 seconds pump exhaust time) (PMI_RD_WKI_001094). Briefly, before exposure, each row in the cultivation base module of the Vitrocell® 24/48 will be filled with 18.5 mL PBS. Following exposure, an aliquot of 1.2 mL PBS exposed sample (per row) will be collected and mixed with 1.8 mL DNPH solution (15 mM). Subsequently, 150 µL pyridine will be added to quench the chemical derivatization. From this mixture, a 500 µL aliquot will be taken and placed into a LC-MS amber glass vial. After 30 minutes, 485 µL acetonitrile and 15 µL internal standard mixture (24 µg/mL d6-acetone and 21 µg/mL d5-methyl ethyl ketone) will be added to the mixture. Finally, 5 µL of the mixture sample will be injected to an HPLC instrument coupled with a tandem MS (HPLC-MS/MS) to determine the quantity of acetaldehyde, acetone, acrolein, methyl ethyl ketone, formaldehyde, crotonaldehyde, butyraldehyde, and propionaldehyde using an isotope dilution technique.

7.5.5 Maintenance of EpiGingival™ 3D-organotypic Tissue Cultures (Air-Liquid Interface cultures)

Upon arrival, human organotypic gingival tissue cultures (EpiGingival™, MatTek Corporation, Ashland, MA, USA) will be handled under sterile condition under the hood. The tissues will be cultured at the air liquid interface (ALI) in a pre-warmed 37°C EpiGingival™ differentiation media (GIN-100-DM4a) 5.5 mL/well in sterile modified 2 hole tops and 12-well plates according to the supplier's instruction. After three days, the tissue cultures will be fully differentiated and newly fresh supplemented maintenance media (GIN-100-MM, provided by MatTek Corp) 5.5 mL/well will be used to maintain the culture according to the supplier's guidelines. After the exposure to smoke or aerosol the inserts will be placed in a 24 well plates with 0.5 mL/well of GIN-100-MM. After any exposure the tissues will be placed in fresh media.
Throughout the study, the cultures will be regularly monitored using a microscope for morphological characteristics and bacterial or fungal contamination.

7.5.6 Adenylate Kinase (AK) Cytotoxicity Assay

AK activity will be measured from the basolateral medium and apical-administered PBS (for DRA and MPs) of 3D-organotypic tissue cultures, using a BioVision Bioluminescence Cytotoxicity Assay Kit (BioVision, Inc. Milpitas, CA, USA) (PMI_RD_WKI_001048). Briefly, 50 µL of basolateral medium will be transferred to a well of a luminescence compatible 96-well microtiter-plate and mixed with 50 µL Reagent Working Solution. After 5 min of incubation, luminescence will be measured using a FluoStar Omega reader (BMG Labtech GmbH, Ortenberg, Germany). Alternatively, a ToxiLight™ bioassay kit (Lonza, Rockland, MA, USA) will be used to determine cytotoxicity upon aerosol exposure of 3D-organotypic tissue cultures. For this, 20 µL of basolateral medium will be 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 will be measured using a FluoStar Omega reader (BMG Labtech GmbH, Ortenberg, Germany). For a normalization of the AK assay, 3D-organotypic tissue inserts will be treated for 24 h with Triton X100 to 1% final concentration added to the basolateral side, to induce a complete lysis of the cells (= 100% cytotoxicity).

7.5.7 Methyl Thiazoyl Tetrazolium (MTT) Metabolic Assay

700 µL of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (1mg/mL) will be added to each well. Tissues will incubated in MTT at 37°C for 3 h. After the MTT reaction, the tissue inserts will be blotted on dry absorbent paper, cleared of excess liquid, and then transferred to a second 24-well plate containing 1 mL of isopropyl alcohol (0.5 mL on the apical side and 0.5 mL in the basal side medium) in order to stop the reaction and extract the formazan. The 24-well plate will be sealed in a plastic bag and the extraction will be allowed to proceed for 2 h at room temperature in the dark. Afterwards, 200 µL of the formazan extract will be quantified by measuring optical density (OD) at 570 nm using the FluoStar Omega reader (BMG Labtech GmbH, Ortenberg, Germany).

7.5.8 Measurement of Secreted Inflammatory Mediators

Multi-analyte profiling of pro-inflammatory mediators (MAP analysis) will be carried out collecting 200 µL of basolateral medium from 3D-organotypic tissue cultures, taken at different post-exposure times (different post exposure time points after untreated or PBS exposure, whole smoke or aerosol exposure, see Table 4 and Table 6) (PMI_RD_SOP_000361; PMI_RD_WKI_001032; PMI_RD_WKI_001274; PMI_RD_FOR_000803). For the analysis, Luminex® xMAP® technology will be employed (Luminex, Austin, TX, USA) using commercially available assay panels (EMD Millipore Corp., Schwalbach, Germany), as outlined in Table 7 and according to the manufacturer instruction. For this, 25 µL of diluted or non-diluted sample will be used for each detection and the analysis will be run on a Luminex®, 200™ or FLEXMAP 3D®, equipped with the xPONENT software (Luminex, Austin, TX, USA). Data are represented as Median Fluorescent Intensity (MFI) using a 5-parameter logistic or spline curve-fitting method for calculating the analyte concentrations in the sample. For a positive control, EpiGingival™ inserts will be treated (N=3) for 24 h with TNFα and IL-1β (PMI_RD_FOR_000849), each of them given to the basolateral medium to 10 ng/mL final concentration, which induces (the majority although not all) mediators targeted by the MAP analysis.

Table 7. Assay Panels and Various Mediators for the MAP Analysis

PBS Pilot Phase I	
Kit Name	**HCYTOMAG-60K-12**
Mediators	GRO, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-8, IP-10, MCP-1, RANTES, VEGF, TNF-α
All main Phases	
Kit Name	**HCYTOMAG-60K-12**
Mediators	GRO, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-8, IP-10, MCP-1, RANTES, VEGF, TNF-α
Kit Name	**HMMP2MAG-55K-02**
Mediators	MMP-1, MMP-9

7.5.9 Histological Analysis

Tissue inserts will be washed three times with PBS and fixed for 2 h in freshly prepared 4% paraformaldehyde (PFA) (PMI_RD_WKI_001242). Upon completion of the fixation, the fixative will be aspirated and the fixed organotypic cultures will be washed at room temperature both apically and basally 3 times with PBS. Following this process, the fixed cultures will be separated from the inserts by detaching the membrane from the plastic with a forceps and bisected through the middle prior to processing using Leica ASP300S tissue processor (PMI_RD_WKI_001243). Subsequently, the two sections (per insert) will be embedded into a paraffin block (PMI_RD_WKI_001260). Microscopy sections of 5 µm thickness will be obtained using a microtome (PMI_RD_WKI_001262) and mounted on glass slides which will be then transferred to the automated slide stainer Leica ST5020 for staining with Hematoxylin & Eosin (PMI_RD_WKI_001266). Digital images of each slide will be generated using the Hamamatsu NanoZoomer slide scanner (PMI_RD_WKI_001314).

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

The activity of CYP1A1/CYP1B1 (combined) will be 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 CYP-Glo substrate luciferin-6' chloroethyl ether (Luciferin-CEE), which is a substrate for both, CYP1A1 and CYP1B1, will be added to the basolateral medium, 24 h prior to sample collection. The luciferin product of the CYP reaction will be then detected by mixing 50 µL of the collected medium with 50 µL of Luciferin Detection Reagent. After 30 min incubation at room temperature, luminescence will be measured in a FluoStar Omega reader (BMG Labtech GmbH, Ortenberg, Germany). As a positive control for CYP1A1/B1 induction, three replicates of 3D-organotypic culture inserts will be treated for 48 h with 30 nM TCDD (Sigma-Aldrich, St. Louis, MO, USA), added to a final concentration to the basolateral side of culture inserts. As a negative control, basolateral media from three replicates of untreated culture inserts will be tested for CYP1A1/B1 activity.

7.5.11 RNA/microRNA Purification

Total RNA including microRNA will be isolated after washing the 3D-organotypic culture inserts two times with cold (4°C) PBS, at both basal and apical side. The cells will be then disrupted in 700 µL Qiazol lysis buffer (Qiagen, Hilden, Germany) followed by RNA extraction:
PMI_RD_WKI_001117: Isolation of total RNA including microRNAs from 3D Organotypic tissue inserts using miRNeasy mini kit protocol. The final elution will be done in 30µL RNAse free water.
PMI_RD_WKI_000978: Quality controls following RNA extractions.
The Gene expression workflow will require the use of the following work instructions.
PMI_RD_WKI_001109: Affymetrix IVT PLUS Protocol
PMI_RD_WKI_001125: Fragment analyzer Quality Control
PMI_RD_WKI_001126: Affymetrix-3'array-Cartridge-Hybridization (Nugen PicoWTA, Nugen WB, IVT PLUS)
Hybridization will be performed on Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array.

7.5.12 miRNA profiling

The Flash Tag Biotin HSR kit (Affymetrix, Santa Clara, CA, USA) will be used following:
PMI_RD_WKI_001123: miRNA labeling using the FlashTag Biotin HSR kits
Hybridization will be performed on Affymetrix GeneChip® miRNA V4.0 Array

7.5.13 qPCR array for candidate genes

The expression of genes involved in osmotic stress response will be measured by using the RT² Profiler™ PCR Arrays (Qiagen, Hilden, Germany). The Human Osmotic Stress RT² Profiler PCR Array profiles the expression of 84 key genes involved in the cellular response to changes in osmolarity. Housekeeping genes are included in this panel (ACTB, B2M, GAPDH, HPRT1, RPLP0) and a control to check the genomic DNA contamination (HGDC) and a positive PCR control which is a predispensed artificial DNA sequence detected at the qPCR level.

For this specific panel, the following WKI will be used:
PMI_RD_WKI_001334
: qRTPCR SA Bioscience.
Once the qPCR is run is finished, open the .eds file in the QuantStudio Real time PCR software V1.2 and select among the plates a single threshold for all the genes in Analysis settings. Then uncheck in the export tab and in the Result tab the Skip empty wells and skip omitted wells options. Then press start export button and export your .xlsx file in the Study Folder. The Ct values are then ready for analysis.

7.5.14 Metabolomic assessment

The measurement of the metabolome will be performed in collaboration with Metabolon® (NS, USA). Briefly, tissues will be exposed (same exposure design as MP) to sham and one concentration of 3R4F smoke and THS2.2 aerosol (according to DRA). Five smoking repetitions will be performed and 2 time points considered for analysis (4 and 24 h PE). Whole tissues will be collected and shipped for the analysis. 5 tissues for each exposure condition will be pooled together in order to reach the ideal minimum weight to ensure detectability of the signal. Basolateral media of the tissues will be frozen and stored for future eventual analysis (media from 5 inserts per condition pooled together). Metabolon will run the Discovery HD4™ platform by which they will measure a variety of metabolic pathways (Table 8). The final report delivered will provide the list of named metabolites (known biochemical structure)

and unnamed metabolites, statistical changes between groups, relevant biochemical data and summary of the results. In addition, RAW data will be provided.

Table 8. Pathways analyzed by Metabolon

Amino Acid Metabolism	Cofactor & Vitamin Metabolism	Nucleotide Metabolism	Microbiome Metabolism
Amino Acid catabolism Bioactive intermediates & trace amines Glutathione metabolism Inflammatory mediators Microbiome metabolism Polyamines/ornithine metabolism Urea Cycle	Ascorbate metabolism CoA metabolism FAD metabolism Folate metabolism NAD/NADP metabolism PLP metabolism SAM metabolism Many other cofactors and vitamins (tocopherol, B12, Biotin)	Degradation of nucleotides Deoxyribonucleotides DNA damage FAD metabolism Modified nucleotides Nucleotide Coenzymes Purine and pyrimidine *de novo* synthesis Purine and pyrimidine salvage synthesis Ribose metabolism	2º Bile acids Aromatic amino acids Energy Choline/carnitine Xenobiotics Fatty acids/short chain medium chain Vitamins Polyamines
Carbohydrate Metabolism	**Energy Metabolism**	**Lipid Metabolism**	**Novel Metabolites**
Gluconeogenesis Glucose metabolism Glycogen metabolism Glycosylation pathways Metabolism of other carbon sources Metabolism of sugars (fructose, galactose) Polyol metabolism Pyruvate metabolism	Acyl-carnitines Beta-oxidation Creatine metabolism FAD metabolism Glycolysis Mitochondrial function Pentose phosphate pathway	Bile acids Bioactive lipids Cholesterol Fatty acids Sphingosine Inflammatory mediators Lysolipids Sterols Oxidized lipids (COX, LOX)	Novel drug metabolites Novel xenobiotics Novel microbiota metabolites Novel by-products of non-canonical host metabolism

7.5.15 TPM deposition measurement

The microbalance is used to determine real time deposition of particles on a quartz crystal during a whole smoke exposure run. The method allows the determination of the deposition with a resolution of 10 ng/cm^{2}s. The lowest detection limit is approx. 20 ng/cm^{2}h. In the present study, the microbalance will be applied during each smoke run and it will measure the amount of particles deposited during CS exposure of organotypic tissues with various dilutions of THS2.2 aerosol.
NB: The measurement will give an indication on the amount of delivered particles for a given dose. For the deposition/cm^{2,} the insert surface (around 0.33 cm^2) should be taken in consideration when calculating the deposited amount on each insert because from 100% percent only 33% will deposited on the tissues inserts.

Protocol for the measurement

1) Connect quartz crystal with the microbalance.
2) Open Microbalance Software.
3) Microbalance will be connected with all relevant rows where tissues were exposed.
4) Set the scale to zero and wait a couple of minutes until baseline is set.
5) Start whole smoke exposure according to study plan.
6) After the end of each smoke run set the scale again back to zero for the next measurement.
7) After completing all smoke runs save the files. Microbalance software will generate the raw data file including a graphical display of particle deposition.

8. STATISTICAL AND COMPUTATIONAL METHODS

The analysis and graphical display of the results stored in the study folder under the Cellular Systems Biology folder (\\rd-bsrdata.app.pmi\bsr_data\Cellular Systems Biology), will be performed using SAS v9.2 (SAS Institute).

- Information bias may come from the fact that results would be generated from a specific donor (for the reconstitution of the bronchial tissue inserts). The results obtain in this study may not represent results obtained using other donor.
- Variability due to the different exposure runs will be assessed by conducting at least three exposure runs
- Systematic variations due to spatial and temporal settings during which the exposure runs were conducted, would be solved by implementing paired samples (i.e., at a given concentration and post-exposure time points, results generated from exposed samples will be paired with their respective air control samples.
- When applicable, analysis would be conducted in a blinded manner; for example, during histological evaluation, in which coded samples would be used during evaluation.

8.1 Experimental Unit

For the exposure experiments using organotypic tissue culture models and the Vitrocell®24/48 cultivation and exposure system, the experimental unit will be the exposure run. If a particular endpoint will be measured several times in the same tissue culture insert or in different tissue culture 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 exposure run, then all the average values of a given endpoint will be calculated.
For the controls, endpoints will be measured from tissue inserts that will be 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 will be the study phase, i.e., a single experimental repetition, and the average values of a given endpoint will be calculated from the measurements performed on tissue inserts that will be treated identically with the specific corresponding reagent/chemical.

8.2 Derived Variables

8.2.1 AK Cytotoxicity Assay

Cytotoxicity of the cultures will be derived from AK values. All values of the AK assay readout will be normalized using the average of the positive control (Triton X-100-treated tissue inserts) and negative control (PBS-treated or untreated tissue inserts [unexposed, incubator control]) and percentage cytotoxicity will be calculated as follows:

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

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

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

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

8.2.2 CYP1A1/1B1 Activity Assay

Normalized CYP activity as a percentage (%) would be reported. Each values of CYP would be normalized using the average of the positive control (TCDD + luciferin substrate-treated tissue inserts) and negative control (luciferin substrate-treated tissue inserts).

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

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

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

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

8.2.3 MTT metabolic Assay

Raw OD_{565} absorbance values will be measured, and the following calculations made. Generally, calculations will be performed using an Excel spreadsheet.
The mean OD_{565} value of the extraction solvent (blank) wells will be calculated.
The corrected mean OD_{565} value of the negative control(s) will be determined by subtracting the mean OD_{565} value of the blank wells from their mean OD_{565} values.
The corrected OD_{565} values of the individual test article exposures and the positive control exposures will be determined by subtracting from each the mean OD_{565} value of the blank.

Corrected test article exposure OD_{565} = Test article exposure OD_{565} – Blank mean OD_{565}

The following % of Control calculations will be made:

$$\% \text{ of Control} = \frac{\text{Final corrected } OD_{565} \text{ of each Test Article or Positive Control exposure}}{\text{Corrected mean } OD_{565} \text{ of Negative Control exposure}} \text{ X } 100$$

The individual % of Control values (relative viability) are then averaged to calculate the mean % of Control per exposure. Test article and positive control viability calculations will be performed by comparing the corrected OD_{565} values of each test article or positive control exposure time to the appropriate negative control.

8.2.4 Statistical Tests

8.2.4.1 For continuous Variables

- The comparisons of an exposed sample versus its air control (i.e., the paired-sample from the same exposure run at a given experimental repetition) will be done using a paired T-TEST.
- The comparisons of reference item-exposed versus test item-exposed samples will be done after subtracting the values of the corresponding air controls (i.e., the paired-sample). Then the comparison will be done using a T-TEST corrected for non-equal variance (Satterthwaite correction).

8.2.4.2 For Categorical Variables

- The comparisons of an exposed sample versus its air control (i.e., the paired-sample from the same exposure run at a given experimental repetition) will be done using a Cochran-Mantel-Haenszel test, on the row mean scores differences, with the MODRIDIT score type. The test will be stratified by the exposure run.
- The comparisons of the effects between that of the reference item-exposed versus test-item-exposed samples will be done after subtracting the values of the corresponding air controls (i.e., the paired-sample). Then, the comparisons will be done using an unstratified Cochran-Mantel-Haenszel test, on the row mean scores differences, with the MODRIDIT score type.

8.2.4.3 For Binary Variables

- The comparisons of an exposed sample versus its air control (i.e., the paired-sample from the same exposure run at a given experimental repetition) will be done using an exact McNemar’s test. The test will be stratified by the exposure run.
- The comparisons of the effects between that of the reference item-exposed versus test-item-exposed samples be done after subtracting the values of the corresponding air controls (i.e., the paired-sample). Then, the comparisons were done using a Fisher’s exact test.

8.3 **Descriptive Statistics**

Sample size, mean, standard error mean and/or standard deviation will be reported for each endpoints.

8.4 Missing Values

No imputation for missing values. If there is missing value, the remaining data will be processed as described in this study plan.

8.5 Data Transformation

Data transformation may be conducted when applicable (e.g. data are not normally distributed).

8.6 Hypothesis and Confirmatory Analysis

Not applicable. This is an exploratory study.

8.7 Exploratory Analysis

This is an exploratory study; therefore, significant level is not be defined. Raw p-values will be reported.

8.8 Sample Size Justification / Power Considerations

- Because this is an explorative study without a prior knowledge on the response size associated by the test item THS2.2, power calculation cannot be conducted.
- The sample size will be a minimum of triplicate per phase/study repetition/endpoint or a different number, as indicated. One replicate will be exposed per exposure run in a given main phase (experimental repetition). The main phases will be repeated at least three times to ensure robust measurable signals/results. This knowledge was obtained by the previous study conducted for the assessment of the test item THS2.2.

8.9 Computational Analysis

8.9.1 Sample Randomization

Randomization will be done for RNA extraction and defined prior to the experimental exposures. Array hybridization will be performed as a complete block randomization, where the blocking factor will be defined by both the exposure run ID and the post-exposure time. A single randomization for RNA extraction and hybridization will be performed. When placing sample aliquots on 96-well plates during the analysis, samples within a block will always be analyzed together. Finally, samples within a given block will be hybridized using the same chip lot and same target preparation batch.

8.9.2 Nicotine Analysis

PBS exposed samples are quantified with a ten calibration level of nicotine. Quantitation is performed using isotopic dilution with d3-nicotine as labelled internal standard. Unknown samples are quantified while their concentration fits within the calibration curve. In the case they would be out of the curve, a

dilution step must be performed prior analysis of the batch. Samples are prepared by direct addition of internal standard solution within aliquot of samples to be measured.
The sample measurement involves a calibration curve at the beginning and the end of the analytical sequence, including also QCs to control performance of the system. These last ones are distributed along the sequence to ensure all samples are accurately measured following defined parameters from the validated method. The complete analytical sequence is run by LC-HRAM-MS using positive ionization and data collected in full scan mode monitored by Xcalibur® software(version: SP1.48).
Once analytical sequence is terminated, raw data will be processed with Tracefinder® software (version: 3.1.416.13) using a quantitative method based on the ratio between nicotine and d3-nicotine. The value is then reported to the calibration curve to determine the corresponding concentration. Finally, nicotine amount per samples is reported to the costumer as per request.

8.9.3 RNA Processing and Quality Control (QC) of raw CEL files

The Affymetrix genechip Human Genome U133 Plus 2.0 Array will be used for hybridization, which simultaneously probes the expression of thousands of genes. In accordance to PMI_RD_WKI_001228, PMI_RD_SOP_000346, raw CEL files will be background-corrected, normalized, and summarized using frozen-Robust Microarray Analysis (fRMA) (McCall 2010). Background correction and quantile normalization will be used to generate microarray expression values from all arrays passing quality control checks, which will be conducted 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 plot (RLE), median absolute value RLE (MARLE) and pseudo as well as raw images using R packages will be generated for quality checks (AffyPLM; Bioconductor, Seattle, WA, USA) (Bolstad 2003, Gautier 2004).
CEL files that fulfill at least one of the quality metric rules described below dropped for further analysis.

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

Subsequently, the RLE and NUSE will be recomputed, no more CEL file is removed.

8.9.4 Gene-level Analysis

For each experimental factor combination item, dose and post-exposure, a model for estimating the treatment effect was fitted with LIMMA (Smyth 2004), by including the covariate smoking run as a blocking variable to account for the paired-samples during an exposure experiment (an exposure run comprises of samples exposed to a test item and air exposed controls, see statistical design). The *p*-values for each computed effect will be adjusted across genes using the Benjamini-Hochberg false discovery rate method (FDR). The results will be displayed as volcano plots (x-axis representing the estimated effect and the y-axis representing the –log10(FDR) for each gene). Differentially expressed genes (DEGs) were defined as the set of genes whose FDR is below 0.05.

Reproducibility will be estimated by correlating the fold-changes (exposed samples vs. controls) across experimental repetitions (e.g., experiment 1/2/3 vs. experiment 4/5).

8.9.5 Network-level Analysis

8.9.5.1 Network Perturbation Amplitude

The collection of causal biological networks will be used in the study(s) will be the human network suite CBN v1.3. These networks contain approximately a few dozen and two hundred nodes.
The Network Perturbation Amplitude (NPA) methodology (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 nodes values will be determined by a fitting procedure that infers the values that best satisfy the directionality of the causal relationships (positive or negative signs) contained in the network model, while being constrained by the experimental data (the gene log_2-fold-changes, which will be 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 of the gene expression; L_3 is the sub-matrix of L for the nodes in the network; and L_2 is the sub-matrix corresponding to the edges connecting the network nodes to the downstream gene expression nodes. The differential node values 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 $f^T Q f$, where Q is the signed Laplacian of the network when all of the edge signs have been reversed.
All the details of the methodology is described in a previous publication (Martin 2014).
For the NPA scores, a confidence interval accounting for the experimental variation and the associated *p*-values are computed. In addition, companion statistics are derived to inform on the specificity of the NPA score with respect to the biology described in the subnetwork 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 impacted if all of the three values (the confidence interval, *O, and K* statistics) are below 0.05.

8.9.5.2 Biological Impact Factor

The methodology Biological Impact Factor (BIF) (Martin 2014, Martin 2012) provides a unified and coherent framework for investigating mechanistic effects at each level of granularity of the biological processes represented in the network models. This methodology enables the derivation of the relative BIF, which is a measure of the overall biological impact across all network models. The BIF allows an assessment of the exposures in an objective, systematic, and quantifiable manner, computing a systems-wide and pan-mechanistic biological impact measure for a given substance, mixture, or test item.
The network models are organized into five major families, representing global biological processes: cell stress, cell proliferation, inflammation, tissue repair, and cell fate. Each of them comprised of various subnetwork models, representing the specific processes within a given network family.
An aggregation of the NPA should satisfy the following criteria:

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

To fulfil the first criteria, network scores are aggregated via a weighted sum (as every scoring in NPA is additive). In addition, only networks that have the three statistics *p*-values below 0.05 (*O, *, K*) will be considered (denoted by $\mathcal{N}_i^*$). The BIF is defined as:

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

Where, f denotes the function on the network nodes describing the differential node values; $E(\mathcal{N}_i^*)$ denotes the network edges; and e_0 and e_1 denote the start and the end, respectively, of an edge, e .
For the second criteria, a "fair" BIF would represent the equality of the biological processes (networks) in a given system (e.g., 2D normal human gingival epithelial cells, 3D organotypic tissue cultures, or lung tissue samples from in-vivo studies). If all subnetworks models were perfectly fitted by the gene expression data, the contribution of each subnetwork to the BIF is assumed to be equal. Therefore, the methodology considers that all specific processes represented in the subnetwork models, are equally involved and important for a given network family (e.g., cell stress). A perfect fit of the network will maximize the NPA value while being smooth over the network. Using the notation above, a "perfect" network response (differential node values) is both maximizing the f^TQf with $||f||_2 = 1$ and minimizing the f^TL_3f (the NPA criteria (Hoeng 2014)). Additionally, there is a constraint that L_3f is vanishing for the network nodes that do not have direct downstream gene expression nodes (Hoeng 2014, Martin 2012), as those are expected to be in the image of L^T_2. Such nodes are denoted as *NH*. Therefore, the following optimization problem will be solved:

$$argmax_{f\ s.t.(L_3f)|_{NH}=0} \frac{f^T Q f}{f^T L_3 f}$$

To rebalance the network families, the average over the subnetworks will be considered.
For each edge e = (e_0, σ(e), e_1), the number of occurrence of an edges, *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 $\mathcal{N}_i$ can be defined as:

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

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

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

Therefore, the contribution of each subnetwork $\mathcal{F}$ is:

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

8.9.5.3 Gene-Set Analysis

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

2009). For each test, the *p*-value is adjusted using the Benjamini-Hochberg procedure. The gene-set enrichment are the gene-set statistics mentioned before.

8.9.6 miRNA Analysis

8.9.6.1 Processing and Quality Control (QC) of Raw CEL Files

The arrays raw data contained in the CEL files will be preprocessed through a standard pipeline based on the generic documents PMI_RD_WKI_001228 and PMI_RD_SOP_000346. CEL files will be read using the *read.celfiles* function of the *oligo* package in the Bioconductor suite of microarray analysis tools for the *R* statistical software environment (R CORE TEAM, 2013, Carvalho 2010, Huber 2015). The quality of the array data will be controlled using the *arrayQualityMetrics* package (Kauffmann 2009) and examined according to the following four metrics:

- the distances between arrays at the raw data level,
- the distances between arrays at the normalized data level,
- the Normalized Unscaled Standard Errors (NUSE), and
- the array intensity distributions.

Arrays that will be found to be outliers in at least two of the metrics will be discarded. Subsequently the quality will be iteratively re-examined on the remaining arrays until all will be accepted. In the case of experimental repetitions (also referred to as "main phases" in the studies using organotypic tissue models), the before-mentioned quality control procedure will be applied separately on repeat, which corresponded to two experiments at a time. This approach led to discarding 9 arrays out of 367 in at most 2 iterations. Subsequently, the normalized probe-level data will be obtained by applying robust multi-array (RMA) normalization and summarized using the median polish method at the probe set-level (Bolstad 2003, Irizarry 2003).

8.9.6.2 Probe set Filtering and Calculation of the Detected miRNAs

The Affymetrix GeneChip® miRNA 4.0 arrays will be used for the hybridization. It probes the expression of 30'424 mature miRNAs relevant for 203 species, among which 2'578 miRNAs correspond to Human (Affymetrix, Inc.). 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 human species will be kept in the expression matrix.
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) will be considered to be "detected" (see the "Detection" section in (Affymetrix, Inc. 2011)). A p-value threshold of 0.001 will be chosen to determine the detection calls based on Wilcoxon tests. If a miRNA probe set will be detected in more than 90% of the samples in at least one sample group, then it will be kept for the further analysis; otherwise, it will be discarded.

8.9.6.3 Calculation of Differential miRNA Expressions

According to the experimental design, the experiment consisted of a number of pairwise "treatment vs. control" comparisons (i.e., an exposure run comprises of samples exposed to a test item and those exposed to air (controls), see statistical design). For each comparison, a submatrix will be extracted from the global expression matrix by keeping only the samples belonging to the corresponding treatment or control groups, as well as the miRNA probe sets that will be detected in more than 90% of the samples in at least one of these two groups. Linear models for differential expressions will be applied on all the

resulting submatrices using the moderated *t* statistics implemented in the *limma* package (Ritchie 2015). The models included an additional variable taking into account the exposure runs, which improved the fitting procedure in *limma*. Subsequently, adjusted p-values will be obtained following multiple testing corrections using the Benjamini-Hochberg false discovery rate method (FDR) (Benjamini 1995). The miRNAs below the FDR threshold of 0.05 will be considered to be differentially expressed.
For a given comparison, the results will be displayed as a volcano plot. The x-axis represents the estimated treatment effect (differential expression) of each miRNA and the y-axis representing the corresponding statistical significances given by –log10(FDR).
Moreover, for a given comparison, QQ plots will be generated by first sorting all of the miRNAs according to their unadjusted (raw) p-values in an ascending order. Subsequently, the –log10 transformed of the raw p-values of the resulting quantiles (i.e. the rank divided by total number of miRNAs) will be displayed on the x-axis and the –log10 transformed of the corresponding raw p-values will be displayed on the y-axis. For a given statistical significance threshold, α; the "Z"-shaped boundaries on 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.

8.9.6.4 qPCR analysis

The Ct data contained in the *.xlsx* file generated after completion of the "qPCR array for candidate genes" procedure are imported into the R environment for statistical computing (R CORE TEAM, 2013). The data are converted into objects of the Bioconductor *ReadqPCR* package (Perkins 2012). The optimal ("most stable") choice of reference/house-keeping genes is performed using the *geNorm* method (Vandesompele 2002) implemented in the Bioconductor *NormqPCR* package (Perkins 2012). This reference/house-keeping genes subset is used to generate the ΔCt values by normalizing the Ct values. This step is performed using the function *deltaCq* of the *NormqPCR* package, which essentially consists in subtracting from the Ct values of each sample the arithmetic mean of the corresponding Ct values of the subset of most stable reference/house-keeping genes. The differential expressions ΔΔCt and their significance (p-values) are calculated based on the -ΔCt values by using the standard two-sided t-test implemented in R. The cases of non-detected signal ($Ct \geq 40$) are treated in an appropriate way. No multiple testing corrections are performed on the obtained p-values.

9. ADMINISTRATIVE ASPECTS

9.1 Study Timelines

Proposed experimental starting date:	05-02-2016
Proposed experimental completion date:	Q2
Proposed date for draft study report:	End of Q2

9.2 Names and Addresses

Sponsor	Julia Hoeng Director System Toxicology Philip Morris International R&D Quai Jeanrenaud 5 2000-Neuchatel Switzerland
Test Facility Manager	Nikolai Ivanov Manager Research Technologies Philip Morris International R&D Quai Jeanrenaud 5 2000-Neuchatel Switzerland
Study Director	Filippo Zanetti
Quality Assurance Representative	Fabio Talamo

9.3 Quality Assurance

This is a non-GLP study. Any intended (amendment) or unintended (deviation) change to the study plan will be documented according to applicable study management procedures. The study plan and all related amendments supersede the current version of Standard Operating Procedures (SOP) and/or Work Instructions (WKI) in case of discrepancies are required.

The current study will be performed in compliance with the Systems Toxicology QMS based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

10. ARCHIVING

After completion of the study, the study plan with any amendment, all raw data, the report with any amendment and all further study-related records needed to reconstruct the study will be archived. They will be retained for at least 10 years in compliance with the applicable archiving procedures. Test and reference items can be discarded after their deterioration as, in this case, they would be no longer amenable to further evaluation. If the storage period for paper and electronic records need to be further extended in order to satisfy additional legal or company requirements, the storage location will be specified in a dedicated statement. Paper records will be archived in the archive at Philip Morris Products S.A., Research & Development, PMI Product Testing, Neuchâtel, Switzerland and electronic records will be managed by PMI Product Testing e-archivist on the central archiving server at Philip Morris S.A., Lausanne, Switzerland.

11. ABBREVIATIONS

Abbreviation	
ACTB	Beta Actin
AK	Adenylate Kinase
ALI	Air Liquid Interface
BIF	Biological Impact Factor
B2M	Beta-2 Microglobulin
CYP	Cytochrome
CS	Cigarette Smoke
DEGs	Differentially expressed genes
DNPH	2,4-Dinitrophenylhydrazine
DRA	Dose Range Assessment
FDR	False Discovery Rate Method
fRMA	frozen-Robust Microarray Analysis
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GEX	Gene Expression
GIN-100	EpiGingival™ epithelial tissues
GIN-100-DM4a	EpiGingival™ Differentiation Medium
GIN-100-MM	EpiGingival™ Maintenance Medium
GIN-300-FT-1	EpiGingival™ full-thickness epithelial tissues co-cultured with fibroblasts
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO	Melanoma Growth Stimulating Activity, Alpha
GSA	Gene-set Analysis
HC	Health Canada
HCI	Health Canada Intense
HPLC-MS/MS	High Performance Liquid Chromatography-Mass Spectrometry
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HGDC	(R)-2-Hydroxyglutaryl-CoA dehydratase
HRAM	High-Resolution, Accurate-Mass
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IQ	Installation Qualification
IP10	Interferon gamma-induced protein 10
ISO	International Organization for Standardization
MARLE	Median Absolute Value RLE
MAP	Multi Analyte Profiling (a.k.a, pro-inflammatory mediators measurement using Luminex)

MCP-1	Monocyte Chemotactic Protein 1
MFI	Median Fluorescence Intensity
MMP	Matrix Metalloproteinase
MP	Main Phase, also refers to experimental repetition
MS	Mass Spectrometry
MTT	Methyl Thiazoyl Tetrazolium
NPA	Network Perturbation Amplitude
NUSE	Normalized-Unscaled Standard Error plot
ORA	Over-Representation Analysis
OQ	Operational Qualification
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDSP	Programmable Dual Syringe Pump
PFA	Paraformaldehyde
PQ	Performance Qualification
QC	Quality Control
qPCR	Quantitative PCR
QQ	Quantile Quantile
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RLE	Relative Log Expression Plot
RMA	Robust Multi-Array
RPLP0	Ribosomal Protein, Large, P0
sbPBS	smoke bubbled PBS
SOP	Standard Operate Protocol
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
THS2.2	Tobacco Heating System 2.2
TNF-α	Tumor Necrosis Factor alpha
TPM	Total Particulate Matter
TX-100	Triton X-100
VEGFA	Vascular Epithelial Growth Factor A
WKI	Work Instructions
3R4F	Kentucky University Standard Cigarettes

12. REFERENCES

12.1 Literature

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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_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

PMI_RD_WKI_001334
qRTPCR SA Bioscience (Qiagen)

12.3 **Philip Morris International (PMI) Internal Documents**

PMI Internal Document. Unpublished data. Data storage folder.
https://disco.app.pmi/disco/drl/objectId/0b01d4ec8055c5b1

PMI Internal Document. Unpublished data. Batch release certificate for THS2.2.
https://disco.app.pmi/disco/drl/objectId/0901d4ec80547b2f

PMI Internal Document. Unpublished data. Carbonyl storage folder.
\\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\\AerosolsCharacterization\Carbonyls\Vitrocell

PMI Internal Document. Unpublished data. Nicotine storage folder.
\\rd-bsrdata.app.pmi\BSR_Data\Cellular Systems Biology\ S179800_P1_Repeated_Human_Gingival\Nicotine in PBS

PMI Internal Document. Unpublished data. Delivery date of inserts document.
https://disco.app.pmi/disco/drl/objectId/0901d4ec8056121d

PMI Internal Document. Unpublished data. Exposure plan PBS pilot and DRA
https://disco.app.pmi/disco/drl/objectId/0901d4ec8055c95f

MI Internal Document. Unpublished data. Study documents.
https://disco.app.pmi/disco/drl/objectId/0b01d4ec8055c5b1

MI Internal Document. Unpublished data. Metadata PBS pilot.
https://disco.app.pmi/disco/drl/objectId/0901d4ec8055cb17

MI Internal Document. Unpublished data. Metadata DRA phase.
https://disco.app.pmi/disco/drl/objectId/0901d4ec8055cb16

MI Internal Document. Unpublished data. Metadata main phase 1.
https://disco.app.pmi/disco/drl/objectId/0b01d4ec805600b5

MI Internal Document. Unpublished data. Metadata main phase 2.
https://disco.app.pmi/disco/drl/objectId/0b01d4ec805603ac

MI Internal Document. Unpublished data. Metadata main phase 3.
https://disco.app.pmi/disco/drl/objectId/0b01d4ec805603ad