STUDY PLAN

ADHESION ASSAY – PHASE II AND III

(STUDY NUMBER 160400)

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2 Introduction

2.1 Purpose

The purpose of this study is to assess *in vitro* the impact of MRTPs on the process of adhesion of monocytic cells to an endothelial cell monolayer, a critical step in the initiation of atherosclerosis plaque formation. The study will leverage the "Adhesion assay" developed and optimized with aqueous conventional cigarette smoke extract (smoke-bubbled Phosphate Buffered Saline, sbPBS). This study will investigate the biological impact of an exposure of SMAR NAD, SMAR AD and Zurich sbPBS compared to sbPBS generated with conventional cigarette (CC) 3R4F smoke on the adhesion rate, TNFα release by monocytic cells, cell viability and systems biology data (Gene expression, mRNA).

2.2 Background

Atherosclerosis has been described as an inflammatory disease in which monocytic cells play a key role. (Libby, 2002, Figure 1).

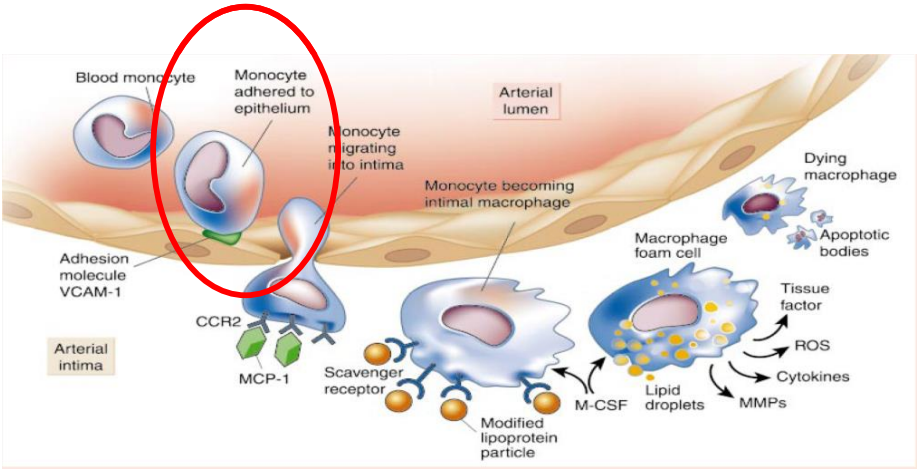


Figure 1: Inflammation in Atherosclerosis (Libby et al., 2002)

The process of monocyte migration and extravasation across the endothelium occurs in several distinct steps, referred to as the multi-step paradigm (Butcher, 1991; Springer, 1994). The first step comprises the rolling of the monocytes over the endothelial cells, mediated by adhesion molecules such as selectin. The second step includes the adhesion and spreading of monocytes on the endothelial cells. This step requires the interaction of several cell surface receptors (e.g., very late antigen 4 (VLA-4), lymphocyte function associated antigen-1 (LFA-1) and adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1),

and intercellular adhesion molecule-1 (ICAM-1). The expression of these surface receptors is triggered by oxidative and inflammatory processes.

The impact of cigarette smoke (CS) on this first step was analyzed in 3 previously performed studies at PMRL (P11571, P12441, P13950 and P160400). The results of these studies demonstrated that the exposure to smoke-bubbled PBS (sbPBS) increased the number of monocytes (i.e. MM6 cells, a human monocytic cell line with characteristics of mature monocytes (Ziegler-Heitbrock et al., 1988) bound to the endothelial cells (e.g., human umbilical vein endothelial cells (HUVECs)). However, sbPBS acted in an indirect manner and increased the expression of the adhesion molecules (vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and endothelial selectin (E-Selectin) on the RNA and protein levels, whereas direct treatment of endothelial cells with same doses of 3R4F sbPBS did not result in an increased expression of adhesion molecules in contrast to the results described by Kalra et al. (1994) or Shen et al. (1996)

Objectives

The objectives of this study are the following:

1) Investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells to endothelial cells. The adhesion assay that will be used for the assessment has been optimized using Mono Mac 6 cells (MM6) and human umbilical vein endothelial cells (HUVECs) (Phase II).
2) Optimize the Adhesion assay using more disease-relevant primary human endothelial cells (e.g Human coronary artery endothelial cells, HCAECs) (Phase III).
3) Once the assay is optimized with relevant ECs, investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells to ECs (Phase III).

3 Experimental Design

3.1 Summary of the Adhesion Assay

The current adhesion assay has been established and optimized using MM6 and HUVEC cells as described in the figure below.

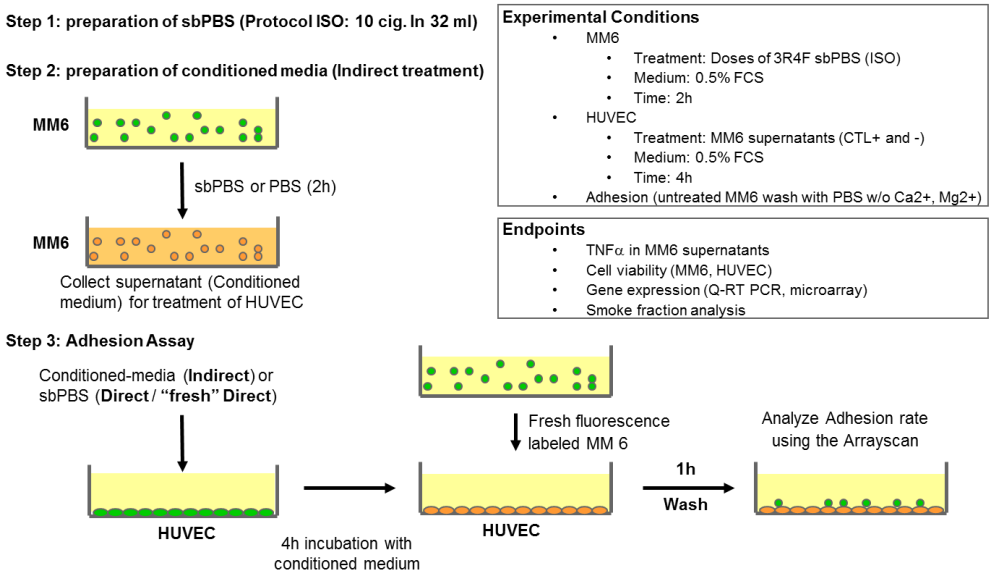


Figure 2: Summary of the adhesion assay

The adhesion assay can be conducted in direct or indirect treatment conditions (see figure 2). The figure below summarizes and defined those conditions and allows distinguishing between direct and "Fresh" direct treatments.

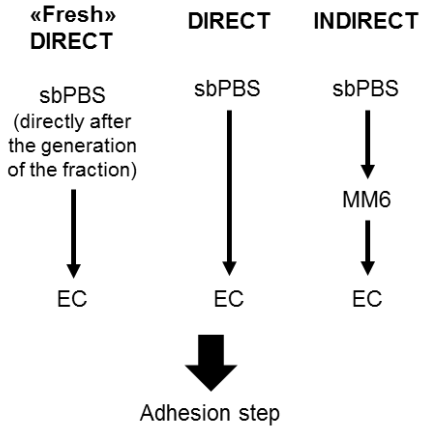


Figure 3: Different treatments used for the adhesion assay

3.2 Summary of results obtained with 3R4F sbPBS in phase I

Most of the adhesion assay experiments have been performed using 3R4F sbPBS generated with ISO smoking regimen. The MM6 and HUVEC cell types were used for the assay.

Indirect treatment

- A dose-dependent release of TNFα by MM6 cells exposed to 3R4F sbPBS was observed for 2h and 4h exposure time (similar response for both time points). At higher doses (0.15, 0.18 puffs per mL), the TNFα release decreases. This observation will be further investigated.
- A similar TNFα release pattern was observed when MM6 cells were incubated in 10% FCS medium compared to 0.5% FCS medium (starvation conditions), however with overall lower levels of TNFα released.
- A dose-dependent adhesion of MM6 to HUVECs treated for 4h with supernatants of 3R4F sbPBS-treated MM6 cells exposed was observed. The adhesion rate reached a maximum using a 0.09 p/mL dose of 3R4F sbPBS. The adhesion of MM6 cells decreases at higher dose (0.15 p/mL).

Direct treatment

- No adhesion of MM6 cell to HUVECs was observed for doses of sbPBS up to 0.09 p/mL. A dose-dependent increase of the adhesion was observed for the doses 0.12 and 0.18 p/mL, however to a lower extent compared to the adhesion rate observed in indirect treatment at 0.09 p/mL (1 experiment has been performed).

3.3 Overview of the experimental design

Indirect conditions: MM6 cells will be exposed to several concentrations of MRTPs such as SMAR NAD, SMAR AD and Zurich (ZRH) sbPBS for 2 h. One concentration of 3R4F sbPBS (maximizing the release of TNFα and adhesion rate) will be used as reference treatment. MM6 supernatants will be collected and frozen at -80°C.

Direct conditions: 3R4F and MRTPs sbPBS will follow the same process of dilution in culture medium, incubation at 37°C for 2 hours, and storage at -80°C, as the one used to prepare sbPBS-treated MM6 supernatants.

For the adhesion assay, HUVECs are treated for 4 h with thawed conditioned-media obtained in direct and indirect conditions.

"Fresh" direct conditions: HUVECs will be directly treated with freshly generated sbPBS (diluted in medium) for 4 h before the adhesion assay.

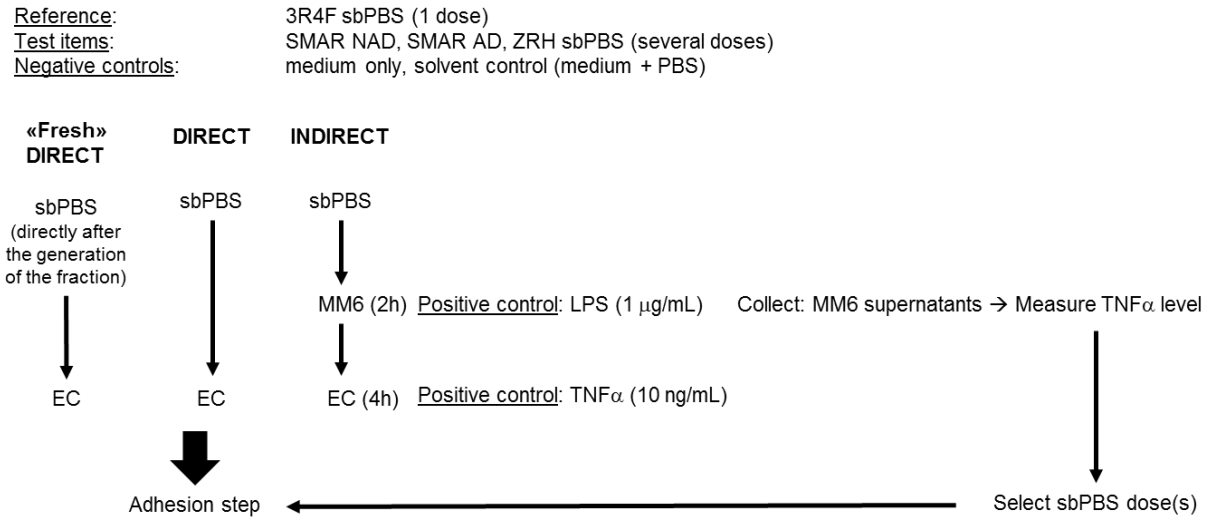


Figure 4: Summary of the overall experimental design (endothelial cells, EC will be HUVEC in phase II and more disease-relevant human primary endothelial cells in phase III)

3.4 Experimental protocols

3.4.1 Cell culture

HUVECs

HUVEC (Human Umbilical Vein Endothelial Cells, PromoCell) will be cultured according to the SOP CV-42 HUVECs will be cultivated in growth medium supplemented with 2% fetal calf serum FCS for 3 days (e.g. from Monday to Wednesday) and starved 24h before performing the adhesion assay (e.g. Thursday). The starvation medium contained 0.1% FCS (Endothelial Cell Growth Medium Kit 500 ml consists of a 500 ml bottle of Basal Medium and the Supplement Pack a set of individual vials with pre-measured supplements, C-22110 PromoCell, Heidelberg).

Disease-relevant primary endothelial cells HCAECs and HAOECs

HCAECs and HAOECs will be used as disease-relevant primary endothelial cells for the adhesion assay. A cell culture protocol will have to be optimized in the context of this study. Details of optimization experiments are described in section 6.

MM6 cells

Mono Mac 6 (MM6) is a human monocytic cell line with characteristic of mature monocytes (Ziegler-Heitbrock et al., 1988) and will be cultivated according to the SOP CV 30.

3.4.2 Generation of conditioned media with (for indirect treatment) or without (for direct treatment) MM6 cells

Conditioned-media with MM6 cells

Before exposure to sbPBS, MM6 cells will be seeded in 6-well plate specifically designed for suspension cells. The cell concentration will be adjusted to 2 million cells/mL using a starvation medium (0.5% FCS) and 2mL/well will be added. Then, cells will be incubated for 2 h ± 10 min at 37°C. Fifteen minutes before the end of the incubation time, sbPBS dilutions will be prepared using the MM6 starvation medium. Then, 2mL/well of the sbPBS dilutions will be added and cells will be incubated 2 h ± 10 min at 37°C. Following exposure, cells will be centrifuged for 7 min at 800g, the supernatant will be collected and immediately stored at -80°C for adhesion experiments described in section 4.4.4. The cell pellet will be also kept and given to the genomic team for further analysis.

MM6 supernatants will be collected as follows: 500µl of MM6 supernatant will be collected in 2 mL sterile Eppendorf tubes for TNFα detection and LDH assay, and the rest will be collected in 15 mL Falcon tubes for the adhesion assay.

TNFα levels will be measured in the supernatant using an ELISA assay (see section 4.4.12).

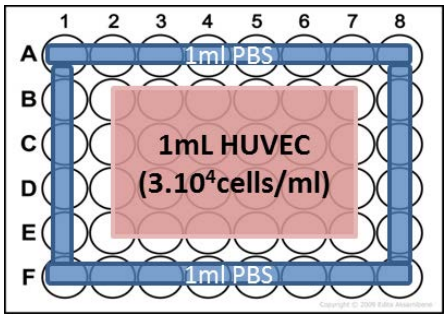
Conditioned-media without MM6 cells

In parallel, sbPBS dilutions will be also prepared for direct treatment (see section 4.7). In this case, the MM6 starvation medium will be incubated at 37°C for 2 h in 6-well plate but without MM6 cells. Then, sbPBS dilutions will be added, incubated for 2 h, collected and stored at -80°C until the adhesion assay experiment.

3.4.3 Culture and preparation of HUVECs for the assay

For the adhesion assay, HUVEC cells can be used until passage 7 maximum.

HUVECs cultivated in flasks will be trypsinized, centrifuged, resuspended in a growth medium (2% FCS) and counted with a Casy cell counter. The cell concentration will be adjusted to 3E4 cells/mL and 1 mL will be added to each well in a 48-well plate coated with Collagen A. Only the inner wells will be used and border wells will be filled with 1mL of PBS.



The cells will be cultivated for 3 days until they reach 100% confluence and then, will be starved (0.1% FCS) for 24 h ± 1 h. After the starvation, the medium will be aspirated and the cells will be washed with 800 µL/well of PBS containing Ca^{2+} and Mg^{2+}.

3.4.4 Indirect treatment of HUVECs

For the indirect treatment, HUVEC cells will be treated with the thawed conditioned-media obtained with MM6 cells. 500 µL/well of MM6 supernatant will be added in triplicate and the cells will be incubated at 37°C for 4 h ± 15 min. As a positive control, 500 µL of TNFα (10 ng/mL final concentration) will be added to 3 wells seeded with HUVECs. As a negative control, 500µL of the solvent control (MM6 starvation medium with PBS) dilution will be also added in triplicate to HUVEC cells.

3.4.5 Direct treatment of HUVECs

For the direct treatment, HUVECs will be treated with the thawed conditioned-media obtained without MM6 cells (following the same experimental conditions as the ones used to generate sbPBS-treated MM6 supernatants). 500 µL/well of conditioned-media will be added in triplicate and the cells will be incubated at 37ºC for 4 h ± 15 min. As a positive control, 500µL of TNFα (10 ng/mL final concentration) will be added to 3 wells seeded with HUVECs. As a negative control, 500µL of the solvent control (MM6 starvation medium with PBS) dilution will be also added in triplicate to HUVECs.

3.4.6 “Fresh” Direct treatment of HUVECs

HUVECs will be directly treated with freshly generated 3R4F or MRTPs sbPBS fractions.

500 µl of sbPBS diluted in the MM6 starvation medium (0.5% FCS) will be added to each well in triplicates and the cells will be incubated at 37°C for 4 h ± 15 min. As a positive control, TNFα (10 ng/mL final concentration) will be added to 3 wells seeded with HUVECs. As a negative control, the solvent control (MM6 starvation medium with PBS) dilution will be also added in triplicate to HUVECs.

3.4.7 Staining of MM6 cells for adhesion experiments

MM6 cells will be pooled, counted and centrifuged for 7min at 400g. The cell pellet will be re-suspended in the MM6 starvation medium containing 1:200 CMTMR (stock solution CMTMR-dye: 1mM in DMSO). Cell concentration will be adjusted to 10E6 cells/mL approximately and MM6 cells will be incubated for 15 min at 37°C with the dye. After incubation, cells will be centrifuged and washed twice with PBS without calcium and magnesium (critical point). Then, the cell pellet will be resuspended in the MM6 starvation medium and the cell concentration will be adjusted to 2E6 cells/mL. The cells can be stored in the incubator for 1h before being used for the adhesion assay.

3.4.8 Determination of adhesion of MM6 cells to HUVECs (ArrayScan VTI HCS reader):

After treatment with conditioned media (see section 4.4.4, 4.4.5, 4.4.6), HUVECs will be washed once with PBS^+ (PBS containing calcium and magnesium) and 500 µL of the stained MM6 will be added to each well containing HUVECs. After 30 ± 5 min incubation at 37°C, 500µL of Hoechst dye diluted 1:500 in the MM6 starvation medium will be added for an additional 15 min ± 2 min.

Remaining non-adherent cells will be removed by three washes with PBS^+ (Turn the plate upside down and knock out the PBS onto blotting paper).

Thereafter, cells will be fixed with 500 µL/well of glutaraldehyde (10% in PBS) for 15 ± 2 min at 37°C, washed once with PBS^+ (with calcium and magnesium) and wells will be re-filled with 300 µL PBS^+. At this stage, it will be possible to store the plate overnight at 4°C protected from the light for later readout.

The adhesion of human monocytic MM6 to endothelial cells will be analyzed using the ArrayScan VTI HCS Reader according to SOP FU 51.

3.4.9 Image acquisition

Images will be acquired using the ArrayScan VTI HCS Reader, an integrated system capable of automated acquisition and analysis of large populations of individual cells, following the manufacturer's instructions. Images will be acquired using the XF93 filter set (Omega Optical, Brattleboro, VT) with 1 image for the Hoechst stain and 1 image for CMTMR Orange. Data capture, extraction, and calculation of adhesion will be done in 20 picture fields per well and with a 10 x objective (0.3 NA).

3.4.10 Image analysis

The Cellomics software uses an object identification algorithm based on intensity thresholds between adjacent pixels. Identified objects within an imaged field can subsequently be accepted or rejected for analysis on the basis of object characteristics, such as area, shape, and fluorescent intensity. Once a primary object is identified and accepted as valid, an area around (or within) the object can be defined for use in the subsequent quantitation procedure. The analysis of the determined regions of interest is refined by

pre-packaged generic and adaptable image analysis software modules named BioApplications. To determine the adhesion of MM6 cells, e.g the Target Activation BioApplication software (version 4, Cellomics) will be used.

The Target Activation BioApplication is an automated image analysis algorithm for the ArrayScan VTI platform. The BioApplication allows automatic quantification of fluorescent stained cells that adhere onto a cell lawyer with markers defining the nucleus and the cytoplasm. A 2-channel protocol was created to simultaneously investigate adherence of MM6 cells on top of a confluent HUVEC cell layer. Nuclei will be detected in the first channel (Hoechst), the cytoplasma stain CellTracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) will be detected in the second channel (Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)). Selection criteria for adherent MM6 cells is a double staining with Hoechst 33342 in the first channel and CMTMR Orange in the second channel (TRITC filter).

3.4.11 TNF-alpha protein quantification

The TNF-alpha level in the supernatant of 3R4F or MRTPs sbPBS-treated MM6 cells will be determined using a R&D Systems ELISA Development kit (Human TNF-alpha DuoSet, Ref: DY210) according to the manufacturer's instructions (http://www.rndsystems.com/Products/dy210/AssayProcedure).

4 Test and Reference Items

4.1 Test and Reference Items

Items to be tested within the present study will be the following MRTPs: ZRH, SMAR-AD and SMAR-NAD.

The Reference Cigarette 3R4F will be used to generate aqueous cigarette smoke extract (3R4F sbPBS) and compared to aqueous aerosol extract generated from SMAR NAD, SMAR AD and Zurich platforms.

Table 1: Reference and test items

Description	Pack Code	Type	Batch number	Short Name
ZRH_CH FPD 4.2	-	test	Cigarette batch Number : B-05771	ZRH_4.2
SMAR-Alu		test	SBAV_ANA_REP_CHEMICAL AND	SMAR_AD

Disk			ASSESSMENT OF THE SMAR-5492 FOR BATCH RELEASE.doc http://disco.app.pmi/disco/drl/objectId/0901d4ec80342665	
SMAR-non Alu Disk		test	SBAV_ANA_REP_ CHEMICAL ASSESSMENT OF THE SMAR-5410 FOR BATCH RELEASE.doc http://disco.app.pmi/disco/drl/objectId/0901d4ec8033cec1	SMAR_NAD
3R4F	3R4F	reference	NA	3R4F_HC 3R4F_ISO

Remark : to be sure that the SMAR test item was still stable a light batch release was performed in April 2013

http://disco.app.pmi/disco/drl/objectId/0901d4ec8036bbfd

ZRH test item composed of a device (CH FDP4.2) and a sensorial media (Sens Med-1684).

ZRH_4.2 test item will be provided by PHILIP MORRIS International, Neuchâtel, Switzerland, with the production of 2000 sensorial media. The production is not finalized yet. The sensorial media will be packed in cartons which will contain a bag with sensorial media shrink-wrapped in aluminium foil. The aluminium bag will be labelled with the production lot. No. and the quantity and the production date.

5 boxes with **SMAR-AD** test items and 4 boxes with **SMAR-NAD** test item, each box containing 4000 items, were provided by PHILIP MORRIS Neuchâtel in May 2012. SMARs are generated in one production batch and are taken from the 5th batch of the COPD II study (Batch Release Certificate 007 SMAR-5410 COPD II.pdf http://disco.app.pmi/disco/drl/objectId/0901d4ec8033d012).

Reference Cigarettes **3R4F** (30.000 cigarettes) were purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (for specifications see www.2r4f.com/3r4f.pdf, Table 3). They were packed 20 cigarettes/soft pack. The packaging capacity was 5000 cigarettes per carton. They were received at PMI R&D Neuchâtel July 2012.

Reference Cigarettes 3R4F, SMAR-AD, SMAR-NAD and the sensorial media are stored in a cooling chamber at 5 ± 3 ºC with uncontrolled humidity conditions in the original packaging, before conditioning. 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.

Table 2: Specifications of the Reference Cigarette 3R4F

Parameter	Value
Physical Analysis Data	
butt length (mm)	35
cigarette length (mm)	84
total cigarette weight (g)	1.05
resistance to draw (cm water column)	13.35
circumference (mm)	24.9
paper permeability (Coresta units)	24
paper additive type (citrate) (%)	0.60
FTC Smoke Analysis	
puff count (puffs/cig.)	9.0
TPM (mg/cig.)	11.0
FTC tar (mg/cig.)	9.4
nicotine (mg/cig.)	0.73
CO (mg/cig.)	12.0
Blend Summary	
flue-cured (%)	35.41
Burley (%)	21.62
Maryland (%)	1.35
Oriental (%)	12.07
Reconstituted (Schweitzer Process) (%)	29.55
glycerine (%)	2.67
isosweet (sugar) (%)	6.41

Reference: University of Kentucky (www.2r4f.com/3r4f.pdf)

Remarks: The smoke analysis data were generated under FTC conditions

4.1.1 Preparation, Dosing and Analysis of Smoke Fractions

As state above, the sensorial media for ZRH, SMAR AD, SMAR NAD and the Reference Cigarette 3R4F will be conditioned according to ISO standard 3402 (1999), between 7 and 21 days at 22 ± 1 °C and a relative humidity of 60 ± 3 % before being used for smoke generation. The test atmosphere for smoke generation will be 22 ± 2 °C and 60 ± 5 % relative humidity. The mainstream smoke for the reference item will be generated under the ISO and HC regimen. The sbPBS preparartions will be generated under Health Canada Intense (HCI) smoking regimen. For the HCI smoking protocol, 3R4F cigarettes will be 100 % vent-blocked by taping (SOP AC 226). The sensorial media as well as the SMAR test items will not be taped because there are no ventilation holes in the filter region. The smoking regimens are given in Table 3.

Table 1: Smoking Regimens

Short Name	**Puff Volume** (ml)	**Puff Duration** (s)	**Puff Frequency** (min^{-1})	**Puff Count**
3R4F_HC	55	2.0	2	to a butt length of 35 mm = X puffs
3R4F _ ISO	35	2.0	1	to a butt length of 35 mm = x puffs
ZRH_4.2	55	2.0	2	12 (predefined)
SMAR_AD	55	2.0	2	12 (predefined)
SMAR_NAD	55	2.0	2	12 (predefined)

Sample generation for 3R4F

The 3R4F cigarettes will be smoked on the 20-port rotary smoking machine Borgwaldt RM20H. For the HC smoking regimen, the machine will be used in the 10-port mode due to technical reasons. The smoking machine settings are summarized in Table 4.and Table 5

Table 2 Smoking Machine Settings for RM20H, Puff Parameter Sets

Short Name	Set No.	Puff Profile No.	Puff Volume (ml)	Puff Time [1] (s)	Piston Sweep Time [a] (s)	Puff Fre-quency [a] (min^{-1})	Period, Duration (s)	Pause, Time (s)	Volume Correction Factor
3R4F_HC	2	0 [2]	55	2	1.9	2	30	28	variable [3]
3R4F_ISO	2	0 [4]	35	2	1.9	1	60	58	variable [5]

Table 3 Smoking Machine Settings for RM20H, Cigarette Parameter Set

Short Name	Set No.	Cigarette Length (mm)	Butt Length (mm)	Lighter Capacity (W)	Light Twice [6]	Number of Puffs [7]
3R4F_HC/ISO	0	84	35	70	0	to a butt length according to ISO (35 mm)

Sample generation for SMAR

Both SMAR test items (SMAR_AD, SMAR_NAD) will be smoked on 30-port rotary smoking machines (type PMRL-G, SM2000/SMAR) used in a 15-port mode with active sidestream smoke exhaust equipped with programmable dual syringe pumps. The SM2000/SMAR is equipped with 2 lighters (heat coils) for the lighting of the SMAR. The smoking machine cabinet is supplied with conditioned air and connected to an exhaust ventilation system to stabilize the temperature conditions in the handling area of the SMAR_AD/SMAR_NAD.

[1] The following phrases may be used in parallel: Puff Time – Duration; Piston Sweep Time – Intermission; Puff Frequency – Time

[2] Zero corresponds to the bell-shaped profile.

[3] The volume correction factor will be adjusted to meet the puff volume (see SOP-G_AW_004).

[4] Zero corresponds to the bell-shaped profile.

[5] The volume correction factor will be adjusted to meet the puff volume (see SOP-G_AW_004).

[6] setting whether to light twice: 0 for *no*, 1 for *yes*

[7] Setting 0 is for butt-length-controlled, other settings for predefined puff counts.

The SMAR items will be electrically lit using heating coil lighters (supplied by the New Smoking Articles Department). A pneumatic actuator will automatically move the SMAR with its charcoal heat source into the heating coil lighter during the lighting period (insertion depth 7 mm = charcoal end in line with first coil spiral). The SMAR will be lit for 25 s at 50 W and immediately inserted into the carousel of the smoking machine. As there are 2 lighters on the carousel, only 2 SMARs can be inserted into the carousel during 1 rotation, i.e., complete loading of the carousel needs 8 rotations when 15 ports are used.
To reduce condensation the SMAR SM will be equipped with a temperature controlled insulation at 37°C in the concentrated aerosol path

All samples from the SMAR are collected immediately before the pump outside of the carousel at a distance of 60 cm from the suction port for technical reasons.

Sample generation for ZRH

Aerosols derived from Zurich test items with the tobacco heating system (THS™, FPD4.2) are generated using 30-port rotary smoking machines (Type: SM_SDDS_ZRH) and a Programmable Dual Syringe Pump (PDSP). In this study always the same smoking machine will be used. The Smoking machine (SM) is equipped with up to 30 Functional Prototype Devices (type FPD4.2) of the Cigarette Holder (CH) and corresponding Smoking Device Docking Station (SDDS) as an electrical interface between the CH and the SM. The SM is controlled by a Programmable Logic Control system (PLC) allowing either an automated or a manual operation of the SM. The SM_SDDS_ZRH machines will be equipped with a temperature-controlled insulation kit (surface warming system) at 41°C in the concentrated aerosol path, with the goal of reducing aerosol condensation. This comprises water warmed surfaces for all components (i.e. tubes, PDSP valve, pump unit, and manifold to dilution) from the suction port of the SM up to a manifold assembled to the dilution system.

The sensorial media (Sens Med-1684) of the ZRH test article will be smoked with a bell-shaped puff profile under HCI smoking regimen with a predefined puff count of 12 puffs. The sensorial media will be electrically heated in the CH with a defined heating pattern. The used heating pattern is stored in CH and details will be given in the final report. All detailed SM and PDSP setting for achieving the HC smoking regime will be compiled in the final report.

Experimental details for the preparation of sbPBS: see table 6

Table 6: Experimental details for the preparation of sbPBS

Short Name	**Number of Test Items to be Smoked for sbPBS**	**Amount of PBS (ml)**
ZRH	30	25
SMAR_AD	10	25
SMAR_NAD	10	25
3R4F_HC	6	36
3R4F_ISO	10	32

4.1.2 Analytical Determination of Carbonyls in sbPBS

For each sbPBS batch, the trapped amount of carbonyls in the sbPBS will be determined in order to check the reproducibility of the smoke fraction generation.

5 Experimental Groups and Dosing

5.1 PHASE II: Investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to endothelial cells (HUVECs).

5.1.1 Experiment 1 phase II (1 week): SMAR NAD sbPBS assessed in direct and indirect conditions

1) Generation of 3R4F (ISO and HC) and SMAR NAD (HC) sbPBS using standard smoking regimen.
2) Generation of conditioned media using 3R4F (1 dose) or SMAR NAD (several doses) sbPBS with (indirect) or without (direct) MM6 cells (2 h exposure in starved medium 0.5% FCS). Positive control used with MM6 cells: LPS (1 ug/ml). Negative controls: only medium, solvent control (medium with PBS).
3) Treatment of HUVECs with conditioned media (indirect and direct) for 4 h (HUVECs in starved medium 0.5% FCS for 24 h prior treatment). Positive control: TNFα (10 ng/mL). Negative controls: only medium, solvent control (medium with PBS).

4) Adhesion assay.

Collect: MM6/HUVEC supernatant and cells

Endpoints: TNFα in MM6 supernatant

Adhesion rate

Cell viability (LDH) for MM6 and HUVECs

Gene expression levels (e.g. adhesion molecules) will be measured upon needs (qRT-PCR)

Adhesion assay

Plate 1

CTL- (med.+PBS)	CTL- (med.+PBS)	CTL- (med.+PBS)	CTL+ TNFalpha	CTL+ TNFalpha	CTL+ TNFalpha
Only medium	Only medium	Only medium	Solvent control	Solvent control	Solvent control
3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)	3R4F HC (0.09p/mL)	3R4F HC (0.09p/mL)	3R4F HC (0.09p/mL)
SMAR NAD Dose 1	SMAR NAD Dose 1	SMAR NAD Dose 1	SMAR NAD Dose 2	SMAR NAD Dose 2	SMAR NAD Dose 2

Plate 2

SMAR NAD Dose 3	SMAR NAD Dose 3	SMAR NAD Dose 3	SMAR NAD Dose 4	SMAR NAD Dose 4	SMAR NAD Dose 4
Solvent control	Solvent control	Solvent control	3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)
3R4F HC (0.09p/mL)	3R4F HC (0.09p/mL)	3R4F HC (0.09p/mL)	SMAR NAD Dose 1	SMAR NAD Dose 1	SMAR NAD Dose 1
SMAR NAD Dose 2	SMAR NAD Dose 2	SMAR NAD Dose 2	SMAR NAD Dose 3	SMAR NAD Dose 3	SMAR NAD Dose 3

SMAR NAD: Dose 1: 0.045; Dose 2: 0.09; Dose 3: 0.12; Dose 4: 0.18 (all doses expressed as puff/mL)

Blue: Indirect treatment

Orange: Direct treatment

5.1.2 Experiment 2 phase II (1 week): SMAR AD sbPBS assessed in direct and indirect conditions

Same experimental design as the experiment 1 phase II.

5.1.3 Experiment 3 phase II (4 days): 3R4F sbPBS assessed in "fresh“ direct conditions

1) Generation of 3R4F (ISO and HC) sbPBS using standard smoking regimen.
2) Treatment of HUVECs with several doses of freshly prepared 3R4F sbPBS for 4 h (HUVECs in starved medium 0.5% FCS for 24 h prior treatment). Positive control: TNFα (10 ng/mL). Negative controls: only medium, solvent control (medium with PBS).
3) Adhesion assay

Collect: HUVEC supernatants and cells

Endpoints: Adhesion rate

Cell viability (LDH) for HUVECs

Gene expression levels (e.g. adhesion molecules) will be measured upon needs (qRT-PCR)

Adhesion assay

Plate 1

CTL- (med.+PBS)	CTL- (med.+PBS)	CTL- (med.+PBS)	CTL+ TNFalpha	CTL+ TNFalpha	CTL+ TNFalpha
Only medium	Only medium	Only medium	Solvent control	Solvent control	Solvent control
3R4F ISO (0.015p/mL)	3R4F ISO (0.015p/mL)	3R4F ISO (0.015p/mL)	3R4F ISO (0.045p/mL)	3R4F ISO (0.045p/mL)	3R4F ISO (0.045p/mL)
3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)	3R4F ISO (0.09p/mL)	3R4F ISO (0.12p/mL)	3R4F ISO (0.12p/mL)	3R4F ISO (0.12p/mL)

Plate 2

3R4F ISO (0.15p/mL)	3R4F ISO (0.15p/mL)	3R4F ISO (0.15p/mL)	3R4F ISO (0.18p/mL)	3R4F ISO (0.18p/mL)	3R4F ISO (0.18p/mL)
3R4F HC (0.015p/mL)	3R4F HC (0.015p/mL)	3R4F HC (0.015p/mL)	3R4F HC (0.045p/mL)	3R4F HC (0.045p/mL)	3R4F HC (0.045p/mL)
3R4F HC	3R4F HC	3R4F HC	3R4F HC	3R4F HC	3R4F HC

(0.09p/mL)	(0.09p/mL)	(0.09p/mL)	(0.12p/mL)	(0.12p/mL)	(0.12p/mL)
3R4F HC (0.15p/mL)	3R4F HC (0.15p/mL)	3R4F HC (0.15p/mL)	3R4F HC (0.18p/mL)	3R4F HC (0.18p/mL)	3R4F HC (0.18p/mL)

Purple: Fresh direct treatment

5.1.4 Experiment 4 phase II (4 days): SMAR NAD and AD sbPBS assessed in "fresh“ direct conditions

1) Generation of 3R4F (ISO and HC), SMAR NAD (HC) and SMAR AD (HC) sbPBS using standard smoking regimen.
2) Treatment of HUVECs with several doses of freshly prepared 3R4F, SMAR NAD or SMAR AD sbPBS for 4 h (HUVECs in starved medium 0.5% FCS for 24 h prior treatment). Positive control: TNFα (10 ng/mL). Negative controls: only medium, solvent control (medium with PBS).
3) Adhesion assay

Collect: HUVEC supernatants and cells

Endpoints: Adhesion rate

Cell viability (LDH) for HUVECs

Gene expression levels (e.g. adhesion molecules) will be measured upon needs (qRT-PCR)

Adhesion assay

Plate 1

CTL- (med.+PBS)	CTL- (med.+PBS)	CTL- (med.+PBS)	CTL+ TNFalpha	CTL+ TNFalpha	CTL+ TNFalpha
Only medium	Only medium	Only medium	Solvent control	Solvent control	Solvent control
3R4F ISO Dose 1	3R4F ISO Dose 1	3R4F ISO Dose 1	3R4F HC Dose 1	3R4F HC Dose 1	3R4F HC Dose 1
SMAR NAD Dose 1	SMAR NAD Dose 1	SMAR NAD Dose 1	SMAR NAD Dose 2	SMAR NAD Dose 2	SMAR NAD Dose 2

Plate 2

SMAR NAD	SMAR NAD	SMAR NAD	SMAR NAD	SMAR NAD	SMAR NAD

Dose 3	Dose 3	Dose 3	Dose 4	Dose 4	Dose 4
SMAR NAD Dose 5	SMAR NAD Dose 5	SMAR NAD Dose 5	SMAR AD Dose 1	SMAR AD Dose 1	SMAR AD Dose 1
SMAR AD Dose 2	SMAR AD Dose 2	SMAR AD Dose 2	SMAR AD Dose 3	SMAR AD Dose 3	SMAR AD Dose 3
SMAR AD Dose 4	SMAR AD Dose 4	SMAR AD Dose 4	SMAR AD Dose 5	SMAR AD Dose 5	SMAR AD Dose 5

Doses for SMAR will be defined based on the results obtained in the two first experiments.

Purple: Fresh direct treatment

5.2 PHASE III: 1) Optimize the Adhesion assay using more disease-relevant primary human endothelial cells

In the context of atherosclerosis, more relevant primary human endothelial cells will be used to optimize the adhesion assay. Human coronary or aortic artery endothelial cells (HCAEC and HAOEC respectively) are good candidate cell types since the development of atherosclerotic plaques frequently take place in coronary and aortic arteries. These cells unlike HUVECs seem to be sensitive to 24h-starvation procedure using cell culture medium supplemented with 0.5% FCS since they rapidly die. Therefore, it will be important to conduct some experiments optimizing the conditions for the adhesion assay with these cells.

5.2.1 Experiment 1 phase III part 1 (3 weeks): Optimization of cell culture conditions for HCAEC and HAOEC (similar conditions to HUVECs and morphological readout)

In order to ensure good cell viability, confluence, morphology to run the assay, cell culture conditions for primary endothelial cells will be optimized.

Different cell densities will be tested to seed the cells. Cell proliferation and morphology will be checked every day with the microscope. Photos will be captured. The cell viability can be monitored using the supernatant by LDH assay.

5.2.2 Experiment 2 phase III part 1 (4 days): Impact of FCS percentage (in medium) and starvation duration on the primary endothelial cell viability

ECs (HCAEC and HCAOEC) will be incubated in cell culture medium supplemented with decreased FCS percentage (from 10 to 0.5%) for a 24 h-starvation period. The starvation period will also vary.

Starvation period (h)	2% FCS (CTRL -)	1.5% FCS	1% FCS	0.8% FCS	0.5% FCS	0.3% FCS	0.2% FCS	0.1% FCS
24	3	3	3	3	3	3	3	3
6	3	3	3	3	3	3	3	3
2	3	3	3	3	3	3	3	3
Triton (CTRL +)	3							

The supernatant will be collected and LDH assay will be performed to assess cell viability.

Negative control: normal cell culture conditions (no starvation)

Postive control: Triton (kill the cells)

Endpoints: cell viability (LDH)

A percentage of FCS and duration of starvation period will be selected for the adhesion assay based on the cell viability which should exceed 80%.

5.2.3 Experiment 3 phase III part 1 (1 week): Adhesion assay tested with new experimental conditions for primary endothelial cells: HCAECs (3R4F sbPBS)

The adhesion assay will be conducted in direct and indirect treatment using HCAECs.

3R4F smoking protocol used will be ISO and HC.

Same experimental design as Exp24 CW27 (performed on week: 1-5th of July 2013 by Alexandra Laurent)

5.2.4 Experiment 4 phase III part 1 (1 week): Adhesion assay tested with new experimental conditions for primary endothelial cells: HAOECs (3R4F sbPBS)

The adhesion assay will be conducted in direct and indirect treatment using HAOECs.

3R4F smoking protocol used will be ISO and HC.

Same experimental design as Exp24 CW27 (performed week: 1-5th of July 2013 by Alexandra Laurent)

5.3 PHASE III: 2) Investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to disease-relevant primary endothelial cells

Same experimental designs as the ones of phase II with adaptation of the protocols depending on the results obtained with the experiments done in phase III part 1.

The cell type between HCAECs and HAOECs giving the best results for the adhesion assay (experiments 3 and 4 phase III part 1) will be selected to perform the assessment of MRTPs.

5.3.1 Experiment 1 phase III part 2 (1 week): SMAR NAD sbPBS assessed in direct and indirect conditions

Same experimental design as the experiment 1 phase II.

5.3.2 Experiment 2 phase III part 2 (1 week): SMAR AD sbPBS assessed in direct and indirect conditions

Same experimental design as the experiment 2 phase II.

5.3.3 Experiment 3 phase III part 2 (1 week): SMAR NAD and AD assessed in „fresh“ direct conditions

Same experimental design as the experiment 3 phase II.

5.4 PHASE III: 3) Investigate the impact of Zurich compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to disease-relevant primary endothelial cells

The experiments will be done when stable ZRH sbPBS could be generated.

5.4.1 Experiment 1 phase III part 2 (1 week): ZRH sbPBS assessed in direct and indirect conditions (disease-relevant primary endothelial cells).

Same experimental design as the experiment 1 phase II.

5.4.2 Experiment 2 phase III part 2 (1 week): ZRH assessed in "fresh“ direct conditions (disease-relevant primary endothelial cells)

Same experimental design as the experiment 3 phase II.

5.4.3 Experiment 3 phase III part 2 (1 week) - Optional: ZRH sbPBS assessed in direct and indirect conditions (HUVECs).

Same experimental design as the experiment 1 phase II.

5.4.4 Experiment 4 phase III part 2 (1 week) - Optional: ZRH assessed in "fresh“ direct conditions (HUVECs).

Same experimental design as the experiment 3 phase II.

Remark: depending of the results obtained for experiments 1 and 2 phase III part 2, a decision will be made to continue or not with experiments 3 and 4 phase III part 2.

5.5 Generation of transcritpomics data

For the experiments phase III part 2, cell lysates from MM6 and primary endothelial cells will be collected to extract mRNA to generate transcriptomics data. The subset of samples to be used will be determined in relation to the TNFα and adhesion results. From previous experiments, the number of MM6 cells (4E6 cells per well) used for the experiment enables to extract a largely sufficient amount of mRNA for transcriptomics, while for HUVECs, 2 wells (48-well plate) per replicate condition have to be pooled to get sufficient amount of mRNA.

6 Procedures

Table 4 Standard Operating Procedures

SOP ID	Title	Deviation/Addition/Remarks
AC 123	Herstellung wasserlöslicher Rauchfraktionen (Preparation of Water Soluble Smoke Fractions)	-
CM 19	Elektronische Zählung von Zellen mit dem Casy 1 Cell	-

	Counter und Analyzer System, Modell TTC (Electronic Cell Count with Casy 1 Cell Counter and Analyzer System, Model TTC)	
CRS 4	Mycoplasma Test (Mycoplasma Test)	-
CV 30	Subkultur der humanen monozytischen Mono Mac 6 (MM6) Zellinie (Subculture of the Human Monocytic Mono Mac 6 (MM6) Cell Line)	-
CV 42	Kultivierung von adhaerenten Zellen (Subculturing of Adherent Cells → General procedure of HUVEC cell culture preparation)	-
CRL G4	CASY Cell Counter and Analysis System	
FU 51	In-Vitro Assay zur Bestimmung der Adhäsion von humanen Monozyten auf humanen Endothelzellen (In Vitro Assay for the Determination of the Adhesion of Human Monocytes on Human Endothelial Cells)	
Wiki site	Determination of eight carbonyls in aerosols with HPLC-MS/MS	Changes in procedure due to lab relocation will be given in the report
Wiki site	Determination of Nicotine in TPM on Glass Fiber Filters with GC-FID/GC-NPD according to DIN ISO	Changes in procedure due to lab relocation will be given in the report

Template for Adhesion Assay

CTL- (med.+PBS)	CTL- (med.+PBS)	CTL- (med.+PBS)	CTL+ TNFalpha	CTL+ TNFalpha	CTL+ TNFalpha

7 Administrative Aspects

7.1 Study Timelines

PHASE	EXP	PURPOSE	DURATION	DATE	REMARK
II		**Investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to endothelial cells (HUVECs)**			
II	1	SMAR NAD sbPBS assessed in direct and indirect conditions & chemical analysis of 3R4F (ISO), 3R4F (HC) and SMAR NAD sbPBS	1 w	22-26 Jul	Need: 3R4F (ISO), 3R4F (HC) and SMAR NAD (HC) sbPBS
II	2	SMAR AD sbPBS assessed in direct and indirect conditions & chemical analysis of 3R4F (ISO), 3R4F (HC) and SMAR AD sbPBS	1 w	05-09 Aug	Need: 3R4F (ISO), 3R4F (HC) and SMAR AD (HC) sbPBS
II	3	3R4F sbPBS assessed in "fresh" direct conditions & chemical analysis of 3R4F (ISO) and 3R4F (HC) sbPBS	4 d	12-16 Aug	Need: 3R4F (ISO) and 3R4F (HC) sbPBS
II	4	SMAR NAD and AD sbPBS assessed in "fresh" direct conditions & chemical analysis of 3R4F (ISO), 3R4F (HC), SMAR NAD and SMAR AD sbPBS	4 d	19-23 Aug	Need: 3R4F (ISO), 3R4F (HC), SMAR NAD and SMAR AD (HC) sbPBS
III part 1		**Optimize the Adhesion assay using more disease-relevant primary human endothelial cells**			
III part 1	1	Optimization of cell culture conditions for HCAEC and HAOEC (similar conditions to HUVECs and morphological readout)	3 w	27-13 Sep	
III part 1	2	Impact of FCS percentage (in medium) and starvation duration on the primary endothelial cell viability	4 d	16-20 Sep	
III part 1	3	Adhesion assay tested with new experimental conditions for primary endothelial cells: HCAEC (3R4F sbPBS) & chemical analysis of 3R4F (ISO) and 3R4F (HC) sbPBS	1 w	23-27 Sep	Need: 3R4F (ISO) and 3R4F (HC) sbPBS Same design as Exp24 CW27 (Alex)
III part 1	4	Adhesion assay tested with new experimental conditions for primary endothelial cells: HAOEC (3R4F sbPBS) & chemical analysis of 3R4F (ISO) and 3R4F (HC) sbPBS	1 w	01-04 Oct	Need: 3R4F (ISO) and 3R4F (HC) sbPBS Same design as Exp24 CW27 (Alex)
III part 2		**Investigate the impact of MRTPs compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to disease-relevant primary endothelial cells**			
III part 2	1	SMAR NAD sbPBS assessed in direct and indirect conditions & chemical analysis of 3R4F and SMAR NAD sbPBS	1 w	07-11 Oct	Need: 3R4F (HC) and SMAR NAD (HC) sbPBS
III part 2	2	SMAR AD sbPBS assessed in direct and indirect conditions & chemical analysis of 3R4F and SMAR AD sbPBS	1 w	14-18 Oct	Need: 3R4F (HC) and SMAR AD (HC) sbPBS
III part 2	3	SMAR NAD and AD sbPBS assessed in "fresh" direct conditions & chemical analysis of 3R4F, SMAR NAD and SMAR AD sbPBS	4 d	21-25 Oct	Need: 3R4F (HC), SMAR NAD and SMAR AD sbPBS
		Investigate the impact of Zurich compared to 3R4F as aqueous smoke/aerosol fraction (sbPBS) on the adhesion of monocytic cells (MM6) to endothelial cells		On Hold	
III part 2	1	ZRH sbPBS assessed in direct and indirect conditions (disease-relevant primary endothelial cells) & chemical analysis of 3R4F and ZRH sbPBS	1 w	?	depends on generation of stable ZRH sbPBS (3R4F: HC)
III part 2	2	ZRH assessed in "fresh" direct conditions (disease-relevant primary endothelial cells) & chemical analysis of 3R4F and ZRH sbPBS	4 d	?	depends on generation of stable ZRH sbPBS (3R4F: HC)
III part 2	3	ZRH sbPBS assessed in direct and indirect conditions (HUVECs) & chemical analysis of 3R4F and ZRH sbPBS	1 w	?	depends on generation of stable ZRH sbPBS (3R4F: HC)
III part 2	4	ZRH assessed in "fresh" direct conditions (HUVECs) & chemical analysis of 3R4F and ZRH sbPBS	4 d	?	depends on generation of stable ZRH sbPBS (3R4F: HC)

Proposed experimental starting date:	End of July 2013
Proposed experimental completion date:	End of October (if no ZRH) – November (if ZRH tested) 2013
Proposed date for draft study report:	

7.2 Names and Addresses

Sponsor	Manuel Peitsch Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

Test Facility	Philip Morris Products S. A Research & Development Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Study Director	Carine Poussin Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Deputy Study Director	Hector De Leon Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Quality Assurance Officer	NA

7.3 Quality Assurance

This study will not be conducted in accordance with Good Laboratory Practice (GLP).

8 Archiving

After finalization of the study, the study plan, amendments, raw data, test and reference items, retention samples, specimens collected, the report, and all study-related records will be stored for a maximal period of one year at the archive at Philip Morris Products S.A, Research & Development. Thereafter, all records and materials will be transferred to the external GLP-certified service archive Iron Mountain Deutschland GmbH, Harpener Hellweg, 44805 Bochum, Germany.

Electronic records will be archived on the central archiving server at Philip Morris Products S.A, Research & Development, which is managed by the local IT specialists.

Electronic and paper records will be archived for at least 10 years after finalization of the final report according to the specific archiving SOPs.

9 References

10 Abbreviations

Abbreviation	
CC	Conventional cigarette
CS	Cigarette Smoke
sbPBS	Smoke bubbled PBS
SMAR NAD	SMAR non-alu disc
SMAR AD	SMAR alu disc
ZRH	Zurich
MRTPs	Modified risk tobacco product
MM6	Mono Mac 6
HUVECs	Human umbilical vein endothelial cells
HCAECs	Human coronary artery endothelial cells
HAOECs	Human aortic endothelial cells
LDH	Lactate dehydrogenase

For complete definition, refer to PMI OPS Glossary and PMI RD Glossary.

11 Appendix

Table of Product references

Products	**Supplier**	**Ref number**
CMTMR dye CellTracker™ Orange CMTMR (5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine)-Mixed Isomers	Invitrogen	C2927 – 1mg
Hoechst	Sigma	14533 – 100mg

bisBenzimide H 33342 trihydrochloride for fluorescence, ≥97.0%		
Glutaraldehyde Glutaraldehyde solution Grade II, 25% in H2O	Sigma	G6257 - 100ml
TNF-α TNF-α Recombinant Human	Invitrogen	PHC3015 – 10µg
48-well plates – Collagen A coated Corning® Costar® cell culture plates 48 well, flat bottom	Sigma	CLS3548-100EA
6-well plate for suspension cells X100 Plaque F6 PS Suspen.st	Fisher	W1789Z
PBS Dulbecco's Phosphate Buffered Saline - Without MgCl2 and CaCl2	Sigma	D8537-6X500ml
PBS^{+} Dulbecco's Phosphate Buffered Saline - With MgCl2 and CaCl2, liquid, sterile-filtered, suitable for cell culture	Sigma	D8662-6X500ml
DMSO Dimethyl Sulfoxide	Sigma	D8418-100ml
LPS Lipopolysaccharides from Escherichia coli 0111:B4	Sigma	L2630
ELISA – Human TNFa Human TNF-alpha DuoSet	R&D Systems	DY210
ELISA – Substrate Reagents Substrate Reagent Pack (8 vials Color A, 8 vials Color B)	R&D Systems	DY999
ELISA – Stop solution Stop Solution 2N Sulfuric Acid (15 x 6 mL)	R&D Systems	DY994
ELISA – Reagent diluent Reagent Diluent Concentrate 2 (10X, 5 x 21 mL)	R&D Systems	DY995
ELISA – 96w plate Corning® 1 x 8 Stripwell™ 96 well plates	Sigma-Aldrich	CLS2592-25EA
ELISA – Sealing tape Corning® microplate sealing tape	Sigma-Aldrich	CLS3095-100EA
HUVEC - Growth medium Endothelial Cell Growth Medium (Ready-to-use)	Promocell	C-22010
HUVEC – Starvation medium Endothelial Cell Growth Medium Kit	Promocell	C-22110
HUVEC cells Human Umbilical Vein Endothelial Cells (HUVEC)	Promocell	Order n°: C-12200 Lot n°: 1052602

		Newborn / male / Caucasian
MM6 cells Acute monocytic leukemia	Ziegler-Heitbrock	s. DSMZ ACC 124
RPMI 1640 (for MM6 media)	Sigma	R0883
OPI Media Supplement (for MM6 media)	Sigma	O5003
FBS low LPS (for MM6 media)	PAA	A15-102
NEAA (100x) (for MM6 media)	PAA	M11-003
L-Glutamine (200mM) (for MM6 and HUVEC media)	PAA	M11-004
Penicillin/Streptomycin (100x) (for MM6 and HUVEC media)	Ruwag	P11-010
HAOEC cells	Tebu-Bio	
HCAEC cells	Promocell	