STUDY PLAN
IN VIVO EVALUATION OF THE EFFECTS OF ZURICH MRTP (DEL/C3/FR1/RDO) ON THE RESPIRATORY AND CARDIOVASCULAR SYSTEMS OF APO LIPOPROTEIN E-DEFICIENT MICE
(STUDY NUMBER 15015)

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Author(s)	PHILLIPS, Blaine
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1 Review and Approval

This document has been approved by:

Name	Justification	Date / Signature
Phillips, Blaine	Approved by Study Director	31Aug16
Chua, Joanne	Approved by Test Facility Management	31 Aug 16

Note, this version of the study plan has been modified from the original study plan in order to address minor formatting changes required to align with the 'Global Submit Publishing Standards'.

2 Introduction

Cigarette smoking is a strong causative element to the development and progression of a series of diseases, including cardiovascular disease (CVD) and chronic obstructive lung diseases such as COPD/emphysema. Significant health benefits have been associated with smoking cessation. However, the underlying mechanisms of both disease progression, and the impact of cessation are largely unknown. This study will examine both COPD and CVD endpoint development in Apolipoprotein E-deficient (ApoE-/-) mice exposed to either cigarette smoke, or an aerosol from Zurich MRTP (referred to as ZRH) over an 8 month period of time. In addition, a comparison of cessation or switching to ZRH after 2 months of cigarette smoke exposure will be assessed.

The animals will be exposed to cigarette smoke or to an aerosol from ZRH (600 µg/l TPM or nicotine equivalent for ZRH) for 3 hours per day, five days per week, for up to 8 months. Previous studies (Study 13882) have shown that this exposure regime and timeframe is sufficient to induce symptoms of both emphysema/COPD and CVD in the ApoE-/- mice, and is well tolerated by the animals.

The test atmosphere will be monitored throughout the study in order to demonstrate constant exposure to 600 µg/l TPM (for cigarette smoke) or to equivalent nicotine exposure concentrations. The following analytical parameters will be assessed at defined intervals: TPM, carbon monoxide, puff volume, formaldehyde, acetaldehyde, acrolein, nicotine, and particle size distribution.

The animals will be randomly allocated to the following groups:

i) Sham – exposed to filtered air

ii) 3R4F – aerosol from reference cigarettes (600 µg/l TPM target dose)

iii) ZRH – aerosol from an electrically heated stick from the Zurich development platform (concentration will be matched to nicotine levels in the 3R4F exposure conditions)

iv) Cessation – 2 months 3R4F, followed by filtered air

v) Switching to ZRH – 2 months 3R4F, followed by aerosol from ZRH

vi) Insulin resistance (IR) – an additional group of mice will be allocated for the study of the effect of cigarette smoke, or ZRH aerosol on the progression of insulin resistance.

At the end of the exposure period (1, 2, 3, 4, 6, or 8 months) – organs, tissues and blood will be collected for molecular analysis (proteomics, transcriptomics/genomics, and lipidomics). Pulmonary pathological and functional changes will be assessed by standard histopathology or morphometric analyses as well as by lung function testing, which will be performed in selected groups. Additional endpoints will examine pulmonary inflammation, atherosclerotic plaque development, biomarkers of exposure and the effects of the aerosols on metabolic parameters (glucose and insulin levels, as well as metabolic analyte levels in the plasma).

All experimental procedures will be performed according to PMIRL-S QMS procedures, unless otherwise specified.

3 Objectives

The objectives of this study are to:

- Investigate the effects of ZRH MRTP on atherosclerosis- and respiratory-related endpoints (i.e. emphysema) as compared to 3R4F- and sham-exposed control groups at 1, 2, 3, 6 and 8 months.
- Assess the effects of switching from conventional cigarettes (2 months exposure to 3R4F smoke) to ZRH MRTP *vs*. cessation or sustained 3R4F smoke exposure on atherosclerosis- and respiratory -related endpoints at 3, 6, and 8 months.
- Demonstrate the potential of heating (MRTPs) versus burning (conventional cigarettes) to reduce exposure-related risks for development of the smoke related diseases.
- Investigate the effects of conventional cigarette smoke or ZRH MRTP aerosol on the development and progression of IR.

ApoE-/- mice will be used as a model for cigarette smoke-exposed diseases/pathological conditions such as atherosclerosis, emphysema/COPD and IR.

4 Experimental Design

4.1 Test and Reference Items

4.1.1 Test item- MRTP Zurich FPD 4.2 sensorial medium

The test item is Zurich FPD 4.2 sensorial medium (Table 1). The sensorial media (hereafter referred to as 'stick') is a special cigarette that contains different elements (tobacco plug, transfer section and mouth piece) assembled with an over-wrapping of cigarette paper. The sticks will be provided by Philip Morris International (PMI), Neuchâtel, Switzerland. Aerosol specifications (Batch quality control or batch release conditions) will be stored in the Project Management Folder (PMF) as batch release certificates.

The ZRH sticks will be produced at PMI (Neuchâtel) in 3 batches, as the stability of the sticks is stability tested only up to 6 months (less than the 8 month study period (section 4.1.3)). Proposed production volumes and delivery schedules are as follows;

Batch 1: 60,000 sticks, delivery CW27, 2013 (received, and in storage)
Batch 2: 360,000 sticks, delivery CW32 (planned)
Batch 3: 250,000 sticks, delivery CW52 (planned)

The sticks will be packaged in mini princess packs (packs of 2 collation of 10 sticks each). The packaging is labeled with product identification (Del/C3/FR1/Rdo), pack lot number and the production date. The batches of ZRH sticks will also be distinguished by the use of cork tipping paper. All sticks will be stored in the original

packaging in room 2024 with controlled room temperature (from 2 to 10 °C), but uncontrolled humidity conditions. The cigarettes will be conditioned in room 3103 according to ISO standard 3402 (1999), for between 7 to 21 days at a temperature of 22 ± 1 °C and 58 ± 3% relative humidity before being used for aerosol generation.

Table 1 **Identification Details for test item- Sensorial Media**

Description	Short Name	Batch Number
Del/C3/FR1/Rdo	ZRH	Batch 1: [i]B-05771

[i]: Number for batch 2 and 3 will be available in the batch release form, stored in the PMF

As part of the smoking system, the sensorial medium is inserted into a cigarette holder that heats the tobacco plug. The cigarette holder includes a battery, controlling electronics, a heating element and the cigarette extractor. The cigarette holders will be provided by Philip Morris International (PMI), Neuchâtel, Switzerland. Batch quality control or batch release certificate will be stored in the PMF.

The daily usage of ZRH cigarette holders will be documented by the aerosol team. A total of 600 cigarette holders were requested for use during the study exposure period. The sum of operating days for each batch of cigarette holder will be tabulated at the end of the study.

Table 2 **Identification Details for Cigarette Holders**

	Cigarette Holder
Product description	FPD 4.2
Product code	DV-000067.RD
Batch Number	B-06143

4.1.2 Reference Item – 3R4F Conventional Cigarettes

The 3R4F cigarettes will be purchased from the University of Kentucky (for specifications see University of Kentucky, http//www.ca.uky.edu/refcig), packaging: soft packs of 20 cigarettes (10 soft packs/carton, and 25 cartons/box). All cigarettes will be stored in the original packaging in room 2025 with controlled room temperature (from 2 to 10 °C), but uncontrolled humidity conditions. The cigarettes will be conditioned in room 3102 for >7 to <21 days prior to smoking, at a temperature of 22 ± 1 °C and 58 ± 3% relative humidity.

4.1.3 Stability of Test and Reference Items

The stability of the reference cigarettes are described in the link in section 4.1.2 above. The ZRH sticks have previously been stability tested for a period of 6 months (Stability data to be stored in PMF). As this study is for an 8 month exposure period, the sticks will be prepared in batches such that they are used within the 6 month period.

4.1.4 Treatment Administration

Whole-body exposure chambers (type WBEC85) will be used in this study as it is the most practical and reproducible means to expose mice to conventional cigarette smoke (3R4F) or MRTP (ZRH) aerosols. The target concentration of mainstream smoke from the 3R4F conventional cigarettes is 600 µg/l TPM. The exposure using the aerosol derived from the ZRH (MRTP) will be dose-matched to the nicotine levels in the 3R4F aerosol (see 4.1.6). Therefore, during the preliminary, or test-smoking – the TPM/nicotine ratios will be determined, and the ZRH dose set based on these details. As nicotine/TPM ratios are subject to subtle variations due to stick batch variability (three batches will be used throughout the study period), aerosol generation conditions or the presence of animals in the chamber, the nicotine levels may be adjusted to compensate at the discretion of the study director.

For the conventional cigarette, the aerosol generated from 1 smoking machine will be diluted with filtered, conditioned air to the target concentration of 600 µg/l TPM for delivery to one exposure chamber. For the ZRH groups, 2 smoking machines will be combined to produce an aerosol with matched nicotine concentrations to the 3R4F conventional cigarette mainstream smoke. The aerosol from either the test or reference item will be conveyed via glass tubing, from the smoking machines to the exposure chambers. The total flow rate through the whole-body exposure chambers will be adjusted to approximately 80 l/min. For the sham group, mice will be exposed to filtered, conditioned fresh air, whilst the other exposure conditions will be the same as those for the smoke-exposed mice.

4.1.5 Test Atmosphere Generation and Characterization

The 3R4F and ZRH sticks will be smoked according to the Health Canada Intensive Smoking Protocol (Health Canada, 1999) which is based on the ISO standards 3308 (2000) with the exceptions of the puff volume (55ml), puff frequency (one puff every 30 seconds) and ventilation block. In addition, several minor deviations from the ISO standards 3308 (2000) are necessary for technical reasons (Table 3).

Aerosol from 3R4F is generated on 30-port rotary smoking machines with active side stream exhaust (type PMRL-G, SM2000) and equipped with Programmable Dual-port Syringe Pump (PDSP). Aerosol from ZRH sticks is generated using 30-port carousel Smoking Machines Smoking Device Docking Station Zurich (SM SDDS ZRH) equipped with cigarette holder and a PDSP. The SDDS serves as an interface to ensure synchronization between the cigarette holder and the smoking machine. The SDDS ZRH smoking machine was developed by

PMI R&D Neuchatel, Switzerland. The serial number and certificate of conformity are stored in PMF. The MRTP smoking machines will also include a temperature-controlled insulation kit (Tube Warming System) in the undiluted aerosol pathway, with the goal of reducing aerosol condensation. The smoking machine and chamber layout for both 3R4F and ZRH exposure conditions are depicted in Figure 1 and Figure 2, respectively.

Prior to the start of the study, performance verification (PV) will be performed on each of the inhalation setups. Each inhalation setup shall be smoked at the target concentrations using the assigned smoking machines. The purpose of performing PV on the inhalation setups includes:

1. To determine the process air flow rates that are required to achieve the target concentrations.
2. To determine the equivalent nicotine levels for the TPM target concentrations of the 3R4F groups.
3. To demonstrate that the inhalation setups are capable of achieving the targeted concentrations.

The inlet temperature of the test atmosphere in the exposure chamber will be maintained at 22 $\pm$ 2 °C and the relative humidity between 30 and 70%. Air flow and relative humidity through the exposure chamber will be monitored continuously and recorded in Citect during the exposure period. The flow through the exposure tubes will be calculated for each of the groups and will be targeted at 1.5 l/min per tube but must not reach levels less than 1.0 l/min per tube.

Puff count for Zurich FPD 4.2 is set to 12 puffs due to device configuration. The 3R4F cigarettes will be smoked to a butt length of 35 ± 1 mm; The butt length and puff count for 3R4F will be determined monthly while static burning rate will be determined at least once during the inhalation period. In order to characterize the test atmosphere and to check the reproducibility of aerosol generation and dilution, several analytical parameters will be determined at the breathing area of the animals. Particle size distribution will be performed using the PIXE impactor protocol.

4.1.6 Target aerosol concentrations and acceptable variance

The target aerosol concentration is defined as the nicotine concentration of 3R4F at 600 µg/l TPM during the test smoking period (expected to be approximately 27.5 µg/l nicotine as described in section 4.1.4).

4.1.6.1 Daily fluctuations and accepted limits

According to the OECD 412 Testing Guideline, which is applied for standard safety toxicological assessment, the acceptable variation for inhalation studies is up to 20% of the chamber mean concentration for solid or gas aerosols. Based on the in-house experience with aerosols from ZRH test items, the following limits for the exposure are defined. The total mean chamber concentration (calculated monthly, excluding the initial dose adaptation phase) should be within ±10% of the target, with allowable daily fluctuations of up to ±20% for the 3R4F chambers, and ±20% for the ZRH chambers. Each occurrence of deviation outside of these target ranges will trigger a non-conformance incidence report.

4.1.6.2 Machine breakdown, or low aerosol delivery

The ZRH smoking machines involve multiple mechanical processes and manipulations of the sticks, holders, etc., which lead to the occasional jamming or breakdown incidents. In such cases, when one or both machines are not functioning, the chamber aerosol concentrations will drop. This may be reflected by reduced TPM or nicotine levels should such 'breakdown' occur during a sampling period. During breakdowns, the following plan will be used to ensure accurate delivery for a total 3 hour exposure period:

i) One machine not functioning – simple problems which can be resolved in less than 10 minutes – The machine will be repaired, the disruption noted in the log sheet, and SD informed. There will be no change to exposure timings.

ii) One or both machine (s) not functioning, complex problems which can't be resolved in less than 10 minutes – the exposure will be stopped, and the animals provided with fresh air. The exposure will then continue in full (i.e., to complete the full 3 hours) once the repair is completed. Disruption noted on log sheet, SD informed.

iii) One machine not functioning, irreparable – exposure will be stopped and fresh air delivered to animals. Exposure will resume in full (i.e., to complete the full 3 hours) once a replacement machine is installed. Disruption noted on log sheet, SD informed.

4.1.6.3 Change to nicotine/TPM ratio

In a long-term study, the TPM/nicotine ratio may slightly change due to cigarette/stick batch, change in exposure conditions (replacement parts, machines, etc.) or the presence of animals in the chamber. In such cases, the exposure will continue by pegging to the nicotine levels, despite potentially changing TPM concentrations.

Note, as the animals in the chamber may influence the TPM/nicotine ratios, the SD and aerosol manager may adjust the target nicotine peg after the adaptation period is complete, and the aerosol levels are stable.

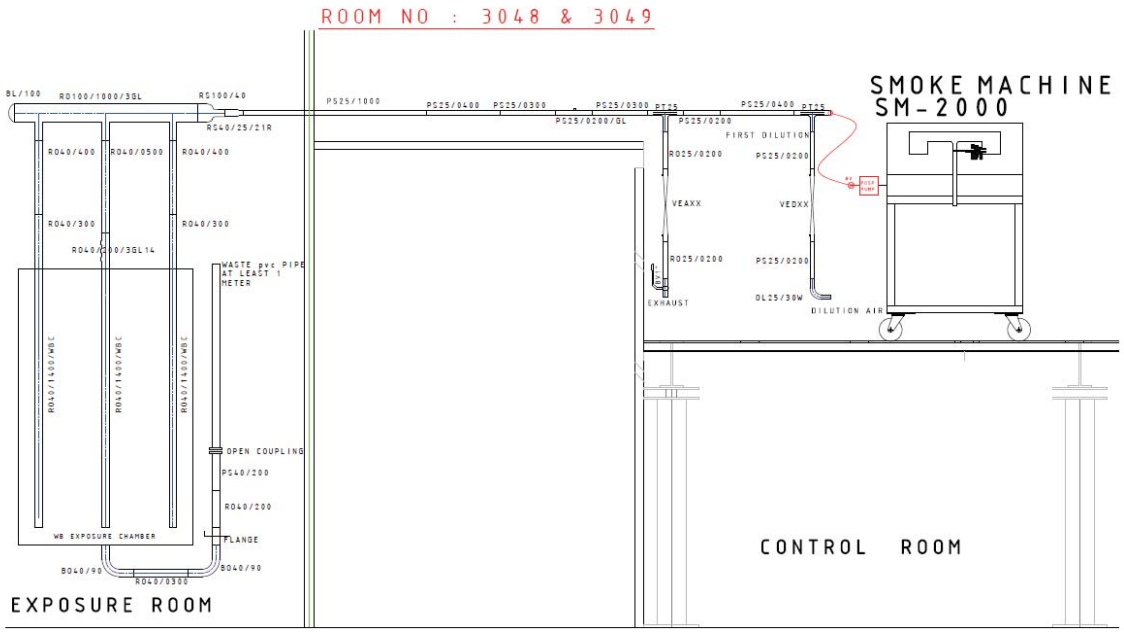


Figure 1. **Smoking machine and chamber layout for 3R4F groups.**

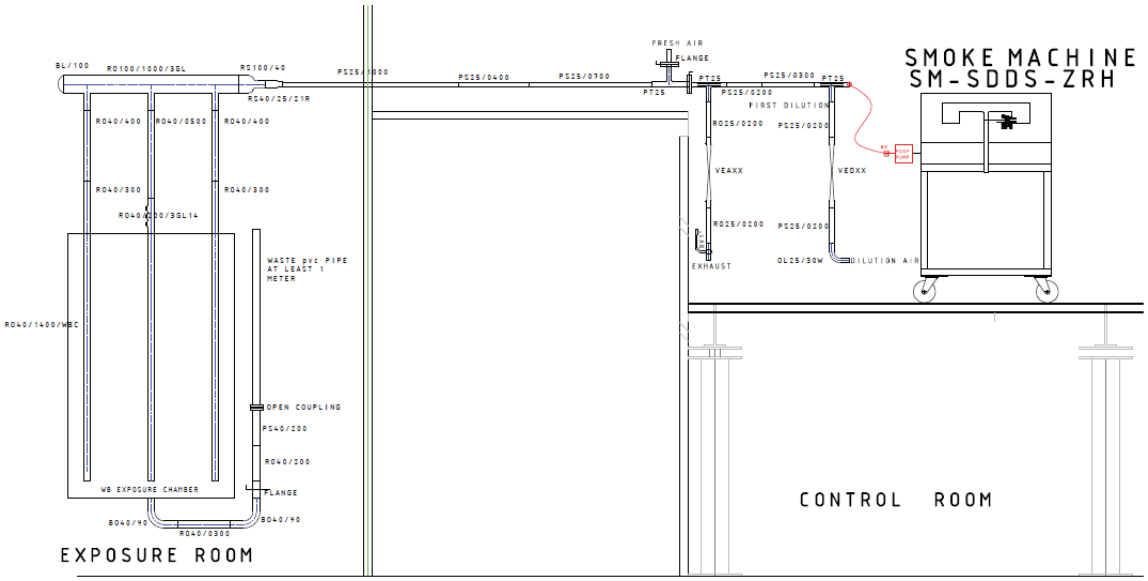


Figure 2. **Smoking machine and chamber layout for ZRH groups.**

Table 3 [i]**Deviations from ISO standards 3308 (2000) for Zurich FPD 4.2**.

Parameter	ISO 3308 (2000)	Recommended condition	Explanation for deviation
Puff count	rounded to the nearest one-tenth of a puff	only whole puffs	device configuration
Restricted smoking	restricted smoking	Free/ Open end smoking	device configuration
Cigarette holder	Labyrinth seal and perforated disc (washer)	Single seal and no washer	device configuration
Puff termination & counting	Butt length sensor/ 0.1 puff counting resolution	Fixed number of puffs/ only whole puffs counted	device configuration
relative humidity during smoking	60 ± 5%	The air supplied to the smoking machine meets the requirements	The conditions inside the smoking machine are unmonitored
temperature during smoking	22 ± 2 °C	The air supplied to the smoking machine meets the requirements	The conditions inside the smoking machine are unmonitored
Air velocity around sticks	170 - 230 mm/sec	The ZRH stick is enclosed within the cigarette holder	airflow around the stick is controlled by the device

[1]: Deviation for puff count, relative humidity and temperature is also applicable for 3R4F.

Table 4 **Analysis of Aerosol**

Parameter	Assay Principle	Determination schedule SHAM	Determination Schedule 3R4F	Determination Schedule ZRH
TPM	Gravimetry after trapping on Cambridge filters	≥ 1 time/week	During every exposure block	During every exposure block
Carbon monoxide	Nondispersive infrared photometry of gas/vapour phase	Continuously	Continuously	Continuously
Nicotine	Capillary gas chromatography after trapping on sulfuric acid-impregnated silica gel	≥ 1 time/week	During every exposure block	During every exposure block
Formaldehyde, acetaldehyde, and acrolein	Reversed phase high-performance liquid chromatography (HPLC) of 2,4-dinitrophenylhydrazine (DNPH) derivatives after trapping in DNPH solution	NA	≥ 1 time/week	≥ 1 time/week
Relative humidity	Capacitive	Continuously; only sham group	N/A	N/A
Temperature	Thermistor probe Pt100	Continuously	Continuously	Continuously
(Static) puff volume	Soap bubble flow meter	Before and after daily smoke generation	Before and after daily smoke generation	Before and after daily smoke generation
Flow rate through exposure chamber	Pressure difference over a Venturi tube	Continuously	Continuously	Continuously
Particle size distribution	Impactor	≥1 time/month	≥1 time/month	≥1 time/month

4.2 Test System

It has been previously demonstrated (PMRL Study 13882) that ApoE-/- mice are a suitable murine model system to study atherosclerosis, as well as emphysema development and smoke-induced physiological effects from cigarette mainstream smoke at exposure concentrations of 600 µg/l TPM. This model system will therefore be utilized in this follow-up study of cigarette smoke, cessation, and MRTP switching on the effects on atherosclerotic and emphysema markers. Female ApoE-/- mice (TC-ApoE-F) bred under specific pathogen-free conditions will be obtained from Taconic, UK. The mice will be approximately 6 - 8 weeks old at arrival (8-10 weeks at exposure start) and their health status will be verified using the health check certificate provided by the breeder. Additional health checks will be tested at Harlan, UK. The health checks will be performed according to the following schedule:

Month 0: Live animals (6 sentinel animals, one per group) sent for analysis (Health screen type 310M for mice, Harlan, U.K.).

Month 3: Serum (6 sentinel animals, one per group, non-terminal) sent for analysis (Health screen type SM246 for mice, Harlan, U.K.).

Month 6: Serum (6 sentinel animals, one per group, non-terminal) sent for analysis (Health screen type SM246 for mice, Harlan, U.K.).

Month 8: Live animals (6 sentinel animals, one per group) sent for analysis (Health screen type 310M for mice, Harlan, U.K.).

The study will run in an AVA-licensed (Agri-Food & Veterinary Authority of Singapore) facility where care and use of the animals will be in accordance with the National Advisory Committee for Laboratory Animal Research (NACLAR) Guideline 2004.

Following arrival of the mice, the boxes and containers will be carefully examined prior to transportation into our animal holding rooms. Shipping boxes in which the seal is breeched will be rejected from the study. Other damage will be assessed and accepted at the discretion of the study director with input from the veterinarian. Prior to the start of the exposure, the mice will be acclimatized to the facility for a period of at least 2 weeks.

Mice will be individually identified by subcutaneously implanted transponders. They will be kept and exposed in the animal laboratory (rooms 3046 - 3051) with restricted access and under specific pathogen-free conditions. The laboratory air will be filtered, conditioned fresh air. Positive pressure will be maintained inside the laboratory. The room temperature will be maintained at 22 ± 3 °C and the relative humidity between 30 and 70%. The light/dark cycle will be 12 /12h.

A maximum of eight mice will be housed per cage. Cage-enrichment (Igloo, and Nylabone™ for gnawing) will be provided to each cage during the non-exposure period. The bedding material will be autoclaved softwood

granulate. Gamma-irradiated pellet diet will be provided on the cage top from cage lids, and filtered tap water from water bottles will be supplied ad libitum for each cage. No diet will be available during exposure periods.

Bedding material (Lignocel® BK 8-15, J. Rettenmaier & Sohne, GmbH + Co KG.) will be analyzed by the supplier for aflatoxins, selected pesticides, heavy metals, polychlorinated biphenyls, and pentachlorophenol. There will be two types of food used in this study. A gamma-irradiated pellet diet (T2914C irradiated rodent diet for the ApoE switchin arm, or TD.88137 for HFD groups for IR arm, both from Harlan, U.K.) will be provided on the cage top from cage lids. The T2914C will be screened by the supplier, with guidance from the panel of dietary components and contaminants outlined by the NTP (National Toxicology Program, 2000), which describes the analysis for levels of aflotoxins, nitrosamines, heavy metals, chlorinated hydrocarbons, organophosphates, PCB's and bacterial contaminants. The HFD (TD.88137) will be screened by the supplier according to FDA and EPA recommendations (21 CFR 58, *1979,* Federal Register, Part 2, 27334-27375, Part 4, 44054-44093) standards. The results and constituents assessed of all screens for diet will be stored in the PMF. The animal drinking water will be screened once every two weeks for microbiological quality. Each batch of diet and bedding will be monitored for microbiological quality before first usage and every month thereafter for the sterile conditions.

To check the hygienic conditions within the animal laboratory rooms, samples of the laboratory air will be collected every two weeks during animal housing. To check the effectiveness of laboratory disinfection and sanitation, impression samples will be taken prior to the introduction of the mice and weekly thereafter. From each sample, the bacterial plate colony count will be determined.

4.3 Experimental Groups and Dosing

4.3.1 Allocation of Animals to Treatment Groups

A total of 904 mice will be allocated to exposure groups for the main arm of the study named 'ZRH ApoE switching '(groups 1 through 5 in Figure 3, column A to E). In addition, 75 mice will be allocated at the same time to groups 601 – 613, and will comprise the insulin resistance groups (IR). In order to allow replacement of mice which may become moribund or die during the study period, up to 8 reserve mice will be exposed in each exposure group from the ZRH ApoE switching study arm (these are represented by the 40 animals in the 'G' column of Figure 1), and 24 mice for the IR arm. These reserves will be used for replacement should any study animals die during the study period, and will also be used for blood sampling for measurement of biomarkers of exposure, or for optimization of methods and dissection workflows. The allocation to groups will be performed within one week of exposure start in order to obtain similar mean body weights for all groups at the start of the smoke exposure. The group sizes have been used routinely at PMIRL-S and shown to deliver acceptable statistical interpretation of the data while keeping the animal numbers in line with expectations of the animal welfare policy.

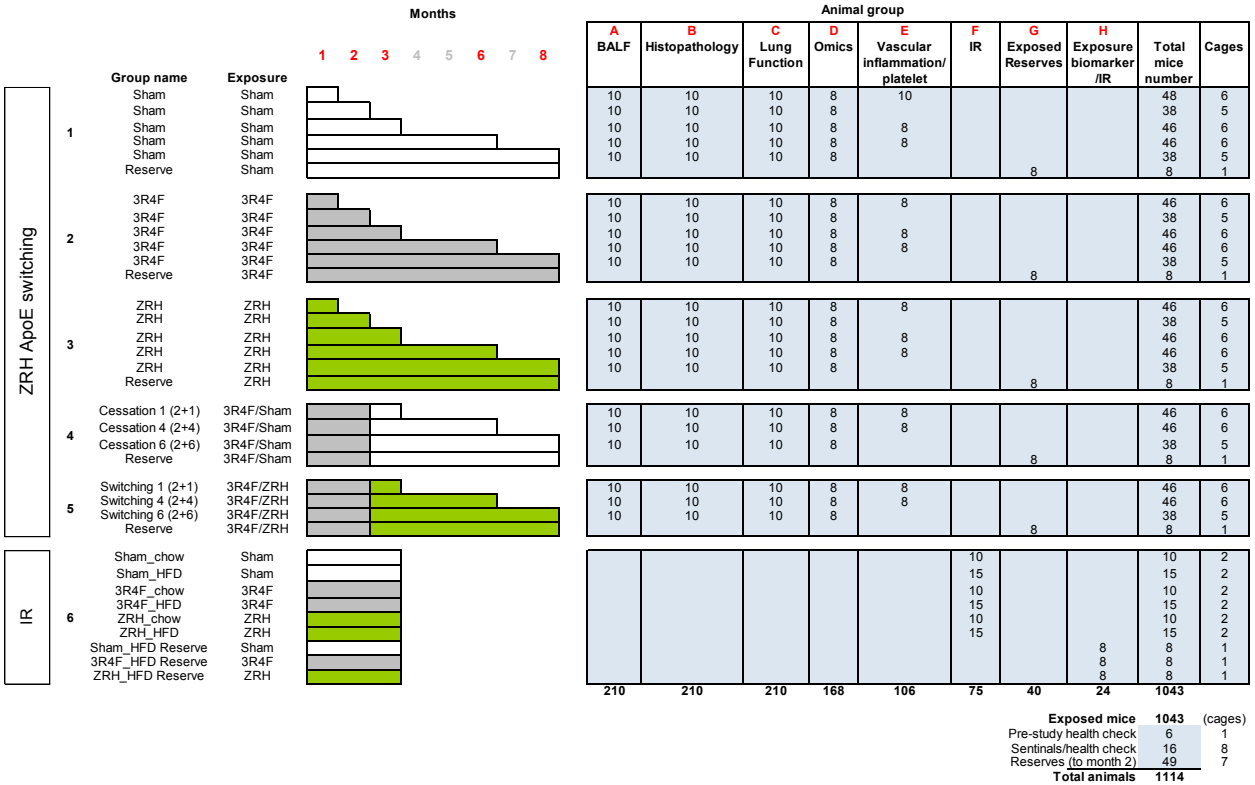


Figure 3 **Schematic representation of the study set-up.** Depicted are the study arms ZRH ApoE Switching, and Insulin Resistance (IR), exposure groups, the dissection timepoints, the distribution of the mice to each dissection point, total mice per group, and number of cages per group. More detailed description in the text (4.3.1).

4.3.2 Test groups

This study will contain two experimental arms, though with many shared sampling methods, times, and endpoints (Figure 3). These are briefly summarized as follows:

4.3.2.1 **ZHR ApoE Switching**: Timecourse analysis of the effect of conventional cigarette smoke (3R4F), MRTP (ZRH), switching or cessation on respiratory and atherosclerotic pathologies development and progression.

This study phase is composed of an 8 month timecourse, with 714 mice allocated to fresh air (sham), cigarette smoke, ZRH, 2 months cigarette smoke – then cessation, or 2 months cigarette smoke – switch to ZRH. The timepoints for dissection will be at month 1, 2 and 3 (bracketing the switch/cessation timepoint), then month 6 and 8. The group sizes, and dissection endpoints are depicted in Figure 3.

4.3.2.2 **Insulin Resistance (IR)**: Effect of conventional cigarette smoke or ZRH aerosol on the development and progression of insulin resistance.

The second arm consists of a three and a half month exposure to fresh air, 3R4F cigarette smoke or ZRH aerosol (same dose as time course), with longitudinal sampling periods for the assessment of insulin resistance markers. Some of the animals will be fed a high fat diet (HFD), and all animals will be assessed for fasting glucose levels measured monthly. The endpoints will consist of combined atherosclerosis and metabolic endpoints.

4.3.2.3 Reserve animals

A total of 111 mice are available as reserve animals. As depicted in Figure 3, these will be utilized as follows:

- Exposed reserves – 8 reserve mice (1 cage) per exposure group will be exposed along with each of the 5 different groupings of the ZRH ApoE switching arm of the study (40 mice total). These mice may therefore be available to replace any moribund animals from the respective group during the 8 month study period.
- Pre-study health check – 6 mice will be sent for health assessment (Health screening type 3M, refer to section 4.2).
- Sentinels – 16 animals will be used as sentinels. Two mice will be housed and exposed to the bedding from 1 of the 8 groupings as follows:
  i) ZRH ApoE Switching – 1 sentinel cage per group 1 through 5.
  ii) IR – 1 sentinel cage for Sham, 3R4F, and ZRH (so 3 cages total).
  - the sentinel animals will be used for all health screening planned in the study.
- Non-exposed reserves – the remaining animals will not be exposed, with a planned treatment as follows:
  i) Replacement animals for any moribundity or mortality during animal transport to the Singapore facility.
  ii) Replacement animals for any moribundity or mortality up to 1 week into the exposure period.
  iii) Method training (up to 20 animals): histoprocessing of liver, pancreas, lung, nasal area and plaque area measurement.
  iv) Method training (up to 5 animals, exposed to 3R4F): 'omics dry run.
  v) Method training (up to 20 animals, including up to 10 animals exposed to 3R4F to induce inflammation): vascular inflammation and platelet aggregation analysis.
  vi) Method training (up to 5 animals): blood collection from tail or facial vein.
  Note – study replacement will be the top priority for the reserve animals, therefore the numbers for method training may be adjusted at the study director's discretion if fewer reserve animals are available.

Table 5 **Exposure Groups**

Short Name	Group Code	TPM Concentration (µg/l)	Daily Exposure Duration (h)	Exposure Duration (months)
Sham_1m	101	0	3	1
Sham_2m	102			2
Sham_3m	103			3
Sham_6m	106			6
Sham_8m	108			8
3R4F_1m	201	600 µg/l	3	1
3R4F_2m	202			2
3R4F_3m	203			3
3R4F_6m	206			6
3R4F_8m	208			8
ZRH_1m	301	nicotine equivalent	3	1
ZRH_2m	302			2
ZRH_3m	303			3
ZRH_6m	306			6
ZRH_8m	308			8
Cessation_3m	403	600 µg/l 3R4F Then fresh air		3
Cessation_6m	406			6
Cessation_8m	408			8
Switching_3m	503	600 µg/l 3R4F Then nicotine equivalent ZRH		3
Switching_6m	506			6
Switching_8m	508			8
Sham_chow	601	fresh air or 600 µg/l cigarette smoke/ZRH (nicotine equivalent)		3
Sham_HFD	611			3
3R4F_chow	602			3
3R4F_HFD	612			3
ZRH	603			3
ZRH_HFD	613			3

4.3.3 Exposure

Mice will be whole-body exposed to fresh, conditioned air or to the aerosol generated from either the test or reference item: cigarette mainstream smoke of the reference cigarette 3R4F at concentrations of 600 µg TPM/l or to aerosol from ZRH to matched nicotine levels (relative to 3R4F), 3 hours per day, 5 days/week for between 1 and 8 months, depending on the necropsy timepoint (Table 5, Figure 3). The mice that are scheduled to be dissected must be exposed at least two consecutive days before necropsy (therefore animals dissected on Monday or Tuesday will undergo a weekend exposure prior to dissection).

The exposure phase will begin with an adaptation period lasting for 6 days: target 3R4F TPM concentration will be 150 µg/l on study days 1 - 2, 300 µg/l on study days 3 - 4, and 450 µg/l on study days 5 - 6. From study day 7 and

onwards, the target concentration will be 600 μg/l (nicotine equivalent). The equivalent nicotine levels will be used for the adaptation period for the ZRH-exposed mice. During daily administration, animals will be exposed to filtered fresh air for 30 minutes after completing the first 1 hour of mainstream smoke exposure, and for 60 minutes after completing the second exposure block. This is to control the increasing carboxyhaemoglobin (HbCO) levels which will accumulate in the mice during the high 3R4F exposure (Figure 4).

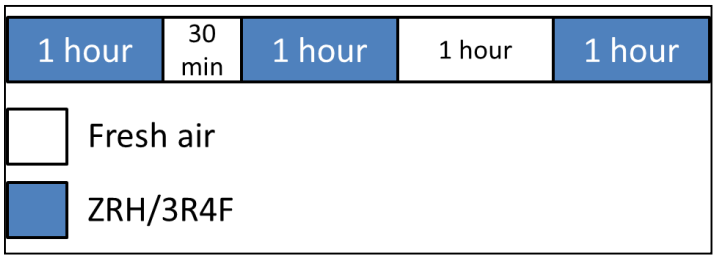


Figure 4. **Exposure regime.** Mice will be exposed a total of 3 hours per day, but with fresh air 'breaks' during the exposure period.

Food and cage enrichment will be removed during exposure, but the animals will continue to have free access to water. The position of the cages within the exposure chamber will be changed once per week (during the cage change) according to a rotation schedule. The cages will be taken out from exposure chambers after exposure and put back on racks.

At the first month of the study, a total of 7 inhalation chambers will be used to expose all of the groups. As the animal numbers decrease while the study is progressing, cages will be systematically removed and exposure chambers will be removed from the study when no longer required (Table 6, Table 7, and Table 8).

Table 6 **Total number of animals exposed/housed by month**

	Study month							
Mice	1	2	3	4	5	6	7	8
sham	257	209	309	184	184	184	92	92
3R4F	531	485	171	92	92	92	46	46
ZRH	255	209	309	184	184	184	92	92
Unexposed (reserve)	71	65	0	0	0	0	0	0
total	1114	903	789	460	460	460	230	230

Table 7 **Total number of cages of mice exposed/housed by month**

	Study month							
Cages	1	2	3	4	5	6	7	8
sham	34	28	41	24	24	24	12	12
3R4F	70	64	23	12	12	12	6	6
ZRH	34	28	41	24	24	24	12	12
Unexposed (reserve)	16	15	0	0	0	0	0	0
total	138	120	105	60	60	60	30	30

Table 8 **Total number of chambers used by month**

	Study month							
Chambers	1	2	3	4	5	6	7	8
sham	2	2	2	1	1	1	1	1
3R4F	3	3	1	1	1	1	1	1
ZRH	2	2	2	1	1	1	1	1
total	7	7	5	3	3	3	3	3

Table 9 **Exposure chambers and numbering**

Short Name	Chamber #
SHAM1	1
SHAM2	2
3R4F1	3
3R4F2	4
3R4F3	5
ZRH1	6
ZRH2	7

4.4 Procedures

4.4.1 Parameters to Monitor General Condition and Health

General condition and health of the mice and smoke uptake/exposure will be monitored throughout the study (Table 10). This includes body weight measurements, which will be performed twice per week, as well as group and individual observations. Animals with identified ailments or injuries will be tracked daily on a special observations list until they are fully recovered or removed from the study.

Table 10 **General condition and health**

Parameter	Method	Frequency (ZRH-ApoE switching)	Frequency (Insulin Resistance)	Number of Mice	Remarks
[i]Body weight	Balance	1 time/week	1 time/week	All mice	Individually
In life observations	Group observations	Daily	Daily	All mice	After exposure
	Individual observations according to checklist	Daily	Daily	2 cages per group per day	
Food consumption	Gravimetry	[ii]At discretion of SD	Weekly	All groups	NA
Mortality	Observation	Daily	Daily	all mice	-

[i]: Note, body weight frequency may be increased at the discretion of the SD in order to track the health status of the animal colony.

[ii]: Food consumption is not planned for the ZRH ApoE switching groups, but may be requested based on the body weight development of the groups over time

4.4.2 Parameters to Monitor Smoke Uptake and Exposure

In addition to the aforementioned analysis of the aerosols in the breathing zone of the animals uptake of aerosol components will be measured. This is monitored by measuring HbCO levels in the bloodstream immediately after the 3rd exposure block (see Figure 4), as well as by the quantification of the 5 main nicotine metabolites in the urine for an approximately 16 hour period after exposure. Urine will also be assessed for additional biomarkers of exposure in study delegation phase 5.

4.4.2.1 Blood sampling for HbCO determination

The HbCO concentration in the blood and the nicotine metabolites in the urine will be tested three times per exposure group (ZRH ApoE switching) throughout the course of the study (Table 11 and

Table 12). The animals in the HFD groups of the IR arm will be sampled a single time for HbCO levels.

Table 11 **Biomarkers of aerosol exposure**

Parameter	Method	Frequency		Number of Mice per group	Remarks
		ZRH ApoE switching	IR		
Blood HbCO	Spectrophotometric measurement	3 times/study (2nd, 4th and 7th month)	[1]1 time per study, 2nd month. HFD animals only from exposed reserve mice	12 (ZRH ApoE Switching) 8 (IR)	Individually
Urine Nicotine + major metabolites	HPLC	4 times/study (month 2, 3, 6, 8)	NA	8 x 4	groups of 4 mice per metabolic cage, not including exposure period
Urine smoke metabolites	Method to be determined (ABF)	4 times/study (month 2, 3, 6, 8)	NA	8 x 4	Note, same urine sample as nicotine metabolites

[1] As blood collection may impact the glucose and metabolic parameters of the animals, we instead will take from exposed reserve mice fed a high fat diet. The blood collection timing will correspond to the 'ZRH ApoE switching' groups, so that the regular chow animals may be used for comparative purposes.

Table 12 **Blood sampling by month for HbCO analysis**

		Month							
Short Name	**Group Code**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sham_1m	101								
Sham_2m	102		12						
Sham_3m	103								
Sham_6m	106				12				
Sham_8m	108							12	
3R4F_1m	201								
3R4F_2m	202		12						
3R4F_3m	203								
3R4F_6m	206				12				
3R4F_8m	208							12	
ZRH_1m	301								
ZRH_2m	302		12						
ZRH_3m	303								
ZRH_6m	306				12				
ZRH_8m	308							12	
Cessation_3m	403								
Cessation_6m	406				12				
Cessation_8m	408							12	
Switching_3m	503								
Switching_6m	506				12				
Switching_8m	508							12	
Sham_chow	601								
Sham_HFD	611		8						
3R4F_chow	602								
3R4F_HFD	612		8						
ZRH_chow	603								
ZRH_HFD	613		8						

4.4.2.2 Urine sampling for Nicotine and exposure metabolites determination

Urine samples will be collected overnight after exposure (Table 13), split into 2 samples and stored ≤ -70°C. One of the urine samples will be used for the analysis of nicotine metabolites in urine three times per group by the method of HPLC after 1,3 diethyl-2-thiobarbituric acid derivatization at PMIRL-S. These sampling timepoints were selected in order to bracket the 2 month switching timepoint (month 2 and 3) as well as to test the metabolites levels at later timepoints (month 6 and 8) to demonstrate consistency in test aerosol uptake in different points of the study.

Additional biomarkers of aerosol exposure will be performed at test site 5 (ABF), where the following biomarker analysis will be performed:

i) HPMA (hydroxypropylmercuric acid)

ii) NNAL (total) ((4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)

iii) SPMA (S-phenylmercapturic acid)

iv) CEMA (2-cyanoethylmercapturic acid)

v) 4-HNE (4-hydroxynonenal)

vi) MDA (Malondialdehyde)

vii) 8-OH-dG (8-hydroxy-2'-deoxyguanosine)

viii) Eicosanoids:
- Tetranor PGE-M
- 2,3-dinor-8-iso-PGF2a
- 8-iso-PGF2a
- 2,3-dinor-TBX2
- 11-dehydro-TBX2
- 12-HETE

Table 13 **Urine Sample Collection**

		Month							
Short Name	**Group Code**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sham_1m	101								
Sham_2m	102		8 x 4						
Sham_3m	103			8 x 4					
Sham_6m	106						8 x 4		
Sham_8m	108								8 x 4
3R4F_1m	201								
3R4F_2m	202		8 x 4						
3R4F_3m	203			8 x 4					
3R4F_6m	206						8 x 4		
3R4F_8m	208								8 x 4
ZRH_1m	301								
ZRH_2m	302		8 x 4						
ZRH_3m	303			8 x 4					
ZRH_6m	306						8 x 4		
ZRH_8m	308								8 x 4
Cessation_1m	403			8 x 4					
Cessation_4m	406						8 x 4		
Cessation_6m	408								8 x 4
Switching_1m	503			8 x 4					
Switching_4m	506						8 x 4		
Switching_6m	508								8 x 4

Remarks: 8 x 4 means 8 metabolic cages with 4 mice per cage

4.4.3 In life assessment of insulin resistance

Limited parameters will be monitored during the in life phase of the 'insulin resistance' groups (601 - 613). This will include the collection of blood samples after 6 hours fasting period (but not more than 7 hours fasting).

Table 14 **Parameters to monitor insulin resistance**

Parameter	Method	Minimum Volume	Frequency	Number of Mice per group	Remarks
Blood glucose (I)	Enzymatic detection (glucometer)	10 µl, whole blood	3 times per study, + pre-exposure baseline	≥10	6 hours fasting, Target 120 µl blood collected
Insulin	Insulin Elisa	20 µl, plasma			
HbA1C	HbA1C Elisa	20 µl, plasma			

4.4.4 Necropsy

The endpoints of the study will be oriented towards the examination of endpoints related to inflammation state, development and progression of emphysema/COPD, atherosclerosis, and IR. This will logistically be performed by splitting the animals into 6 dissection streams (A through F), depicted in Table 15. The details of each dissection stream, and the methods utilized will follow. Note, due to the length of the study, necropsies will be performed as 28 day month cycles, in order to standardize the timing between the dissection endpoints.

Table 15 **Number of Animals From Each Exposure Group, and Per Necropsy Phase**

Short Name	Group Code	A (BALF)	B (Histo-pathology)	C (Lung Function)	D (Omics)	E Vascular Inflammation/platelet	F IR	Total mice
Sham_1m	1	10	10	10	8	10		48
Sham_2m	2	10	10	10	8			38
Sham_3m	3	10	10	10	8	8		46
Sham_6m	6	10	10	10	8	8		46
Sham_8m	8	10	10	10	8			38
3R4F_1m	101	10	10	10	8	8		46
3R4F_2m	102	10	10	10	8			38
3R4F_3m	103	10	10	10	8	8		46
3R4F_6m	106	10	10	10	8	8		46
3R4F_8m	108	10	10	10	8			38
ZRH_1m	201	10	10	10	8	8		46
ZRH_2m	202	10	10	10	8			38
ZRH_3m	203	10	10	10	8	8		46
ZRH_6m	206	10	10	10	8	8		46
ZRH_8m	208	10	10	10	8	8		46
Cessation_1m	303	10	10	10	8	8		46
Cessation_4m	306	10	10	10	8			38
Cessation_6m	308	10	10	10	8			38
Switching_1m	403G	10	10	10	8	8		46
Switching_4m	406G	10	10	10	8	8		46
Switching_6m	408G	10	10	10	8			38
Sham_chow	601						10	10
Sham_HFD	611						15	15
3R4F_chow	602						10	10
3R4F_HFD	612						15	15
ZRH_chow	603						10	10
ZRH_HFD	613						15	15
Total		210	210	210	168	106	75	979

The endpoint necropsy consists of 6 separate dissection streams. The tissues and methods will be performed as follows:

A) BALF (10 mice per group)

- Free lung cells – Quantification and differential count (section 4.4.5)
- BALF – Inflammatory mediators (study phase 4, Myriad/Rules-based Medicine, RodentMAP analysis)
- BALF – MMP activity
- Aortic arch – Plaque size (section 4.4.8.4)

- Thoracic aorta - Histopathology (paraffin embedding)
- Blood – White blood cell counts (Sysmex)
- Serum – Serum inflammatory mediators (study phase 4, Myriad/Rules-based Medicine, RodentMAP analysis)

B) Histopathology (10 mice per group)

- Left lung – Histopathology/stereological assessment/immunohistochemistry (section 4.4.10, 4.4.12)
- Right lung (all lobes) – Histopathology/morphometry back-up
- Liver (left lobe) – Histopathology (H&E, PAS, PASD, IHC (4.4.12))
- Liver (left lobe) – Histopathology (cryosections; Oil Red O)
- Nose (3 levels) – Histopathology (H&E, AB-PAS)
- Blood – Glucose, HDL, LDL, VLDL (Singapore) (section 4.4.8)
- Plasma – Oxysterols (section 4.4.8.1)

C) Lung Function (10 mice per group)

- Lung function analysis (section 4.4.9)
- Serum – Proteomics (section 4.4.7.4)

D) Omics (8 mice per group)

- Aortic arch – Plaque size (section 4.4.8.4)
- Nasal epithelium – Transcriptomics (left and right nostril) (4.4.6)
- Lung (left) – Transcriptomics/genomics
- Lung (right lung c) – Lipidomics
- Lung (right lung a and b) - Proteomics
- Liver (left lobe) – Transcriptomics
- Liver – Genomics
- Liver - Proteomics
- Liver (left lobe) – Lipidomics
- Liver (left lobe) – Histopathology – (cryosection Oil red O)
- Liver (left lobe) – Histopathology (H&E, PAS, PASD, IHC (4.4.12))
- Liver (remaining lobes) - back-up tissue (frozen)
- Heart (left ventricle, apex) – Storage (frozen)
- Thoracic aorta – Transcriptomics,
- Abdominal aorta – Lipidomics (test site 3)
- Blood – Transcriptomics
- Blood - Genomics
- Plasma – Lipidomics(test site 3)
- Serum – Proteomics

E) Vascular inflammation and platelet aggregation

- Aortic arch and descending aorta – Cell dissociation/FACS (section 4.4.13)
- Blood – Platelet aggregation/FACS (section 4.4.14)

F) IR endpoints (10 to 15 mice per group)

- Mesenteric fat – Transcriptomics

- Liver (left lobe) –Histopathology (H&E, PAS, PASD)
- Liver (left lobe) – Histopathology (cryosections, oil red O)
- Liver (left lobe) – Transcriptomics
- Liver (left lobe) – Protein (Western blotting for validation of biomarkers)
- Liver (left lobe) - Lipidomics
- Pancreas – Histopathology (H&E)
- Blood – Glucose, HDL, LDL, VLDL (Singapore)
- Plasma – Lipidomics (Zora Biosciences)
- Plasma – Metabolic MAP (study phase 4, Myriad/Rules-based Medicine, Metabolic MAP analysis)
- Muscle (soleus and gastrocnemius as single tissue piece) – Transcriptomics
- Aortic arch – Morphometric evaluation of total plaque area
- Thoracic aorta – Transcriptomics
- Heart (left ventricle, apex) – Transcriptomics

Table 16. Necropsy and endpoint details

Tissue	Dissection group	Target	Application	Location
Free lung cells	A	Inflammation	Differential count	Singapore
Free lung cells	A	Inflammation	Viability	Singapore
BALF (cell free)	A	Inflammation	Inflammatory mediators	Myriad/Rules-based Medicine
BALF (cell free)	A	Back-up	Back-up	Back-up
BALF (cell free)	A	Inflammation	MMP activity	Singapore
Aortic arch	A	Lipidomics	Plaque size	Singapore
Thoracic aorta	A	Histopathology	Immunohistochemistry (paraffin)	Singapore
Blood	A	Haematology	complete blood cell counts	Singapore
Serum	A	Inflammation	Inflammatory mediators	Myriad/Rules-based Medicine
Lung (left)	B	Histopathology	Morphometry and pathology; immunhistochemistry	Singapore
Lung (right)	B	Histopathology	Back-up	Singapore
Nose (3 levels)	B	Histopathology	Histopathology	Singapore
Blood	B	Lipidomics	Lipoprotein analysis	Singapore
Plasma	B	Lipidomics	Oxysterols	Neuchatel
Serum	C	Proteomics	Proteomics	Neuchatel
Aortic arch	D	Lipidomics	Plaques size	Singapore
Nasal epithelium (left)	D	Transcriptomics	Transcriptomics	Neuchatel
Nasal epithelium (right)	D	Transcriptomics	Transcriptomics	Neuchatel
Lung (left)	D	Transcriptomics/genomics	Transcriptomics/genomics	Neuchatel
Lung (right lung a and b)	D	Proteomics	Proteomics	Neuchatel
Lung (right lung c)	D	Lipidomics	Lipidomics	Zora Biosciences
Liver (left lobe 1a)	D	RNA	Transcriptomics	Neuchatel

Table continued

Tissue	Dissection group	Target	Application	Location
Liver (left lobe, 1b)	D	DNA	Genomics	Neuchatel
Liver (left lobe, 1c)	D	Protein	Proteomics	Neuchatel
Liver (left lobe 3)	D	Lipidomics	Lipidomics	Zora Biosciences
Liver (left lobe, 2a)	D	Histolopathology	H&E, PAS, PASD (paraffin)	Singapore
Liver (left lobe 2b)	D	Histopathology	Oil Red O (cryosections)	Singapore
Liver (remaining lobes)	D	Back-up	Back-up	Neuchatel
Heart	D	Back-up	Back-up	Neuchatel
Thoracic aorta	D	Transcriptomics	Transcriptomics	Neuchatel
Abdominal aorta	D	Lipidomics	Lipidomics	Zora Biosciences
Blood	D	Transcriptomics	Transcriptomics	Neuchatel
Blood	D	Genomics	Methylation	Neuchatel
Plasma	D	Lipidomics	Lipidomics	Zora Biosciences
Serum	D	Proteomics	Proteomics	Neuchatel
Aortic arch	E	Inflammation	FACS	Singapore
Blood	E	Platelet aggregation	FACS	Singapore
Mesenteric fat	F	Transcriptomics	Transcriptomics	Neuchatel
Liver (left lobe, 2b)	F	Histopathology	Oil Red O (cryosections)	Singapore/Neuchatel
Liver (left lobe, 2a)	F	Histopathology	H&E, PAS, PASD (paraffin)	Singapore/Neuchatel
Liver (left lobe, 1a)	F	Transcriptomics	Transcriptomics	Neuchatel
Liver (left lobe, 1c)	F	Proteomics	Western blot	Neuchatel
Liver (left lobe, 3)	F	Lipidomics	Lipidomics	Neuchatel
Pancreas	F	Histopathology	H&E (paraffin)	Singapore
Blood	F	Lipidomics	Glucose, HDL/LDL/VLDL	Singapore
Plasma	F	Lipidomics	Lipidomics	Zora Biosciences
Plasma	F	Proteomics	Rodent Metabolic MAP	Myriad/RBM
Muscle (soleus and gastrocnemius combined)	F	Transcriptomics	Transcriptomics	Neuchatel
Aortic arch	F	Lipidomics (Morphometry)	Plaque size	Singapore
Thoracic aorta	F	Transcriptomics	Transcriptomics	Neuchatel
Heart	F	Transcriptomics	Transcriptomics	Neuchatel

4.4.4.1 Partitioning of liver and lungs during dissection

As there are multiple uses for both the liver and lung, the organs will be partitioned as described in Figure 5.

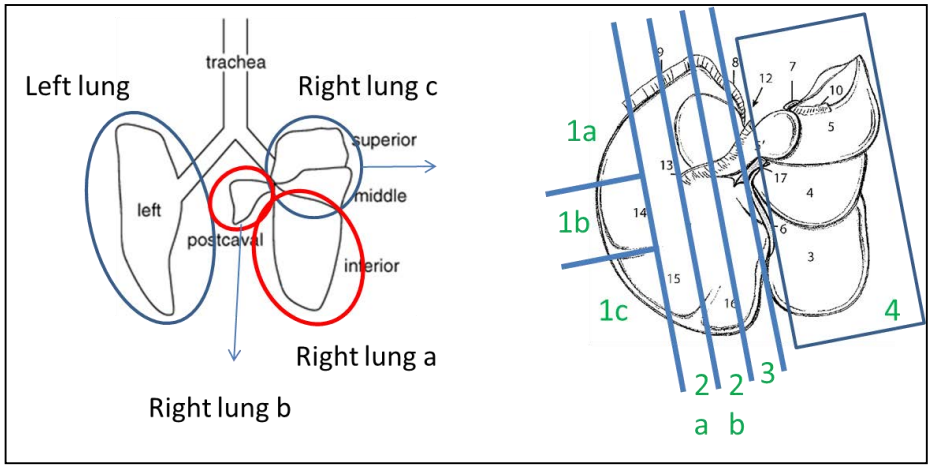


Figure 5. **Liver and lung necropsy for 'omics analysis.** Where described in the necropsy section, lungs may be partitioned into left, and 3 x right lung partitions (lung c: superior & middle; lung a: interior; lung b: postcaval) as depicted (left). The liver will be partitioned into multiple pieces, and described using the numbering system depicted above (right)

4.4.5 Lung inflammation

The parameters listed in Table 17 will be used to assess lung inflammation. These include the analysis of the cellular composition of the BALF (free lung cells), as well as the levels of specific proteins (multi-analyte profile (RodentMAP® v 3.0) – delegated phase 5) or their activities (MMP activity) from the cell-free BALF.

4.4.5.1 Collection of Bronchoalveolar fluid (BALF)

The collection of the BALF will be performed according to standard work instructions (PMI_RD_WKI_000319, 'Prepare lavage samples: Lavage of mouse lung – syringe method'). As there are different downstream processes, the BALF will be split as follows; Cycle 1 will be collected in PBS, and used for protein and enzyme analysis. Cycles 2 – 5 will be collected using PBS + BSA for collection of free lung cells, and processed using flow cytometry.

The BALF supernatant (1st lavage cycle) will be aliquoted into separate tubes for the following applications, in order of priority:

i) RodentMAP 3.0 (300 µl)

ii) MMP activity determination (optimal 150 µl, minimum 120 µl)

iii) Remaining supernatant

The fluid will be stored frozen at ≤ -70 ºC until used for experimentation of shipment on dry ice to the test site (RBM). Any residual supernatant will be stored in a separate tube under the same conditions for potential future analysis.

Table 17 **Lung Inflammation**

Parameter	Biological Material	Method	WKI/SOP #
Collection of free lung cells (FLC)	Lungs of mice	Bronchoalveolar lavage of the lung	'Prepare lavage samples: Lavage of mouse lung – syringe method', PMI_RD_WKI_000319
Determination of cellular viability	BALF (cycles 2 to 5 plus FLC from cycle 1)	Flow cytometric analysis after staining with fluorescein diacetate and propidium iodide	'Perform analysis: Flow cytometric measurement of cell viability with simultaneous determination of cell count by means of BD TruCount beads', PMI_RD_CR_000687
FLC count	BALF (cycles 2 to 5 plus FLC from cycle 1)	Flow cytometrical counting using Trucount® tubes	'Perform analysis: Flow cytometric measurement of cell viability with simultaneous determination of cell count by means of BD TruCount beads', PMI_RD_CR_000687
FLC differentiation	BALF (cycles 2 to 5 plus FLC from cycle 1)	Flow cytometric analysis after staining with cell-type specific antibodies conjugated to fluorochromes	Perform analysis, Flow cytometric differential counting of mouse free lung cells by labeling with CD11c, Ly6G, CD3e, B220 and MHCII. See also Table 18 for revised antibody mixture.
FLC lymphocyte differentiation	BALF (cycles 2 to 5 plus FLC from cycle 1)	Flow cytometric analysis after staining with cell-type specific antibodies conjugated to fluorochromes	'Flow cytometric determination of lymphocyte differentiation in murine bronchoalveolar lavage fluid', PMI_RD_CRI_000229
FLC macrophage activation	BALF (cycles 2 to 5 plus FLC from cycle 1)	Flow cytometric analysis after staining with cell-type specific antibodies conjugated to fluorochromes	'Flow cytometric quantification of activated marker expression in murine alveolar macrophages', PMI_RD_WKI_000648
Inflammatory mediators (Multi-analyte profile)	BALF (cell-free supernatant of cycle 1)	Multiplexed bead array according to the RodentMAP® v 3.0 program	Rules-based medicine (Delegated phase 4)
MMP activity	BALF (cell-free supernatant of cycle 1)	Fluorescence method using fluorescein-conjugated gelatin	'Quantitative determination of matrix metalloproteinase (MMP) activity in rodent bronchoalveolar lavage (BALF)' PMI_RD_WKI_000620.

The BAL cells (FLC, lavage cycles 1, and 2 through 5) will be processed as follows: Cells from cycle 1 will be combined with cycle 2 – 5 for viability and cell quantification. These will then be fixed, and partitioned for each different analytical endpoints (differential count, lymphocyte differentiation, and macrophage activation).

The antibody mixes used will be described in Table 18:

Table 18 **Free lung cell antibody mixes**

Assay	Name	Company	Cat#
Mouse DiffCount	CD11c-APC	Becton Dickinson	550261
	Ly6G (Gr1) - FITC	Becton Dickinson	551460
	CD3 - PE	Becton Dickinson	553064
	B220 - APC - Cy7	Becton Dickinson	552094
	MHCII - PerCP-eFluo710	e-Bioscience	46-4031-82
Lymphocyte differentiation	Ly6G-PE	BD biosciences	553141
	CD4-PerCP	BD biosciences	553052
	CD8a-PE-Cy7	BD biosciences	552877
	CD45R/B220-APC-Cy7	BD biosciences	552094
	CD69-APC	e-Biosciences	17-0691-81
Macrophage activation	CD68-Pe-Cy7	BD Biosciences	553141
	CD54/ICAM-1-PE	BD Biosciences	553253
	CD11b-APC-Cy7	BD Biosciences	557657
	MHCII-PerCP-eFluor710	e-Biosciences	46-4031-82
	[i]Cd11c-APC	Becton Dickinson	550261

[i]: CD11c included as a macrophage and dendritic cell marker in addition to the antibody mix described in the working instruction.

The MMP activity in the BALF supernatants will be determined using a commercially available assay kit which is based on the cleavage of fluorochrome-labeled gelatin by gelatinotytic metalloproteinases (MMP) in BALF (EnzChek® Gelatinase/Collagenase Assay Kit; Invitrogen).

4.4.6 Tissue processing for 'omics analysis (genomics, proteomics, lipidomics)

Tissues for 'Omics analysis (dissection block 'D') will be performed between 16 and 24 hours post exposure. The dissection will be as follows:

i) Anaesthesia – the mice will be anaesthetised using sodium pentobarbital

ii) Blood collection – through the retro-orbital plexus (whole blood for RNA, DNA, plasma for lipids, serum for proteomics)

iii) Perfusion of lung tissue using 0.9% NaCl

iv) Tissue extraction and treatment.

To minimize RNA degradation during the removal process, all surfaces and instruments will be wiped with RNAse away between animals. Tissues will be frozen as quickly as possible, by immersion of the tube in liquid N2, or pouring liquid N2 into the containers.

Note, tissues will be prioritised for time sensitivity. The target is to have the nasal epithelium removed within 15 minutes post-mortem (perfusion time). As all tissues may experience degradation, all tissues should be removed and processed/frozen by 20 minutes post-mortem.

Samples will be stored at ≤-70°C until the completion of the study, after which the samples will be processed.

4.4.7 Blood collection

There are multiple downstream applications requiring blood collection at the necropsy phase, each requiring specific methods of collection and processing.

4.4.7.1 Whole blood for RNA

Blood samples will be collected from the retro-orbital plexus using an uncoated (i.e., no heparin) capillary or PE tube. The blood will be transferred straight to an RNA collection tube of appropriate size for the volume (Qiagen RNA protect animal blood tubes, 100 ul volume planned). The blood must be added up to the 'fill' line on the tubes as closely as possible (avoid overloading the tubes). The tube and blood mixture will be mixed by inversion 10 times, followed by resting overnight (minimum 2 hours) at between 15°C and 25°C. The sample may then be placed at ≤-70°C for storage before further processing or shipment.

4.4.7.2 Whole blood for DNA collection

Blood samples will be collected from the retro-orbital plexus using an uncoated (i.e., no heparin) capillary or PE tube. The blood will be transferred straight into 4 ml EDTA coated tube. The blood will be mixed by inversion 8 times

4.4.7.3 Whole blood for haematology

Blood samples will be collected from the retro-orbital plexus using an uncoated (i.e. no heparin) PE tube. The blood will be collected into EDTA tubes, mixed well, and then placed on a tube rotator until analysis.

4.4.7.4 Serum for proteomics/inflammation

Blood will be collected from the retro-orbital plexus using non-heparinized capillary tubes. The blood will be transferred directly to a serum separator tube (SST) and processed according to our work instructions. Briefly, after centrifugation, the gel will form an interface between the serum and the supernatant. The serum is then transferred to an Eppendorf tube, and frozen at ≤-70°C for storage or shipment. Importantly, there is no heat inactivation as this would denature or inactivate some of the heat labile components in the serum, thereby disrupting the proteomic or lipidomic analysis.

4.4.8 Lipidomics methods

4.4.8.1 Plasma for oxysterols

Plasma will be collected using standard methods. However, immediately after the isolation of the plasma, it will be stabilized with 0.4 µmol/l Probucol and BHT (butyl hydroxy-toluol). The stabilizing solution is prepared by making 4 mM stock solutions of BHT and Probucol (individually) in 100% ethanol. These are then combined 1/100 in ethanol as a 100x stock solution (40 µmol/l). This solution is stable for 1 week when stored at 4°C. The 100x stock solution will then be added to the plasma in a 1/100 dilution (so 1ul stabilizing solution into 100 ul plasma). The samples will then be frozen (freezing on dry ice), then stored at ≤-70°C.

4.4.8.2 Plasma for lipidomics (Delegated phase 3)

Plasma will be isolated according to the recommendations from Zora Biosciences. Blood will be collected into EDTA-plasma tubes, separated as soon as possible after collection, and snap-frozen at -80°C.

4.4.8.3 Oil red o staining

Portions of the liver will be frozen using cryopreservation freezing media, followed by embedding into a Cryomold block. Approximately 10-um thick cryosections will be stained with Oil Red O.

4.4.8.4 Aortic plaque size dissection and area determination

Methods will be described in a study plan amendment

4.4.8.5 Lipoprotein analysis

Methods will be described in a study plan amendment

4.4.9 Lung Function Measurement

All lung function manoeuvres will be performed using equipment (Scireq, Canada). This will be performed according to our standard working instructions, PMI_RD_WKI_000420, 'Perform terminal measurement: Lung function measurement'.

4.4.10 Histoprocessing and Staining

Histological processing of frozen and/or formalin fixed tissues will be performed at PMIRL-S according to internal work instructions. 4-um thick, formalin fixed lung, nose, pancreas, and left liver lobe will be routinely processed and stained with H&E. Additional histochemical staining will be performed for these organs and include; AB-PAS for lung and nose; RF for lung; PAS and PASD for liver; Oil Red O for cryosections of the liver.

Formalin fixed thoracic aortic tissue will be processed and embedded in paraffin blocks.

The left lung lobe will be serially sectioned (4-um thick) at 20 step sections (150-um apart) for an overall assessment of the entire lung lobe.

4.4.11 Histopathology

Histopathological evaluation of the left lung (serial-step sections), nose (3 representative levels), pancreas, and liver will be performed at PMIRL-S according to internal work instructions.

Digitalized (Aperio digital imaging), 4-um serial-step sections of the lung will also be evaluated for pulmonary emphysema. For this measurement, a design-based stereological approach will be used to obtain quantitative data to assess pulmonary emphysema (newCAST, VisioPharm).

4.4.12 Apoptosis/Oxidative Stress in lungs and liver

The occurrence of oxidative stress in the sections of left lung and liver (if tissue available) will be assessed by measuring the level of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OH-dG) by immunohistochemistry. To identify apoptosis in lung cells, sections of left lung of fresh air or cigarette smoke-exposed mice will be subjected to terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay.

4.4.13 Vascular Inflammation

Flow cytometry-based method will be applied to analyze the immune cell composition in aortas of mice exposed to fresh air, 3R4F, ZRH aerosol. In brief, animal will be perfused (with PBS containing heparin) and the aortic arch and the thoracic aorta will be isolated and enzymatically digested according to our standard protocols. After digestion, singe cell suspensions will be labeled with different set of antibodies to differentiate macrophages, neutrophils, dendritic cells, T and B lymphocytes by flow cytometry according to our working instructions.

4.4.14 Platelet aggregation assay

Flow cytometry-based assay will be applied to detect platelet aggregates using whole blood or platelet-reach plasma isolated from mice exposed to fresh air, 3R4F or ZRH aerosol. In brief, two portions of cells from the same donor mouse will be labeled with different fluorochromes of CD9 antibody and, after mixing and upon appropriate stimulation, analyzed for the formation of double-colored aggregates by flow cytometry. This will be performed according to our working instructions.

4.4.15 Insulin resistance (IR)– special methods

4.4.15.1 Longitudinal blood collection (fasting glucose)

Blood/plasma will be collected for assessment of the endpoints described in Table 19. The collection of the blood will be via facial vein bleeding according to our standard working instructions (PMI_RD_WKI_000375) but with the following differences:

i) As inhaled anaesthetics interfere with metabolic parameters, the blood collection will be performed without anaesthetics.

ii) Analgaesics will be administered to the animals, in the form of orally dosed NSAIDS.

Table 19 **Endpoints for longitudinal blood analysis (from 6 – 7 hour fasted animals)**

Primary Endpoint	Method	Timing of the analysis
Whole blood fasting glucose	Glucometer	Immediate in SG
Plasma fasting insulin	ELISA	Outsourced (TBD)
Plasma fasting HbA1C	ELISA	Outsourced (TBD)

4.4.15.1.1 Necropsy – special procedures for insulin resistance study

Procedures for organ extraction will be performed using standard working protocols. The following tissues will also be applied:

- Blood – samples will be collected from the retro-orbital plexus and processed as follows:

Table 20 **Blood samples for metabolic analysis**

Test	Sample	Prefered Volume
Cholestech: Lipid profile (HDL, LDL, VLDL) Glucose	Whole blood	85 uL
RBM (Rat MetabolicMAP v2.0)	Plasma	80 uL
Remainder	Plasma	remainder

- Liver (as described in section 4.4.4) – Liver will be weighed at necropsy, and process for histopathology (H&E, and cryosectioning), protein isolation for future validation work.
- Muscle – soleus + gastrocnemius – the entire muscle complex will be isolated and removed (**Figure 6**). Tissue will be snap-frozen for RNA extraction.

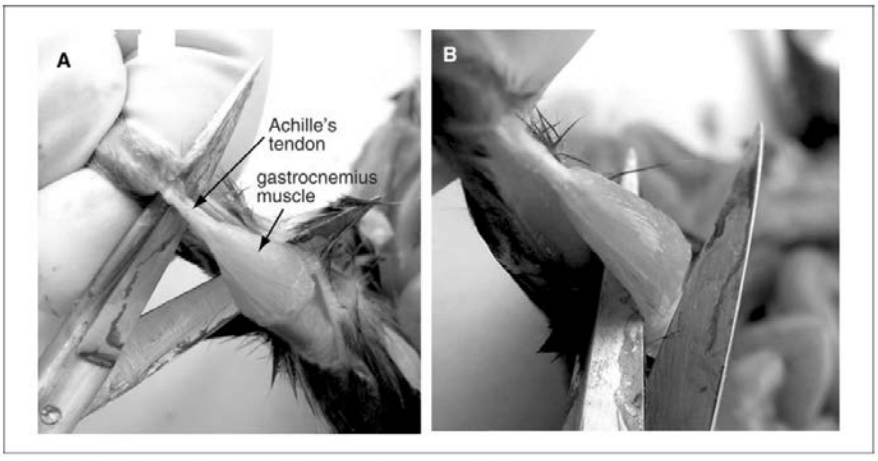


Figure 6. Dissection of gastrocnemius and soleus muscles. Picture taken from : Antal et al. 2007 Tissue collection for systematic phenotyping in the mouse. *Curr. Protoc. Mol. Biol 80:29A.4.1-29A.4.23.*

- Mesenteric fat – This is the fat deposition located within the folds of the intestines (Figure 7). The tissue will be removed and snap frozen in liquid N2 for RNA extraction.

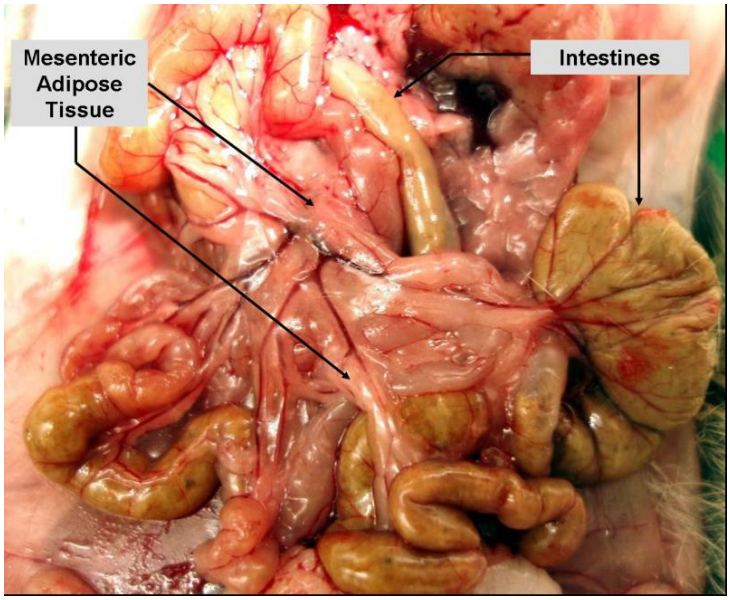


Figure 7. Dissection of mesenteric fat. Picture taken from: http://en.wikipedia.org/wiki/File:MES_Male_1.jpg.

Aortic arch - Atherosclerotic plaque measurements will be conducted in the aortic arch of ApoE mice. The aortic arch will be dissected out, sectioned and cleaned from connective and perivascular fat. All procedures will be conducted under a dissection microscope. The aortic arch will be opened by sectioning the midline of the lesser aortic curvature, from the proximal to the distal end. A short cut from the proximal end of the larger curvature to the bifurcation of the left subclavian artery will be made and the tissue pinned down for microscope image recording. Tissue will then be stained with oil-red O dye (ORO) and another image taken. Plaque area will be determined by planimetry using the ORO-stained images. Procedures for dissection, cutting, image acquisition, ORO staining and planimetric measurements have all been described in detail in WKI documents.

Heart and thoracic aorta - We will also conduct gene expression, transcriptomic analysis of cardiac and aortic tissues. Heart and thoracic aorta will be collected. After the thoracic aorta is excised, a 0.5 cm segment from the proximal end will be sectioned and fixed in paraformaldehyde for paraffin embedding. The rest of the thoracis aorta will be processed for RNA extraction and transcriptomics after the, perivascular adipose and connective tissues are mechanically removed and discarded. This procedure will ensure that only the vascular wall is used for RNA analysis. The apex segment of the heart (left

ventricle), approximately 0.5 cm, will be sectioned transversally and collected in RNAse-free tubes. Both tissues will then be snap-frozen in liquid nitrogen for mRNA extraction and high-throughput hybridization experiments (Affymetrix chips).

5 Statistical Methods

This study is exploratory and there is no formal hypothesis specified a priori. The statistical analysis is then exploratory and no inferential statistical testing will be performed. Nevertheless, 95% confidence intervals and unadjusted p-values will be presented.

The analysis described below will be applied to the biological parameters belonging to the following categories (note that omics data analysis is not covered here and will be described in a separate study plan):

- Lung function measurements
- Histopathology
- Lung inflammation
- Systemic inflammation
- Plaque size
- Oxysterols
- Biomarkers of exposure/oxidative stress/inflammation

Summary statistics will be provided for measurements which do not belong to the above list (test atmosphere characterization, general condition and health, smoke uptake).

5.1 Descriptive Statistics

1) For each treatment group, continuous variables will be presented by showing the number of planned measurements, non-missing and missing observations. When applicable, the number of non-missing observations will further be stratified between values within the limits of quantification, as well as values below the lower limit of quantification (LLOQ) or above the upper limit of quantification (ULOQ). Median, 25 and 75% percentiles, minimum and maximum will be provided for all the groups. In addition, if all the non-missing values of a given group fall within the quantification limits, mean, standard deviation, relative standard deviation, standard error (SE), and 95% confidence intervals will be provided. Graphs will also be provided.

2) For each treatment group, ordinal and categorical variables will be presented by showing the number (and/or percentage) of planned measurements, non-missing observations and missing observations, as well as the number (and/or percentage) of mice falling in each level of the variable of interest (contingency tables). Graphs will also be provided.

5.2 Missing Values

3) Missing values (values < LOD) will be substituted for LOD/2.
4) Values below the LLOQ or above the ULOQ will not be substituted.

5.3 Treatment Comparisons

5) Exploratory comparisons between treatment groups and within timepoints will be performed.

6) For continuous variables, this will be done using a linear mixed model analysis of variance, taking into account:
7) The repeated measurement for an animal (if available),
8) Heterogeniety in covariance structure (allow unequal group variance),
9) Adjustment of degree of freedom.
10) In the presence of value below the limit of detection or deviation from model assumptions, non-parametric methods or data transformation (e.g. Log) will be applied where necessary.

11) For discrete variables:
12) Independent binary data will be analyzed using Fisher exact test
13) Ordinal data will be analyzed using Cochran-Mantel-Haenszel test on row mean score (modified ridit scores)
14) Unordered categorical data with more than 2 levels will be analyzed looking for a general association using the Cochran-Mantel-Haenszel test.
15) Model assumptions will be examined and other non-parametric methods can be applied where necessary.

5.4 Extreme Values

16) If extreme values are identified, they will be discussed with the experimenters. These values will be used in the analysis except if they are clear measurement or manipulation mistakes. In that case, they will be dropped from the analysis and the rationale for excluding these values will be explained in the report.

5.5 Significance Level

17) As the study is exploratory, no specific significance level is required. P-values will be reported as such without considering any adjustment.

6 Administrative Aspects

6.1 Working instructions and SOPs

Title	Document Type	Name (C-Docs)	Version
Manage Envionment, Health Safety & Security	SOP	PMI_RD_SOP_000126	1.0
Implement EHS&S plan : Conduct in offices	WKI	PMI_RD_WKI_000357	1.0
Workplace Incident Reporting	WKI	PMI_RD_WKI_000372	1.0
Implement EHS&S plan : Working clothing / Personal protection outfit	WKI	PMI_RD_WKI_000356	2.0
Implement EHS&S plan : Conduct in labs	WKI	PMI_RD_WKI_000432	5.0
Implement EHS&S plan : Emergency Response Plan (minor change)	WKI	PMI_RD_WKI_000359	3.0
Implement EHS&S plan : Access to restricted area (minor change)	WKI	PMI_RD_WKI_000347	3.0
Implement EHS&S plan : Access to the barrier area (Version jump from 1 to 3 due to withdrawn of WKI in ver 2 stage)	WKI	PMI_RD_WKI_000355	3.0
Implement EHS&S plan : Access to the building	WKI	PMI_RD_WKI_000358	3.0
Implement EHS&S plan : Air handling (HVAC)	WKI	PMI_RD_WKI_000561	2.0
Implement EHS&S plan : Emergency power supply	WKI	PMI_RD_WKI_000354	1.0
Implement EHS&S plan : Manage environmental conditions	WKI	PMI_RD_WKI_000563	5.0
Maintain Water System	WKI	PMI_RD_WKI_000433	2.0
Manage Building Management System	WKI	PMI_RD_WKI_000374	1.0
Implement EHS&S plan : Waste Management	WKI	PMI_RD_WKI_000429	5.0
Manage change control	SOP	PMI_RD_SOP_000154	1.0
Manage lifecycle of laboratory consumables and simple equipment	SOP	PMI_RD_SOP_000127	2.0
Manage lifecycle of complex equipment	SOP	PMI_RD_SOP_000210	2.0
Manage validation	WKI	PMI_RD_WKI_000431	4.0
Manage logbooks & calibration certificates	WKI	PMI_RD_WKI_000360	2.0
Manage Calibration and Maintenance	WKI	PMI_RD_WKI_000567	1.0
Equipment Management	WKI	PMI_RD_WKI_000377	2.0
Installation of software for the SM 2000	WKI	PMI_RD_WKI_000269	1.0
Maintenance and installation SMAR smoking machine	WKI	PMI_RD_WKI_000630	1.0
Maintenance and replacement heatcoils SMAR smoking machine	WKI	PMI_RD_WKI_000631	1.0
Turck Module Configuration	WKI	PMI_RD_WKI_000366	1.0
Maintenance of Zurich Smoking Machine	WKI	PMI_RD_WKI_000800	1.0
Installation of exposure chambers	WKI	PMI_RD_WKI_000260	1.0
Operation and Maintenance of oven and incubator	WKI	PMI_RD_WKI_000583	1.0
Manage microbiology samples	SOP	PMI_RD_SOP_000115	1.0
Screen air conditioning system, using the Air IDEAL sampler	WKI	PMI_RD_WKI_000330	2.0
Screen surfaces	WKI	PMI_RD_WKI_000332	2.0
Screen animal diet	WKI	PMI_RD_WKI_000340	2.0
Screen bedding material	WKI	PMI_RD_WKI_000337	2.0
Screen animal drinking water	WKI	PMI_RD_WKI_000336	3.0
Verify sterilization conditions of the autoclave	WKI	PMI_RD_WKI_000342	3.0
Verify H2O2 disinfection conditions	WKI	PMI_RD_WKI_000344	2.0
Manage area sanitation & pest control	SOP	PMI_RD_SOP_000119	2.0
Sanitization of animal rooms	WKI	PMI_RD_WKI_000334	3.0
Extended sanitation of animal rooms	WKI	PMI_RD_WKI_000427	4.0
Sanitation of corridors, lockers and wash area	WKI	PMI_RD_WKI_000335	4.0
Disinfection of barrier rooms with H2O2	WKI	PMI_RD_WKI_000331	3.0
Perform Pest Control	WKI	PMI_RD_WKI_000430	6.0

Title	Document Type	Name (C-Docs)	Version
Manage material sanitation	SOP	PMI_RD_SOP_000118	2.0
Wash material : Use of the bottle washing and filling station	WKI	PMI_RD_WKI_000339	1.0
Wash material : Use of the cage and rack washer	WKI	PMI_RD_WKI_000341	2.0
Wash material : Use of the cabinet washer	WKI	PMI_RD_WKI_000343	1.0
Autoclave material	WKI	PMI_RD_WKI_000570	2.0
Use of the pass-through box	WKI	PMI_RD_WKI_000345	2.0
Use of the bedding transport system	WKI	PMI_RD_WKI_000365	1.0
Use of the transfer station	WKI	PMI_RD_WKI_000346	2.0
Manage transport	WKI	PMI_RD_WKI_000362	3.0
Handling & Receiving of Deliveries	WKI	PMI_RD_WKI_000764	1.0
Store and Inventorize Equipment, Materials and Consumable	WKI	PMI_RD_WKI_000765	1.0
Manage Specialty Gases	WKI	PMI_RD_WKI_000856	1.0
Sample animal drinking water for chemical parameters	WKI	PMI_RD_WKI_000333	2.0
Key Management	WKI	PMI_RD_WKI_000788	1.0
Manage Chemicals	WKI	PMI_RD_WKI_000564	5.0
Prepare Study	SOP	PMI_RD_SOP_000113	2.0
Content and approval of study plan	WKI	PMI_RD_WKI_000629	4.0
Project Management File (PMF)	WKI	PMI_RD_WKI_000410	3.0
Creation of chronology & time table	WKI	PMI_RD_WKI_000299	1.0
Study plan amendment	WKI	PMI_RD_WKI_000249	3.0
Deviation from study plan, SOP or WKI	WKI	PMI_RD_WKI_000252	2.0
Content, approval and acknowledgment of study report amendment	WKI	PMI_RD_WKI_000573	1.0
Manage Raw Data	SOP	PMI_RD_SOP_000120	3.0
Configuration of data acquisition system Labautomation	WKI	PMI_RD_WKI_000275	1.0
Capture and Process Data	WKI	PMI_RD_WKI_000242	5.0
Manage Samples	SOP	PMI_RD_SOP_000122	1.0
Store and inventorize samples	WKI	PMI_RD_WKI_000246	2.0
Manage Test Sites	SOP	PMI_RD_SOP_000123	1.0
Perform test site selection	WKI	PMI_RD_WKI_000243	1.0
Report and close study	SOP	PMI_RD_SOP_000121	1.0
Termination of a study	WKI	PMI_RD_WKI_000349	1.0
Review study report	WKI	PMI_RD_WKI_000572	2.0
Finalization and approval of study report	WKI	PMI_RD_WKI_000245	2.0
Provantis - Study Protocol Definition	WKI	PMI_RD_WKI_000742	3.0
Provantis Users and libraries administration	WKI	PMI_RD_WKI_000806	1.0
Statistical Analysis with Double Programming	WKI	PMI_RD_WKI_000926	1.0
Perform Statistical Analysis with Provantis template (Minor change)	WKI	PMI_RD_WKI_000924	2.0
Histopathology Workflow	SOP	PMI_RD_SOP_000180	1.0
Gross Trimming	WKI	PMI_RD_WKI_000607	3.0
Use and Maintenance of Tissue Processor	WKI	PMI_RD_WKI_000612	3.0
Decalcification of solid tissue	WKI	PMI_RD_WKI_000678	2.0
Preparation of nasal cavity sections	WKI	PMI_RD_WKI_000677	2.0
Use and maintenance of Embedding Station	WKI	PMI_RD_WKI_000610	3.0
Embedding	WKI	PMI_RD_WKI_000621	3.0
Use and Maintenance of microtome	WKI	PMI_RD_WKI_000616	1.0
Preparation of Step serial sections of Rat or Mouse Lung	WKI	PMI_RD_WKI_000608	1.0
Cutting of paraffin blocks	WKI	PMI_RD_WKI_000609	4.0
Use and Maintenance of Slide Printer	WKI	PMI_RD_WKI_000763	1.0
Preparation of Laryngeal Sections (Rat and Mouse)	WKI	PMI_RD_WKI_000694	1.0
Resorchin Fuchsin Stain	WKI	PMI_RD_WKI_000613	1.0
Alcian Blue-PAS stain	WKI	PMI_RD_WKI_000614	1.0
Haematoxylin and Eosin Stain	WKI	PMI_RD_WKI_000615	1.0
Use and Maintenance of Autostainer Workstation	WKI	PMI_RD_WKI_000658	1.0
Scanning stained slides using Aperio Digital Pathology	WKI	PMI_RD_WKI_000611	1.0
Preparation for Archiving - Histopathology	WKI	PMI_RD_WKI_000659	1.0

Title	Document Type	Name (C-Docs)	Version
Procedure of sending slides and images	WKI	PMI_RD_WKI_000829	1.0
Histopathological Evaluation of Respiratory Tract Organs	WKI	PMI_RD_WKI_000689	1.0
Manage Animals before Exposure	SOP	PMI_RD_SOP_000114	3.0
Manage Animals during Exposure	SOP	PMI_RD_SOP_000155	4.0
Manage Animals during Recovery	SOP	PMI_RD_SOP_000124	1.0
Handle and restraint rats and mice	WKI	PMI_RD_WKI_000324	1.0
Check animals individually	WKI	PMI_RD_WKI_000325	3.0
Perform bodyweight	WKI	PMI_RD_WKI_000305	4.0
Ship animals for health check	WKI	PMI_RD_WKI_000329	1.0
Transfer to dissection	WKI	PMI_RD_WKI_000425	3.0
Handling of moribund animals	WKI	PMI_RD_WKI_000571	2.0
Transfer animals in barrier	WKI	PMI_RD_WKI_000424	5.0
Watering and feeding	WKI	PMI_RD_WKI_000307	3.0
Housing and Cage Change Title in C-Docs: Change cages and Lids	WKI	PMI_RD_WKI_000426	3.0
Accounting of laboratory animals	WKI	PMI_RD_WKI_000315	1.0
Use Provantis to allocate animals to study groups	WKI	PMI_RD_WKI_000735	2.0
Whole-body exposure	WKI	PMI_RD_WKI_000423	3.0
Perform group observations for mice	WKI	PMI_RD_WKI_000311	1.0
Perform individual observations for mice	WKI	PMI_RD_WKI_000313	2.0
Non-terminal testing : Intranasal injection in small rodents	WKI	PMI_RD_WKI_000287	1.0
Oral Gavage: Small Rodents	WKI	PMI_RD_WKI_000622	1.0
Intubation of small rodents	WKI	PMI_RD_WKI_000434	2.0
Perform ophthalmoscopy	WKI	PMI_RD_WKI_000314	2.0
Nose-only exposure	WKI	PMI_RD_WKI_000306	2.0
Observe animals during exposure	WKI	PMI_RD_WKI_000277	2.0
Perform group observations for rats	WKI	PMI_RD_WKI_000310	2.0
Perform individual observations for rats	WKI	PMI_RD_WKI_000312	1.0
Prepare exposure room for next day	WKI	PMI_RD_WKI_000289	2.0
Perform food consumption	WKI	PMI_RD_WKI_000308	1.0
Biological sampling : Urine from rats	WKI	PMI_RD_WKI_000412	3.0
Biological sampling : Urine from mice	WKI	PMI_RD_WKI_000413	2.0
Subcutaneous implantation of a transponder	WKI	PMI_RD_WKI_000327	3.0
Non-terminal testing : Respiratory physiology	WKI	PMI_RD_WKI_000414	3.0
Provantis - In life	WKI	PMI_RD_WKI_000738	3.0
Provantis - Animal Management (CSOR)	WKI	PMI_RD_WKI_000739	1.0
Provantis - Reporting	WKI	PMI_RD_WKI_000741	3.0
Animal Rotation Schedule	WKI	PMI_RD_WKI_000748	2.0
Manage animals in dissection	SOP	PMI_RD_SOP_000125	1.0
Fixate tissues : Fixation solutions	WKI	PMI_RD_WKI_000322	2.0
Fixate tissues : Instillation of lungs	WKI	PMI_RD_WKI_000323	2.0
Prepare samples for micronucleus : Bonemarrow	WKI	PMI_RD_WKI_000316	1.0
Prepare lavage samples : Collection of free lung cells (FLC) from a rat	WKI	PMI_RD_WKI_000318	1.0
Making cytosmears from broncho-alveolar cells (Cytocentrifuge method)	WKI	PMI_RD_WKI_000294	1.0
Prepare lavage samples : Lavage of mouse lung - Syringe method	WKI	PMI_RD_WKI_000319	2.0
Making cytosmears from broncho-alveolar cells (Cytocentrifuge method)	WKI	PMI_RD_WKI_000294	1.0
Perform dissection : Lung perfusion	WKI	PMI_RD_WKI_000304	1.0
Euthenize and dispose animals	WKI	PMI_RD_WKI_000317	4.0
Exsanguinate : Serological health check	WKI	PMI_RD_WKI_000298	2.0
Perform dissection : Histopathological health check	WKI	PMI_RD_WKI_000300	2.0
Perform dissection : Dissection of rat	WKI	PMI_RD_WKI_000301	3.0
Euthenize and dispose animals	WKI	PMI_RD_WKI_000317	4.0
Exsanguinate : Serological health check	WKI	PMI_RD_WKI_000298	2.0
Perform dissection : Histopathological health check	WKI	PMI_RD_WKI_000300	2.0

Title	Document Type	Name (C-Docs)	Version
Perform dissection : Dissection of mouse	WKI	PMI_RD_WKI_000302	2.0
Anesthesize animal : Ketamine anesthesia	WKI	PMI_RD_WKI_000417	2.0
Perform terminal measurement : Lung function measurement	WKI	PMI_RD_WKI_000420	5.0
Blood Collection: Retro-Orbital	WKI	PMI_RD_WKI_000421	2.0
Exsanguinate : Serum and plasma preparation	WKI	PMI_RD_WKI_000625	2.0
Exsanguinate : Blood collection from abdominal aorta or vena cava caudalis	WKI	PMI_RD_WKI_000297	1.0
Blood Collection: Retro-Orbital	WKI	PMI_RD_WKI_000421	2.0
Exsanguinate : Serum and plasma preparation	WKI	PMI_RD_WKI_000625	2.0
Anesthesize animal : Pentobarbital anesthesia	WKI	PMI_RD_WKI_000416	4.0
Non-terminal testing : Intraperitoneal injection in small rodents	WKI	PMI_RD_WKI_000309	2.0
Anesthesize animal : Gas anesthesia	WKI	PMI_RD_WKI_000293	1.0
Biological sampling : HbCO	WKI	PMI_RD_WKI_000568	2.0
Blood Collection: Retro-Orbital	WKI	PMI_RD_WKI_000421	2.0
Blood Collection: Facial Vein	WKI	PMI_RD_WKI_000375	2.0
Blood Collection: Cardiac Puncture	WKI	PMI_RD_WKI_000639	1.0
Blood Collection: Saphenous Vein	WKI	PMI_RD_WKI_000657	1.0
Provantis - Pathology	WKI	PMI_RD_WKI_000747	2.0
Harvest Bone Marrow	WKI	PMI_RD_WKI_000885	1.0
Analytical sampling : Nicotine	WKI	PMI_RD_WKI_000406	3.0
Analytical sampling : Aldehydes	WKI	PMI_RD_WKI_000235	1.0
Calibration of balances	WKI	PMI_RD_WKI_000422	4.0
Check of adjustable pipettes	WKI	PMI_RD_WKI_000364	2.0
Determination of aldehydes	WKI	PMI_RD_WKI_000408	4.0
Definition, Calculation and Use of Quantification and Detection Limits	WKI	PMI_RD_WKI_000247	2.0
Perform analysis : Instrument Control, Data Acquisition and Data Evaluation of Chromatographic Data using Agilent ChemStation	WKI	PMI_RD_WKI_000248	2.0
Perform Analysis: Maintenance of Agilent HPLC and GC system	WKI	PMI_RD_WKI_000373	2.0
Perform analysis : Determination of nicotine in diluted aerosol	WKI	PMI_RD_WKI_000409	5.0
Determination of nicotine in undiluted aerosol	WKI	PMI_RD_WKI_000348	3.0
Determination of Carboxyhaemoglobin in blood with Radiometer NPT 7-series	WKI	PMI_RD_WKI_000250	2.0
Preparation and evaluation of blood smears (Differential Blood Picture)	WKI	PMI_RD_WKI_000296	1.0
Determination of nicotine metabolites in urine	WKI	PMI_RD_WKI_000428	4.0
Perform analysis: Determination of urine parameters by urinalysis	WKI	PMI_RD_WKI_000733	1.0
Perform Analysis: Determination of clotting potential using STA Compact coagulation analyser	WKI	PMI_RD_WKI_000851	1.0
Provantis - Clinical Pathology	WKI	PMI_RD_WKI_000737	2.0
Flow cytometric differential counting of mouse free lung cells (FLC) by labeling with Ly-6G and F4/80	WKI	PMI_RD_WKI_000415	4.0
Perform analysis : Flow cytometric measurement of cell viability with simultaneous determination of cell count by means of BD TruCount Tubes	WKI	PMI_RD_WKI_000419	4.0
Fixate lavage samples : Formaldehyde Fixation of Free Lung Cells (FLC)	WKI	PMI_RD_WKI_000320	1.0
Perform Analysis: Maintenance of BD FacsCanto	WKI	PMI_RD_WKI_000828	1.0
Quantitative Determination of Matrix Metalloproteinase (MMP) Activity in Rodent Bronchoalveolar Lavage Fluid (BALF)	WKI	PMI_RD_WKI_000620	2.0
Use of SDMS-S	WKI	PMI_RD_WKI_000891	1.0
Manual Cell Counting	WKI	PMI_RD_WKI_000890	1.0
Manage Master Schedule	WKI	PMI_RD_WKI_000557	2.0

Title	Document Type	Name (C-Docs)	Version
Competence and Training	BEP	PMI_RD_BEP_000017	1.0
Develop the Qualifications Matrix, Training Catalogue and Annual Training Plan	SOP	PMI_RD_SOP_000157	1.0
Manage Individual Training Plan	SOP	PMI_RD_SOP_000158	2.0
Manage Training	SOP	PMI_RD_SOP_000128	1.0
Manage Training Records and Information	WKI	PMI_RD_WKI_000543	1.0
Manage Training Records	WKI	PMI_RD_WKI_000895	1.0
Manage Resources	SOP	PMI_RD_SOP_000129	1.0
Substitution of personnel, back-up and stand by duty	WKI	PMI_RD_WKI_000558	5.0
Personnel numbers, name, codes and initials	WKI	PMI_RD_WKI_000351	1.0
Supplier Management	SOP	PMI_RD_BEP_000025	1.0
Formulate Needs	SOP	PMI_RD_SOP_000190	1.0
Supplier Selection	SOP	PMI_RD_SOP_000191	1.0
Manage Performance	SOP	PMI_RD_SOP_000192	1.0
Close Out Contract	SOP	PMI_RD_SOP_000193	1.0
Management of Animal Welfare Committee	SOP	PMI_RD_SOP_000132	1.0
Management of IACUC	SOP	PMI_RD_SOP_000131	1.0
Functions of IACUC	WKI	PMI_RD_WKI_000321	3.0
Animal Care and Use Program	WKI	PMI_RD_WKI_000353	3.0
Manage Aerosol Generation	SOP	PMI_RD_SOP_000117	1.0
Determination and adjustment of flow rate	WKI	PMI_RD_WKI_000228	1.0
Calibration and use of Venturi tubes	WKI	PMI_RD_WKI_000229	1.0
Determination of static leak test	WKI	PMI_RD_WKI_000236	1.0
Determination of dynamic leak test	WKI	PMI_RD_WKI_000237	1.0
Set-up of smoke pump	WKI	PMI_RD_WKI_000262	1.0
Determination of puff volume	WKI	PMI_RD_WKI_000232	2.0
Generation of aerosol with a negative pressure smoking machine	WKI	PMI_RD_WKI_000230	1.0
Determination of positive pressure of smoking machine	WKI	PMI_RD_WKI_000264	1.0
Determination of insertor depth	WKI	PMI_RD_WKI_000263	1.0
Adjustment of smoking machine ignition lamp	WKI	PMI_RD_WKI_000231	1.0
Determination of smoke parameters : Butt length	WKI	PMI_RD_WKI_000224	1.0
Determination of smoke parameters : Puff count	WKI	PMI_RD_WKI_000225	1.0
Determination of smoke parameters : Static burning rate	WKI	PMI_RD_WKI_000226	3.0
Cleaning of the smoking machines	WKI	PMI_RD_WKI_000599	1.0
Set-up of the smoking machine	WKI	PMI_RD_WKI_000261	1.0
Check chamber pressure	WKI	PMI_RD_WKI_000303	2.0
Arrange exposure chamber	WKI	PMI_RD_WKI_000227	1.0
Leak rate determination of exposure set-up	WKI	PMI_RD_WKI_000259	1.0
Analytical sampling : CO	WKI	PMI_RD_WKI_000278	1.0
Determination of CO concentration	WKI	PMI_RD_WKI_000233	1.0
Calibration of CO meters	WKI	PMI_RD_WKI_000273	2.0
Sampling and determination of TPM in diluted aerosol	WKI	PMI_RD_WKI_000234	1.0
Determination of smoke parameters in undiluted aerosol : TPM	WKI	PMI_RD_WKI_000598	2.0
Dataflow in Citect	WKI	PMI_RD_WKI_000590	2.0
Provantis - Data Import	WKI	PMI_RD_WKI_000841	2.0
Engineering of DIOS	WKI	PMI_RD_WKI_000363	1.0
Configuration of data acquisition system Citect	WKI	PMI_RD_WKI_000270	1.0
Perform ultrasonic cleaning	WKI	PMI_RD_WKI_000338	2.0
Determination of Climate conditions	WKI	PMI_RD_WKI_000569	3.0
Determination of the relative humidity of the test atmosphere	WKI	PMI_RD_WKI_000239	1.0
Determination of the temperature of the test atmosphere	WKI	PMI_RD_WKI_000238	1.0
Calibration of relative humidity sensors	WKI	PMI_RD_WKI_000272	2.0
Calibration of temperature sensors	WKI	PMI_RD_WKI_000271	2.0
Calibration of pressure transmitters	WKI	PMI_RD_WKI_000274	1.0
Generation of Aerosol from Nebulizer	WKI	PMI_RD_WKI_000827	1.0

Title	Document Type	Name (C-Docs)	Version
Installation of Zurich Smoking Machine	WKI	PMI_RD_WKI_000700	1.0
Generation of Aerosol with Zurich Smoking Machine	WKI	PMI_RD_WKI_000701	2.0
Cleaning of Zurich Smoking Machine	WKI	PMI_RD_WKI_000803	1.0
Droplet Size Determination of Aerosol using PIXE	WKI	PMI_RD_WKI_000848	1.0
Manage Test Items	SOP	PMI_RD_SOP_000116	3.0
Store & inventorize items : Handling of test and reference items	WKI	PMI_RD_WKI_000218	5.0
Store & inventorize items : Cigarette stock management	WKI	PMI_RD_WKI_000219	3.0
Store & inventorize items : Access to test and reference items and samples	WKI	PMI_RD_WKI_000217	1.0
Condition test item : Conventional cigarettes	WKI	PMI_RD_WKI_000223	1.0
Condition test item : Unpacking of cigarettes	WKI	PMI_RD_WKI_000222	1.0
Dispose test items	WKI	PMI_RD_WKI_000220	2.0
Archive Test Items	WKI	PMI_RD_WKI_000221	3.0
PMI R & D Quality Manual	QMA	PMI_RD_QMA_000002	1.0
Operational Quality Manual (OQM)	BEP	PMI_RD_BEP_000014	3.0
Collect Raw Data on Forms (work records)	WKI	PMI_RD_WKI_000240	3.0
Manage archiving	WKI	PMI_RD_WKI_000559	6.0
Storage and disposal of Paper Documents	WKI	PMI_RD_WKI_000361	1.0
OmniRim System - Records and Information Management	WKI	PMI_RD_WKI_000376	1.0
Manage Communications with IS Group	WKI	PMI_RD_WKI_000371	3.0
Internal audit	SOP	PMI_RD_SOP_000134	1.0
External audits	SOP	PMI_RD_SOP_000135	2.0
Plan and perform study- and process-based inspections	WKI	PMI_RD_WKI_000350	1.0
Verify study plan	WKI	PMI_RD_WKI_000411	2.0
Perform QA check on study report	WKI	PMI_RD_WKI_000244	2.0
Document Management	BEP	PMI_RD_BEP_000005	5.0
Manage Quality Documents	SOP	PMI_RD_SOP_000039	6.0
Manage non-conformances & incidents	SOP	PMI_RD_SOP_000156	2.0
Manage SOPs and WKIs and Forms	WKI	PMI_RD_WKI_000216	4.0
Quality Document Change Control	WKI	PMI_RD_WKI_000213	2.0

6.2 Study Timelines

Anticipated arrival of animals:	16 August 2013
Proposed starting date of exposure:	02 September 2013
Proposed end of exposure:	15 May 2014
Proposed experimental completion date:	25 October 2014
Proposed start of delegated phases 1 at PMIRL, Neuchatel (Transcriptomics/Genomics)	01 June 2014
Proposed start of delegated phases 2 at PMIRL, Neuchatel (Proteomics)	01 June 2014
Proposed start of delegated phases 3 at PMIRL, Neuchatel (Lipidomics)	01 June 2014
Proposed start of delegated phases 4 at Myriad/Rules-based Medicine:	01 November 2013
Proposed start of delegated phase 5 at ABF:	01 December 2013
Proposed end of delegated phase 1 at PMIRL, Neuchatel (Transcriptomics/Genomics)	Defined in separate study plan
Proposed end of delegated phase 2 at PMIRL, Neuchatel (Proteomics)	Defined in separate study plan
Proposed end of delegated phase 3 at PMIRL, Neuchatel (Lipidomics)	Defined in separate Business case/study plan
Proposed end of delegated phase 4 at Myriad/Rules-based Medicine:	1 July 2014
Proposed end of delegated phase 5 at ABF:	1 July 2014
Proposed date for final study report (Exposure parameters):	31 December 2014

6.3 Names and Addresses

Sponsor	Peitsch, Manuel VP Biological Systems Research PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland
Test Facility	Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall Singapore Science Park II Singapore 117406
Test Facility Management	Boll, Yin. Manager Support & Technology Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall, Singapore Science Park II, Singapore 117406 Tel: +65-66314524 Fax: +65 64130948 Email: Yin.Boll@pmi.com
Study Director	Phillips, Blaine Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall Singapore Science Park II, Singapore 117406 Tel.: +65-6631-4510 Fax: +65 64130948 Email: Blaine.Phillips@pmi.com
Deputy Study Director	Alberto Oviedo Philip Morris International Research Laboratories Pte Ltd 50 Science Park Road, #02-07 The Kendall Singapore Science Park II, Singapore 117406 Tel.: +65-6631-4591 Fax: +65 64130948 Email: Alberto.Oviedo@pmi.com

Test site for delegated phase 1, 2, and 3	PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland
Principle investigator for delegated phase 1 (transcriptomics/genomics)	Marja Talikka PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland Tel: +41-582422677 Fax: +41(0)58 242 28 11 e-mail: Marja.Talikka@pmi.com
Principle investigator for delegated phase 2 (Proteomics)	Fabio Talamo PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland Tel: +41-582422065 Fax: +41(0)58 242 28 11 e-mail: Fabio.Talamo@pmi.com
Delegated phase 3 (Lipidomics)	Stephanie Boue PHILIP MORRIS INTERNATIONAL CH-2000 Neuchâtel, Switzerland Tel: +41-582422243 Fax: +41(0)58 242 28 11 e-mail: Stephanie.Boue@pmi.com
Delegated phase 4 (Inflammatory mediators)	Ashley Garrett Rules based medicine, Inc 3300 Duval Road Austin Texas. 78759 USA Tel: +1-512-835-8-26 (ext 336) Fax: +1-512-835-4687 E-mail: ashley.garrett@rulesbasedmedicine.com
Delegated phase 5 (Urinary metabolites)	Gerhard Scherer Analytisch-Biologisches Forschungslabor (ABF) Goethestrasse 20 80336 Muenchen Germany

6.4 Multi-Site Arrangements

This study includes analytical endpoints at both Singapore (PMIRL-S) and Neuchatel (PMIRL), and select phases outsourced to external contract research organization. The separation of activities is as follows:

1) PMIRL-S (Singapore)
- Aerosol generation and dilution
- Animal housing and husbandry
- Blood carboxyhaemoglobin
- Urinary nicotine metabolites
- Dissection and necropsy
- Collection of BALF and cells:
  - free lung cell count, viability and differential cell count
  - MMP activity in BALF fluid
- Tissue collection for 'omics analysis
- Histotechnical processing of tissue samples
- Pathological assessment of tissue samples
- Shipment and distribution of samples to test sites
- Blood collection for insulin resistance
- Glucose and cholesterol measurements

2) PMIRL (Neuchatel)
- Genomics/transcriptomics analysis (delegated phase 1)
- Proteomics (delegated phase 2)
- Lipidomics (delegated phase 3 – outsourced)

3) Rules-based Medicine (delegated phase 4)
- Multi-analyte profile of BALF fluid and plasma/serum (raw data provided to test facility)

4) ABF (delegated phase 5)
- Biomarkers of exposure from urine samples (raw data provided to test facility)

5) Test site to be determined
- Insulin and HbA1c analysis from plasma

Storage and shipment of the tissues depends on the nature of the experimentation. Free lung cells will be processed within 48 hours of collection, while the remaining BALF fluid will be frozen and then shipped (dry ice) to Rules Based Medicine. Tissue samples will be frozen until the end of the study, after which they will be processed to either RNA, DNA or protein – these products can then be split, and stored long-term (**section 7**), or shipped to test sites on request from the PIs. Histology samples including liver and pancreas will be fixed in 4% formaldehyde and processed within two weeks post-fixation. Paraffin blocks and all downstream histotechnical products are stable at room temperature indefinitely.

7 Archiving

The study plan, PMF, raw data (from procedures performed in Singapore only), copies of phase reports, the reference item (60 sticks, 3R4F), the test item (60 sticks per batch, ZRH) and the final report will be stored at the test facility for a period of up to 2 years after the study completion date. Thereafter, they will be transferred for long-term storage at an external GLP-certified archive center (Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany). Electronic records will be stored on the 'electronic secure storage share' at Philip Morris Research Laboratories, Singapore (#02-07 Science Park Road, Singapore, 117406). Electronic and paper records will be stored for at least 15 years after study completion date according to local archiving SOPs and WKIs. Histopathological samples will be processed to paraffin blocks within 2 weeks of collection. The blocks will be stored at the test facility (room temperature) for up to 2 years after the study completion date, thereafter, they will be transferred for long-term storage at an external GLP-certified archive center (Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany). Tissue samples will be frozen and stored (≤-70°C) after collection, and will be processed within 2 year of collection, or sent for external storage (site and storage period to be determined, and will be defined in a study plan amendment). RNA, DNA and protein will similarly be stored locally at ≤-70°C before long-term storage (site and storage period to be determined, and will be defined in a study plan amendment).

At the test sites of delegated phases, all raw data of the delegated phases, remainders of the specimen sent to the test site, original phase reports and all delegated phase-related records will be stored at the test site for a maximal period of one year after the finalization of the study, thereafter these records and materials will be transferred to the external GLP-certified service archive Iron Mountain Deutschland GmbH, Harpeer Hellweg, 4408 Bochum Germany.

8 References

Study 13882. Impact of smoking cessation and switching to NGP in ApoE -/- mice. Study Director: an Berges.2011.

Health Canada: T-115, Determination of "Tar", Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, 1999

National Advisory Committee for Laboratory Animal Research (NACLAR), Guidelines on the care and use of animals for scientific purposes. Singapore, 2004

National Toxicology Program (NTP), Specifications for the conduct of studies to evaluate the toxic and carcinogenic potential of chemical, biological and physical agents in laboratory animals for the national toxicology program, Research Triangle Park, NC: National Toxicology Program, 1991, 1992 and Attachment 2, revised in September 2000

University of Kentucky,
Reference Cigarette Program,
College of Agriculture,
www.ca.uky.edu/refcig

9 Abbreviations

Abbreviation	
AB-PAS	Alcian Blue Periodic Acid-Schiff
ApoE	Apolipoprotein E
AVA	Agri-Food & Veterinary Authority of Singapore
BA	Bronchioalveolar Attachments
BALF	Broncho-Alveolar Lavage Fluid
BHT	Butyl hydroxyl-toluol
BSA	Bovine Serum Albumin
CO	Carbon Monoxide
COPD	Chronic Obstructive Pulmonary Disease
CVD	Cardiovascular disease
CW	Calendar Week
DI	Destructive Index
DNA	Deoxyribonucleic Acid
DNPH	2,4-dinitrophenylhydrazine
DUC	Document Unique Code
EDTA	Ethylene diaminetetraacetic acid
EPA	Environmental Protection Agency (USA)
FACS	Flow Cytometry
FDA	Food and Drug Administration
FLC	Free Lung Cells
FPD	Functional Product Design (ZRH stick holder)
GLP	Good Laboratory Practice
H&E stain	Haematoxylin and Eosin stain
HDL	High Density Lipoprotein

HFD	High Fat Diet
HbCO	Carboxyhemoglobin
HPLC	High-Performance Liquid Chromatography
IR	Insulin Resistance
ISO	International Organization for Standardization
Lm	Mean Cord Length
LDL	Low Density Lipoprotein
MMP	Matrix Metalloproteinase
MRTP	Modified Risk Tobacco Product
NA	Not Applicable
NACLAR	National Advisory Committee for Laboratory Animal Research
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
ORO	Oil Red O
p-value (p)	Probability of receiving the value of the test statistic or a more extreme value of the test statistic, if the null hypothesis holds
PE tube	Pressure Equalizer tube
PBS	Phosphate Buffered Saline
PDSP	Programmable Dual-port Syringe Pump
PMF	Project Management Folder
PMIRL-S	Philip Morris International Research Lab in Singapore
PMRL	Philip Morris Research Laboratories
PMI	Philip Morris International
PV	Performance Verification
PUC	Project Unique Code
QMS	Quality Management System
RBM	Rules-Based Medicine
RNA	Ribonucleic Acid

SD	Study Director
SOP	Standard Operating Procedure
SST	Serum Separator
TBD	To Be Determined
TPM	Total Particulate Matter
VLDL	Very Low Density Lipoprotein
WBEC	Whole Body Exposure Chamber
WKI	Work Instruction
ZRH	Zurich

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

10 Appendix

NA

End of Document