STUDY PLAN 150083

90 DAYS OECD (+) ZURICH STUDY PROTEOMICS APPROACH

Program Name	SBAV
Project Name	Animal Clinic
PUC Number	
Work Package Name	OECD Zurich - Proteomics
Study Directors	Ashraf Elamin
Author(s)	Ashraf Elamin, Thomas Schneider and Sophie Dijon
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Table of Contents

Table of Contents 2
1 Review and Approval 3
2 Introduction 3
3 Objectives 4
3.1 Primary Objective. 4
3.2 Secondary Objective. 4
4 Experimental Design 4
4.1 Test and Reference Items. 4
4.2 Test System. 4
4.3 Experimental Groups and Dosing. 4
4.4 Samples to be analyzed by iTRAQ approaches. 5
4.5 Procedure 6
4.5.1 Pre-processing of the lung tissue samples. 6
4.5.2 Sample randomization. 6
4.5.3 Sample preparation of Lung Tissue Slices Extract. 7
4.5.4 Quality Check. 7
4.5.5 Labeled iTRAQ LC MS/MS. 7
4.5.6 Verification Experiments. 8
5 Statistical Methods 8
6 Administrative Aspects 10
6.1 Study Timelines 10
6.2 Names and Addresses 10
7 Archiving 11
8 References & Sources 11
8.1 Literature 11
8.2 Philip Morris International's List of Standard Operating Procedures (SOPs) and Work Instructions 11
8.3 Philip Morris International's Other Internal Documents 11
9 Abbreviations 12
10 Appendix 12

1 Review and Approval

This document has been approved by:

Name	Justification	Date / Signature
Elamin, Ashraf	Approved by Study Director (Supervisor MS-based Proteomics)	30.09.13
Thomas Schneider	Approved by Deputy Study Director (Scientist MS-based Proteomics)	30.09.13
Fabio Talamo	Approved by Manager Proteomics	30.09.13
Nikolai Ivanov	Approved by Manager Research Technologies	03.10.2013
Kogel, Ulrike	Approved by Work Package Leader (OECD +)	30. Sept. 2013
Hoeng, Julia	Approved by SBDAV Program Leader	1.10.2013

2 Introduction

In order to determine the hazard of a harmful effect to individuals or sub-populations resulting from an exposure to the Zurich platform, a battery of chemical and toxicological assays will be conducted. Among these, a 90 days inhalation study was conducted as described in the OECD guideline 413. Its aim is to characterize adverse effects following repeated daily inhalation exposure to different doses of the Zurich product. In addition, different doses of the reference cigarette 3R4F were tested, and a sham group will be used as control.

Data derived from this sub-acute inhalation study aimed to characterize the toxicity of aerosol from a Zurich product prototype by the inhalation route, and provided safety data for the regulatory toxicological assessment and to support the exploratory clinical studies.
Furthermore, additional molecular endpoints were added in order to complement the classical OECD approach with a systems toxicology approach (OECD+). This additional part described in the following business case is the focus of this study plan.

SBDAV_ACL_OECD(+) study BC
http://disco.app.pmi/disco/drl/objectId/0901d4ec8035261f

The reference of the business case is: PMI internal business case *SBDAV_ACL_OECD (+)*, 2012.

3 Objectives

The main objective of the study plan is to complement the basic OECD requirements with investigations on the changes in protein expression levels upon inhalation of 3R4F and Zurich aerosols after 90 and 90 + 42 days compared to sham exposed rats.

3.1 Primary Objective.

- To detect differential proteins in the left lung tissues of male rats using labeled iTRAQ LC-MS/MS approaches.

3.2 Secondary Objective.

- To verify and quantitate selected protein targets in the left lung tissues (and serum if applicable).

4 Experimental Design

4.1 Test and Reference Items.

Test items are described in detail in the section 4.1 of main study plan (15006 THS SP).

Shortly, reference cigarette 3R4F will be purchased from the University of Kentucky. Test item is Zurich FPD 4.2 sensorial medium (batch number B-00829) abbreviated ZRH.

Test and reference items will be administered by nose-only inhalation to ensure maximum reproducibility.

Both 3R4F and ZRH will be smoked at 3 target nicotine concentrations. Test atmosphere will be characterized as detailed in the main study plan.

4.2 Test System.

Experiments are conducted on male and female Sprague Dawley rats bred under specific pathogen-free conditions purchased from Charles River, USA (at 8-week age). For more details see the section 4.2 of the main study plan (15006 THS SP).

4.3 Experimental Groups and Dosing.

All experimental groups included in the main study are described in the section 4.3 of the main study plan (15006 THS SP).

Only some of the groups are subjects of the analyses included in this particular “omics” study plan (OECD+ part). They are summarized in Table 1 and Figure 1

Table 1: Experimental groups and dosing

Short Name (main study plan)	Group Code	Target nicotine (µg/L)	Time Point (days)	
			90	90+42
			male	male
SHAM	11	0	6	5
3R4F – low	12	8	6	6
3R4F – medium	13	15	6	6
3R4F – high	14	23	5	5
ZRH – low	15	15	6	6
ZRH – medium	16	23	5	6
ZRH – high	17	50	6	-

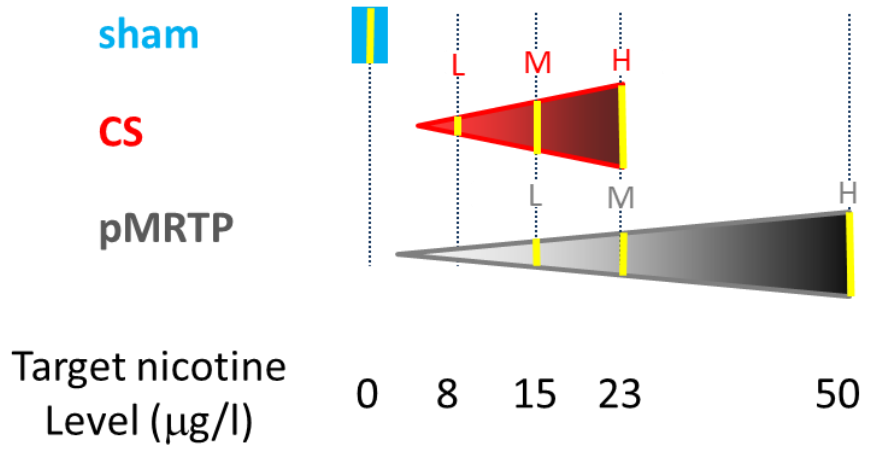


Figure 1: Schematic Representation of Groups Included in the Study with their Target Nicotine Level.

4.4 Samples to be analyzed by iTRAQ approaches.

In the iTRAQ approach, 90 days samples (left lungs from male rats only) will be processed and analyzed in a first series of experiments. 90 + 42 days samples (left lungs from male rats only) will be processed and analyzed in a second series of experiments. The experimental details are summarized in the following paragraphs.

For the 90 days samples (left lungs from male rats only), six or five (depending on the treatment sample) biological replicates of all treatment groups will be analyzed using iTRAQ 8-plex. Reporter-ion channel 113 will be used for Sham, 114 for 3R4F low, 115 for 3R4F medium, 116 for 3R4F high, 118 for Zurich low, 119

for Zurich medium, 121 for Zurich high and 117 for a reference mix containing an aliquot of all samples processed for the time point at 90 days. In case of missing samples (e.g. only 5 samples are available for 90 days 3R4F high treatment group and Zurich medium treatment group) the reference mix will be added to the missing reporter-ion channel. For the 90 + 42 days samples (left lungs from male rats only), six biological replicates of all treatment groups will be analyzed using iTRAQ 8-plex. Reporter-ion channel 113 will be used for Sham, 114 for 3R4F low, 115 for 3R4F medium, 116 for 3R4F high, 118 for Zurich low, 119 for Zurich medium, 121 for a reference mix containing an aliquot of all samples processed for the time point at 90 + 42 days and 117 for a reference mix containing an aliquot of all samples processed for the time point at 90 days. In case of missing samples (e.g. only 5 samples are available for Sham and 3R4F high treatment group) the reference mix from 90 + 42 days will be added to the missing reporter-ion channels.

Each biological replicate will be processed twice (2 technical replicates) and each technical replicate will be injected twice on the Q-Exactive as described in PMI_RD_WKI_001034, PMI_RD_WKI_001038, PMI_RD_WKI_001036.

4.5 Procedure

4.5.1 Pre-processing of the lung tissue samples.

Sliced left lung tissue samples will be used for protein extraction. All samples from each experimental group will be cryo-sliced according to the scheme represented in Figure 2. To ensure a proper representation of the full lung tissue, serial sections from the three areas of the organ (represented in the figure as Parenchyma, Whole Lung and Main Bronchus) will be collected in one tube. The Tissue Research (TR) Laboratory will cryo-slice the left lungs into 40 µm slices and collect the aliquot for protein extraction in empty tubes. At least a total of 20 slices will be provided for protein extraction.

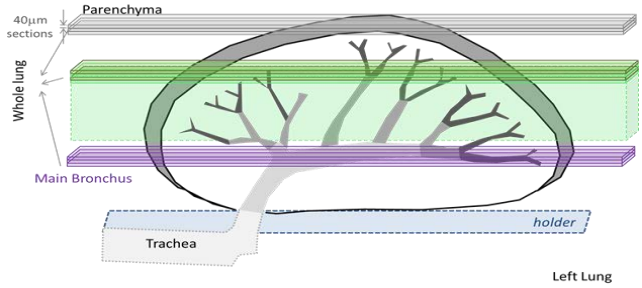


Figure 2. Schematic representation of the left lung tissue processing

4.5.2 Sample randomization.

The order for the analysis of lungs samples and the assignment of the different samples to iTRAQ sets will be randomized according to a plan supplied by statisticians (Gregory Vuillaume and Florian Martin).

4.5.3 Sample preparation of Lung Tissue Slices Extract.

The slices (> 20) from the left lungs will be homogenized in the lysis buffer (BioRad) using the Tissue Mixer II (Qiagen) for 2 minutes at a frequency of 30 Hertz. The homogenized mixture will be split into four aliquots (for iTRAQ LC-MS/MS and verification experiments) and each aliquot is subjected to acetone precipitation by adding 4 x volume of pre-chilled acetone for 2 hour in -20 °C followed by centrifugation at 16,000 x g at 4 °C. The precipitates will be resuspended in 200 µl of 0.5 M triethylammonium bicarbonate (TEAB) buffer containing 1 M urea and 0.1% SDS. Protein concentrations of the samples are determined using the Bradford assay according to supplier's instructions. Procedures are described in PMI_RD_WKI_001043.

4.5.4 Quality Check.

The quality control procedure to check the sample proteins' stability before performing the experimental analyses will consist of 2 steps: (i) determination of protein concentrations and (ii) determine sample integrity by checking the protein-band profile using 1D-PAGE according to PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

4.5.5 Labeled iTRAQ LC MS/MS.

4.5.5.1 Labeling of peptides and MS/MS analysis.

1. Labeling with iTRAQ reagents. An amount of 50 µg of each iTRAQ aliquot prepared according to 4.5.3 will be subjected to reduction of cysteines using tris-(2- carboxyethyl) phosphine (TCEP) and blocking using methyl methane-thiosulfonate (MMTS). iTRAQ labeling procedure is described in PMI_RD_WKI_001034, PMI_RD_WKI_001038, PMI_RD_WKI_001036.
2. The samples will be digested with trypsin (Promega) to break down the proteins into small peptides followed by labeling with iTRAQ reagents.
3. After labeling, the samples will be mixed together.
4. The labeled tryptic peptides will be separated by 1D LC column separation and analyzed by Q-Exactive ESI-MS/MS (Thermo Scientific) to obtain the mass lists as described in PMI_RD_WKI_001034, PMI_RD_WKI_001038, PMI_RD_WKI_001036
5. Xcalibur and RawMeat software is used to evaluate the spectra and mass-accuracy as described in PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

4.5.5.2 Protein database searches

Protein database searches and quantification will be performed with Proteome Discover (Thermo Scientific). The generated mass lists will be matched to protein databases to obtain identification of proteins. Changes in differences in protein abundance between samples will be calculated from the intensities of the corresponding iTRAQ-reporter ions in each sample. The procedure for database searching for protein identifications is described in the PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

4.5.6 Verification Experiments.

Analysis in lung samples will be performed on protein aliquots for verification prepared according to **4.5.3.** For serum, proteins will be extracted as described in PMI_RD_WKI_001043. For both sample types an amount of 100 µg will be used for these experiments.

The procedure for biomarkers verifications using selected reaction monitoring (SRM) is as follows:

1. One to four tryptic peptides representative of the selected protein in the sample mixture will be selected according to their MS properties (abundance, length and content of proline residues), uniqueness (no interfering isoforms), possible presence of post-translational modifications and/or propensity for chemically induced modifications, and susceptibility for missed cleavage sites (avoid non-cleavable and missed cleaved tryptic peptides). The peptides will be selected using the Pinpoint software (Thermo Scientific).
2. Samples will be trypsin digested as separated using 1-D LC as described in PMI_RD_WKI_001407, PMI_RD_WKI_001272, PMI_RD_WKI_001402.
3. An MS scan performed on the Q-Exactive (Thermo Scientific) will confirm the presence of the peptide(s) in the spectrum and the MS/MS will ensure that these selected peptides can be fragmented. This is the proof of feasibility check that it will work and further confirms the presence of the protein.
4. After the peptides pass the proof of feasibility check, the tryptic digested samples as described in step 2, will be injected again to perform relative quantitation. Multiple protein biomarkers can be monitored at the same time. Synthetic peptides will be ordered 2 per target protein to perform absolute quantifications.
5. The pinpoint software will confirm and verify the presence of the target of interest, and the absolute quantification as compared to synthetic peptides of the target.

5 Statistical Methods

The proteins identified by the label-based iTRAQ-approach are then confirmed by SRM. This section applies to these specific measurements. The analysis below is performed separately for all proteins measured by SRM.

Population for analysis

For the 90 day sample set, 6 samples for Sham, 3R4F low dose, 3R4F medium dose, ZRH low dose, and ZRH high dose, and 5 samples of 3R4F high dose and ZRH medium dose are available. Each sample is coming from a different rat. For each protein measured by SRM, 40 (= 5 * 6 + 2 * 5) samples will thus be used in the statistical analysis.

For the 90 + 42 day sample set, 6 samples for 3R4F low dose, 3R4F medium dose, ZRH low dose and ZRH medium dose and 5 samples for Sham and 3R4F high dose are available (see Table 1), Each sample is coming from a different rat. For each protein measured by SRM, 34 (= 6 * 4 + 5 * 2) samples will thus be used in the statistical analysis.

Treatment of special values

No missing data will be imputed in the analysis, and it will be shown in any summaries as missing. For values reported as below limit of quantification (LOQ), a value of LOQ/2 will be used in the analysis.

Descriptive statistics

For all measurements, the following summary statistics will be computed for each of the 7 study groups: number of missing observations, number of observations below LOQ, number of observations used to compute the statistics, mean, standard deviation, relative standard deviation (%), standard error on the mean, minimum, median, maximum.

Inferential statistics

Note that the study is exploratory and no adjustment for multiple testing is performed. Therefore all p-values and confidence intervals should be interpreted descriptively. The various group means will be compared by linear models followed by pairwise comparisons. If strong deviations to the assumptions of these methods are observed (e.g. variance inhomogeneity between groups), data will be transformed or non-parametric methods will be used.

6 Administrative Aspects

6.1 Study Timelines

Proposed experimental starting date:	September 30th 2013
Proposed experimental completion date:	iTRAQ 90 days samples: October 28th 2013 iTRAQ 90+42 days samples: December 20th 2013 Verification :Q1-2, 2014
Proposed date for draft study report:	Q3, 2014

6.2 Names and Addresses

Sponsor	Manuel Peitsch Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Philip Morris Products S. A Research & Development Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Study Director	Ashraf Elamin Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Deputy Study Director	Thomas Schneider Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Quality Assurance Officer	N/A Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

7 Archiving

After finalization of the study, study plan, amendments, raw data, test and reference items, retention samples, specimens collected, the report, and all study-related records will be stored for a maximal period of one year at the archive at ITSC. Electronic records will be archived on the central archiving server at ITSC, which is managed by the local IT and ITSC specialists.

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

8 References & Sources

8.1 Literature

NA

8.2 Philip Morris International's List of Standard Operating Procedures (SOPs) and Work Instructions (WKIs)

LC-MS for proteomics analysis: PMI_RD_WKI_001038

iTRAQ 8-plex: PMI_RD_WKI_001034

Sample purification for LC MS/MS: PMI_RD_WKI_001036

Protein Extraction from Murine Lung Tissue and Acetone Precipitation: PMI_RD_WKI_001043

Determination of Protein concentration using 660 nm: PMI_RD_WKI_001104

Determination of Protein concentration using Bradford assay: PMI_RD_WKI_001103

Quality control of protein samples using LabChip instrument: PMI_RD_WKI_001131

Sample purification for PRM: PMI_RD_WKI_001407

Parallel reaction monitoring: PMI_RD_WKI_001272

Creation of heavy-labeled peptide pool for PRM: PMI_RD_WKI_001402

8.3 Philip Morris International's Internal Documents

Philip Morris International. Unpublished on file data. Internal business case. SBDAV ACL OECD (+): 90 DAY INHALATION STUDY (OECD 413) INCLUDING ADDITIONAL ENDPOINTS (OECD+). 2012.

15006 THS SP: Philip Morris International. Unpublished on file data. Internal Study Plan; "90 DAY OECD INHALATION STUDY ON SPRAGUE-DAWLEY RATS TO CHARACTERIZE TOXICITY OF MAINSTREAM AEROSOL FROM ZURICH FPD 4.2 CIGARETTES AND COMPARISON TO 3R4F MAINSTREAM AEROSOL TOXICITY". Study number 15006. 2013.

9 Abbreviations

Abbreviation	
BSA	Bovine serum albumin
CAN	Coded animal number
DTT	Dithiolthreitol
ESI	Electrospray ionization
IAA	Iodoacetamide
LC	Liquid Chromatography
MMTS	Methyl methane-thiosulfonate
MS	Mass spectrometry
MRTP	Modified Risk Tobacco Products
PAGE	Polyacrylamide gel electrophoresis
PMIRL-S	Philip Morris International Research Lab in Singapore
PMRL	Philip Morris Research Laboratories
PVDF	Polyvinylidene fluoride
PTMs	Posttranslational modifications
SRM	Selected reaction monitoring
TCEP	Tris-(2- carboxyethyl) phosphine
TEAB	Triethylammonium bicarbonate
ZRH	Zurich

10 Appendix

N/A