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6.1.4 Clinical Risk Endpoints

6.1.4.1 Introduction: Clinical and Non-Clinical Programs to Assess Biological and Functional Endpoints

The FDA recommends in its MRTP Application Guidance that "applicants conduct human studies to assess the full range of the human health risks related to the use of the tobacco products, including exposure to tobacco-related compounds" (FDA, 2012a). Furthermore it is recommended to conduct "human studies that show the product's use will result in a significant reduction in harm and the risk of tobacco-related disease to individual tobacco users" (risk modification order; (FDA, 2012a), p. 17) or "Nonclinical and/or human studies that demonstrate that use of the product is expected to result in a measurable and substantial reduction in morbidity or mortality to individual tobacco users based on the effects of the product on an endpoint that is reasonably likely, based on epidemiological, therapeutic, pathophysiologic, or other evidence, to predict an effect on reducing harm or disease" (exposure modification order; (FDA, 2012a), p. 17).

PMP has developed an MRTP assessment framework (Smith et al, 2016) that utilizes what is known from the literature about smoking cigarettes and has incorporated both the epidemiological evidence and mechanistic understanding into its approach. Epidemiological studies inform on the causal relationship between smoking cigarettes and risk of developing disease, as well as the reduction in these risks related to smoking cessation. Exposure to cigarette smoke leads to both a time- and dose-dependent increase in the risk of developing smoking-related diseases, such as cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD) and lung cancer (Stratton et al, 2001). Following smoking cessation, the accumulated risks begin to diminish and continue to reduce over time. The cause-and-effect relationship for these diseases are based on sound epidemiological evidence, which is conceptually depicted in Figure 1.

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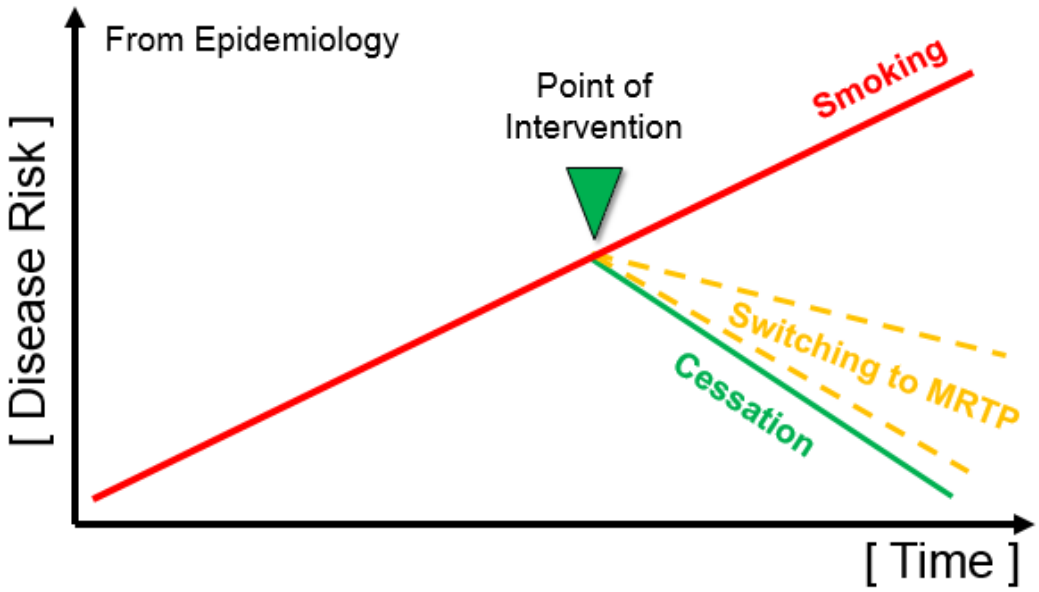


Figure 1 Conceptual Framework for MRTP Assessment

Conceptual depiction of the cumulated risk of smoking and the effect of cessation over time. These represent the two boundaries for the assessment of an MRTP: 1) comparing switching to an MRTP with continued smoking and 2) benchmarking switching against smoking cessation, the "gold standard". Note that the straight lines used in this figure are for illustration purposes only, as the accumulation of disease risk and the reduction upon cessation and switching to an MRTP follow different trajectories for specific diseases.

The Institute of Medicine (IOM) defined smoking cessation as the 'gold standard' for assessing risk reduction, stating that the closer the risks and exposures associated with the use of a candidate MRTP are to smoking cessation, the more confident a regulator can be of achieving a net public health benefit (Stratton et al, 2001).

Smoking-related diseases have a complex etiology and involve several mechanisms which affect several organ systems (USDHHS, 2010b). Chronic exposure to cigarette smoke induces molecular changes in the exposed organism and disrupts multiple biological processes. This in turn causes alterations at the cell and tissue level that result in physiological changes that eventually manifest as disease (Figure 2).

As smoking-related diseases often take decades to manifest, conducting long-term epidemiological studies is not practical to assess the risk of a candidate MRTP prior to market introduction. Therefore, an approach which leverages short-term data for long-term risk assessments is needed. Towards this end, the epidemiology-driven framework described above, supported with a disease mechanism-based approach to MRTP risk assessment, has been developed to compress the time component required for adverse outcomes to manifest.

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Figure 2 Chronic Exposure to Cigarette Smoke Affects a Number of Biological Networks Associated with Smoking-Related Diseases in a causal chain of events known as Adverse Outcome Pathway

PMP's clinical and non-clinical MRTP assessment programs are based on this conceptual framework, with the fundamental objective being to demonstrate that upon switching to a candidate MRTP the risk of developing smoking-related diseases is reduced and approaches that of smoking cessation. They were developed using the available scientific literature on the epidemiology of smoking-related diseases as well as the mechanistic understanding of the impact of smoking and smoking cessation. The programs were aligned so that evidence generated in the non-clinical assessment program on biomarkers of exposure and mechanistic markers could be corroborated and expanded with the data generated for related clinical risk endpoints. Table 1 summarizes the assessments performed in both the non-clinical and clinical assessment programs and provides the relationship between the biomarkers of exposure, the mechanistic markers and clinical risk endpoints.

Since chronic exposure to cigarette smoke leads to the multifaceted perturbation of a number of biological networks, which trigger multiple adverse effects causally linked to smoking–related diseases, a single clinical risk endpoint or biomarker is not sufficient as a surrogate measure for the multiple adverse health effects associated with smoking or to fully demonstrate reduction of risk. Therefore, our evaluation of the effects of exposure is based on an assessment that integrates multiple sources of evidence to demonstrate that our candidate MRTP significantly reduces the risk of smoking-related diseases for smokers who switch to our candidate MRTP when compared to smokers who continue to smoke cigarettes.

Within the identified disease networks, several mechanistic markers and clinical risk endpoints have been identified that are related to the pathophysiological mechanisms of smoking-related diseases. Many of these mechanistic markers and clinical risk endpoints have been shown to favorably change in the short to mid-term (e.g., within one week to one year) following smoking cessation and were therefore considered for evaluation and inclusion in our assessment program.

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Table 1 The Nonclinical and Clinical Assessment Programs

Risk Parameters	Endpoints/Biomarkers	*In vitro* Studies	Rat OECD studies		ApoE THS2.2	A/J Study	Clinical Studies
			THS2.2R	THS2.2M			
Exposure	BoExp	-	x	x	x	x	x (see section 6.1.3)
	Xenobiotic Metabolism	x[a]	x	x	x		x (see section 6.1.3)
	Gene Expression signature	-	-	-	-		x (see section 6.1.3)
Inflammation	General/systemic	-	x	x	x	x	x (WBC; CRP)
	Respiratory inflammation	x[b]	x	x	x	x	-
	Vascular inflammation	x[c]	-	-	x	-	x (sICAM-1, fibrinogen)
Oxidative Stress	Oxidative stress	x[a]	x	x	x	x	x (8-epi-PGF2α; 11-DTXB2)
CVD	CVD risk markers	x[c]	x[e]	x[e]	x	-	x (COHb; , Hb1Ac; HDL-C, LDL-C;total cholesterol; triglycerides; oxysterols ApoB/ApoA1, Blood Pressure)
Respiratory Disease	Lung function	-	x	x	x	x	x (lung function/FEV1; DLCO)
Cancer	Genotoxicity	x[d]	x	x	x	x	x (IARC Class 1/2 BoExp; Urine Ames; CYP1A2 activity)

(a) Organotypic oral, nasal and bronchial and adhesion studies; (b) Organotypic oral, nasal and bronchial studies; (c) Adhesion study; (d) Non-clinical *in vitro* safety toxicology studies (see section 6.1.2); (e) Lipid analysis

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6.1.4.1.1 Non-Clinical Assessment of Biological and Functional Changes

Recent advances in molecular measurement and imaging technologies, mathematical modelling and computational biology enable the integrative analysis of large data sets to quantify the biological impact of exposure to toxicants (Hoeng et al, 2012; Hoeng et al, 2014; Sturla et al, 2014). The integration of these methods with standard toxicological endpoints, such as those defined in the test guidelines of the Organisation for Economic Co-operation and Development (OECD), defines PMP's systems toxicology-informed risk assessment approach. Using computable biological network models of the key mechanisms affected by smoke exposure (Boué et al, 2015), systems toxicology permits the quantification of the biological network perturbation amplitudes (NPA) (Martin et al, 2014) caused by exposure to cigarette smoke and their numerical aggregation into an overall biological impact factor (BIF) (Thomson et al, 2013). This approach was used to conduct a systematic and quantitative mechanism-based comparison of the biological impact of switching to THS compared to continued cigarette smoking as well as benchmarking the impact of switching to smoking cessation in animal models of disease. Systems toxicology was also applied to compare the effects of exposure to THS aerosol with those of cigarette smoke exposure using *in vitro* systems.

PMP has conducted several nonclinical studies using this systems toxicology-based approach using laboratory animals. The aim of these studies is to identify potential hazards and compare the effects of cigarette smoke with those of THS aerosol on mechanisms underlying tobacco smoke-related toxicity and diseases (Table 2).

The two 90-day sub-chronic inhalation studies, as described in the OECD test guideline 413 (OECD, 2009), were conducted in rats to compare the toxicity of aerosols generated from THS and THS Menthol with that of smoke from the 3R4F reference cigarette. A post-exposure period of 42 days was included in both studies to allow for the assessment of the reversibility or delayed occurrence of findings. In-life observations, respiratory physiology, histopathology of the respiratory and non-respiratory organs, and various blood-based parameters were evaluated. To obtain an understanding of the toxicity pathways involved in the response to the exposure, additional molecular endpoints including multi-analyte profiling of bronchoalveolar lavage fluid (BALF), transcriptomics and proteomics analysis of lung and the nasal epithelium were examined using systems toxicology.

Furthermore, to investigate the effects of exposure on hallmarks of COPD and CVD, a study was conducted in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice for an 8-month period. This animal model of disease allows the concomitant investigation of both COPD and CVD related endpoints (Lo Sasso et al, 2016a). Mice were exposed to 3R4F reference cigarette smoke, THS aerosol or fresh air. Furthermore, two additional groups of mice were first exposed for two months to 3R4F smoke and then for 6 months to fresh air to simulate cessation or THS aerosol to simulate switching. Similarly to the 90-day inhalation studies, systems toxicology was used to investigate the effects of exposure, integrating both conventional toxicology parameters, such as physiology and histopathology of respiratory and non-respiratory organs, and molecular measurements comprised of transcriptomics, lipidomics and proteomics.

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These rodent studies were further complemented by a series of *in vitro* studies to comparatively assess the impact of exposure to 3R4F cigarette smoke and THS aerosol on oral, nasal and bronchial organotypic tissues cultured at the air-liquid interface. Various endpoints including the assessment of cytotoxicity and activity of cytochrome P450 (CYP) 1A1/1B1, histological evaluation, and transcriptomics were assessed at different time points following an acute 28-minute exposure. In addition, *in vitro* studies were performed in human coronary artery endothelial cells to study changes in their adhesive properties towards monocytes following treatment with aqueous extracts from 3R4F cigarette smoke and THS aerosol. Alternatively, conditioned media from monocytes pre-incubated with these aqueous extracts were used to challenge endothelial cells. For both approaches, a functional adhesion assay was combined with transcriptomics and inflammatory protein marker data to investigate changes in this model system at the molecular level (Table 2). In addition, a transendothelial migration (TEM) assay was developed and used to examine the potential differential effects elicited by exposure of monocytic THP-1 cells on their capability to traverse an endothelial cell layer using two different approaches, a direct approach stimulating the endothelial cells with freshly prepared extracts, and an indirect approach pre-treating the monocytic cells with the extracts and using the resulting conditioned media to stimulate the endothelial cells.

Table 2 Non-Clinical Studies with Measures of Biological and Functional Changes

Study	Type	Design	Reference
Fraction Impact	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosol fractions)	(Gonzalez-Suarez et al, 2016)
Organotypic oral	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosols)	1743_Organotypic_Buccal_SR (Zanetti et al, 2016)
Organotypic nasal	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosols)	1722_Organotypic_Nasal_SR (Iskandar et al, 2016)
Organotypic bronchial	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosols)	1709_Organotypic_Bronch_SR 1709_Organotypic_Bronch_Pub
Adhesion assay	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosol extracts)	1604_CVD_Adhesion_Assay_Pub (Poussin et al, 2016)
Transendothelial migration assay	*In vitro*	Parallel-group (air (sham), RC[a] and THS2.2 aerosol extracts)	(van der Toorn et al, 2015a)

(table continues)

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Study	Type	Design	Reference
Rat OECD THS2.2	*In vivo*	TG413 (air (sham), RC[a], THS2.2)	15006_150082_150083 THS SR 15006_150082_150083 THS PUB
Rat OECD THS2.2M	*In vivo*	TG413 (air (sham), RC[a], THS2.2M)	15025_176500 THS SR 15025_176500 THS PUB
Apoe$^{-/-}$ Switching	*In vivo*	Parallel-group, switching (air (sham), RC[a], THS2.2, cessation, switching)	15015_CVD_Resp_ApoE_SW_SR 15015_CVD_Resp_ApoE_SW_SR_AA (Lo Sasso et al, 2016a; Lo Sasso et al, 2016b; Phillips et al, 2016; Titz et al, 2016)
A/J Lung Cancer Study	*In vivo*	Parallel-group (air (sham), RC[a] and THS2.2 aerosol)	15020_AJ_QA-checked Interim report

(a): 3R4F reference cigarette

Overall, the data obtained in the non-clinical studies demonstrated that exposure to THS aerosol does not cause systemic toxicity and very low or no toxicity in the respiratory tract and cardiovascular system of laboratory animals. Therefore, and in combination with the *in vitro* toxicology data collected, it is most likely that human adult smokers using THS will not be exposed to additional hazards to those presented by smoking conventional cigarettes. Furthermore, results obtained during the *Apoe*$^{-/-}$ mouse study indicated that switching to THS aerosol exposure resulted in the reversal of respiratory inflammation and halted progression of lung function decline, initial emphysematous changes and the aortic plaque area compared with mice exposed continuously to 3R4F cigarette smoke. Similarly, results from *in vitro* studies showed that exposure of organotypic tissues and endothelial cells to THS aerosol elicited less cytotoxicity and resulted in reduced secretion of inflammatory mediators, monocyte and endothelial cell adhesive properties, as well as a markedly decreased biological impact on cell stress, cell fate and inflammatory networks than exposure to 3R4F cigarette smoke.

6.1.4.1.2 Clinical Assessment of Biological and Functional Changes

Four reduced exposure studies have been conducted as part of the clinical assessment program for THS. Although the primary objective was to assess reductions in the levels of BoExp to HPHCs, the ambulatory studies also collected data on a number of clinical risk endpoints to investigate, as a secondary objective, if the reduction in exposure to HPHCs translated to favorable biological and functional changes (i.e., changes in the same direction as smoking cessation). These studies were conducted among smokers who continued to smoke, smokers who switched to THS to assess the change following a switch to the candidate MRTP, but also included smokers who abstained from smoking (SA) to provide the context and clinical relevance to the extent of the change (i.e.,

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smoking cessation being the gold standard for beneficial effects). Two of the studies only included five days of *ad libitum* use in a confinement setting, but the other two studies were three-month *ad libitum* use studies in an ambulatory setting (Table 3), and therefore allowed an assessment of the effect on the clinical risk endpoints that are reversible within 3 months.

Table 3 Clinical Studies with Measures of Biological and Functional Changes

Study	Country	FSI-LSO [(a)]	Design	Reference
ZRHR-REXC-03-EU	Poland	29-Jun-2013 26-Sep-2013	• Randomized, 3-arm (CC; THS; SA) [(b)] parallel study • 5 days *ad libitum* use • Controlled distribution • Confinement setting	Link CSR
ZRHR-REXC-04-JP	Japan	23-Jul-2013 09-Dec-2013	• Randomized, 3-arm (CC; THS; SA)^2 parallel study • 5 days ad libitum use • Controlled distribution • Confinement setting	Link CSR
ZRHM-REXA-07-JP	Japan	01-Aug-2013 03-Jul-2014	• Randomized, 3-arm (CC; THS; SA)^2 parallel study • 5 days *ad libitum* use (in confinement) • 85 days *ad libitum* use (at home) • Ambulatory setting	Link CSR
ZRHM-REXA-08-US	USA	17-Dec-2013 12-Oct-2014	• Randomized, 3-arm (CC; THS; SA)^2 parallel study • 5 days ad libitum use (in confinement) • 86 days ad libitum use (at home) • Ambulatory setting	Link CSR

(a): **FSI**: first subject in; **LSO**: last subject out
(b): **CC**: conventional cigarettes; **THS**: Tobacco Heating System; **SA**: smoking abstinence

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6.1.4.1.3 Reduced Exposure Studies - Study Design

6.1.4.1.3.1 One-week Reduced Exposure in confinement – ZRHR-REXC-03 and ZRHR—REXC-04

Two randomized, controlled, open-label, 3-arm parallel group Reduced Exposure studies in confinement were conducted to investigate reductions of BoExp to HPHCs following switching from CC to THS for 5 days in an optimal, clinical laboratory setting. A smoking abstinence (SA) group was included providing the benchmark for the observed reduction in exposure to HPHCs. Subjects were randomly assigned to the individual study groups. Product use was directed and monitored by the study staff and participating smokers were controlled for product compliance in a confined setting. The smokers used the THS without restriction (*ad libitum*) during an extended daily time window (16 hours), dual use of CC and THS was not permitted. This design allowed to investigate the intrinsic product performance with regard to reduction of exposure to HPHCs by limiting the influence of factors that might impact HPHCs levels, as the majority of HPHCs can also arise from sources other than cigarette smoke. In both studies, the regular product variant of THS was tested.

Subjective effects associated with smoking (craving, withdrawal symptoms, and product evaluation) were assessed systematically during the study using validated, published questionnaires. Such measures provide insight into product acceptance in terms of ritual, taste, sensory and nicotine delivery and are important to assess the potential for consumer's acceptance. Already from our own market research data, it was recognized that changes in product use behavior can require more than one week of adaption to THS (Section 6.2.2). In this regard, 5 days of THS use in clinical studies in confinement does not capture the aspect of consumption of THS in addition to other tobacco products or to other nicotine-containing products/e-cigarettes (dual use) as subjects were instructed to use the product they have been assigned to.

The studies were conducted in Poland and Japan in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 2008 (WMA, 2008), International Conference on Harmonization (ICH) Good Clinical Practice (GCP) and applicable national regulation and were registered under ClinTrials.gov.

A summary of the methodology and results for the studies ZRHR_REXC-03-EU and ZRHR-REXC-04-JP is provided in Section 6.1.3.

6.1.4.1.3.2 Three-month Reduced Exposure in a confined and ambulatory setting - ZRHR-REXA-07-JP and ZRHM - REXA-08-US

Complementary to the Reduced Exposure studies in confinement, two three-month randomized, controlled, open-label, 3-arm parallel group Reduced Exposure studies comprising sequential confinement and ambulatory periods were conducted in Japan and in the US In both studies, the menthol product variant of THS was tested. The first part of the study in a confined setting was similar to the Reduced Exposure studies described in Section 6.1.4.1.3.1, in that current adult smokers were confined for a week and BoExp to HPHCs were measured as the subjects switched from CCs to the THS or SA. In the

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second phase of the study, subjects were followed over a period of three months in an ambulatory setting which allowed for the detection of changes in BoExp with longer half-lives, such as total NNAL, and to demonstrate that the exposure reduction seen under very controlled conditions can be sustained in an ambulatory, near real-world environment where dual-use of THS and CC or other tobacco products can occur. Furthermore, with the extended ambulatory exposure and follow-up period, the study was long enough to assess the initial changes in some of the clinical risk endpoints that have been shown to be reversible within 2 weeks to 3 months.

The studies were conducted in Japan and in the US in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 2008 (WMA, 2008), ICH GCP and applicable national regulation and were registered under ClinTrials.gov.

A summary of the methodology and results for the studies ZRHM-REXA-07-JP and ZRHM-REXA-08-US is provided in Section 6.1.3.

6.1.4.1.4 Description of the Methodology

6.1.4.1.4.1 Sample collection, Storage and Bioanalytical Methods

The assessment matrix for the clinical risk endpoints measured in PMP clinical studies has been evaluated with regards to validity of the available methods, feasibility and exposure time needed to assure reliable results. Scientifically accepted, standard approaches to assess each of these endpoints have been selected for assessment in these studies.

Detailed procedures for sample collection and handling of samples are described in a separate sample handling manual. Details related to the destruction of samples are provided in Section 7.7 of the Clinical Study Protocol (Appendix A16.1.1 of each Clinical Study Report).

The clinical safety parameters that were assessed were conducted by a central laboratory to ensure consistency and interpretability of the results across the two clinical sites in each of the studies. Whenever possible and appropriate, the bioanalytical assays and laboratory assessments were carried out using validated methods and are documented in the Bioanalytical Report and part of the Clinical Study Report.

Table 4 presents the complete list of the biomarkers of exposure and clinical risk endpoints and timing of collection time for the two reduced exposure studies in an ambulatory setting. In general, these two studies were very similar to one another in design and assess most of the same endpoints, however, there are some slight differences between the two studies that are highlighted in the footnotes of the table below.

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Table 4 Endpoints Collected in the Ambulatory Reduced Exposure Studies (ZRHM-REXA-07-JP and ZRHM-REXA-08-US)

	Baseline Period			Confinement Period					Ambulatory Period			
	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day 30 Visit ± 3 days	Day 60 Visit ± 3 days	Day 90 Visit ± 3 days
Study Day	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**30/31**	**60/61**	**90/91**
BoExp in urine [a]			●	●	●	●	●	●		●	●	●
Creatinine			●	●	●	●	●	●		●	●	●
COHb		●	●	●	●	●	●	●	●	●	●	● ●
Ames mutagenicity test			●					●				●
Vital Signs [b]	●	●	●	●	●	●	●	●	●	●	●	●
Waist circumference	●											●
Spirometry [c,d]	●		●						●			●
Hematology, clinical chemistry, urine analysis [e]			●						●	●	●	●
sICAM-1			●						●	●	●	●
hs-CRP, fibrinogen, homocysteine, LDL & HDL			●							●	●	●

(table continues)

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	Baseline Period			Confinement Period					Ambulatory Period			
	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day 30 Visit ± 3 days	Day 60 Visit ± 3 days	Day 90 Visit ± 3 days
Study Day	**-2**	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**30/31**	**60/61**	**90/91**
HbA1c, Apo A1 & Apo B [(f)]			●									●
8-epi-PGF2α & 11-DTX-B2			●					●		●	●	●
Oxysterols [(g)]			●						●			●
CYP1A2			●					●				●
CYP2A6			●						●			●
Bio-banking [(h)] – transcriptomics			●						●			●
Bio-banking [(h,i)] – nasal epithelial & buccal samples			●						●			●

(a): MHBMA, 3-HPMA, S-PMA, total NNAL, 1-OHP, total NNN, 4-ABP, 1-NA, 2-NA, o-toluidine, CEMA, HEMA, 3-OH-B[a]P, HMPMA, S-BMA, NEQ.
(b): Systolic and diastolic blood pressure.
(c): Spirometry was performed prior to product use. In the ZRHM-REXA-07-JP study it was without bronchodilator, in the ZRHM-REXA-08-US study it was with bronchodilator.
(d): Spirometry in the ZRHM-REXA-08-US study used helium technique with the single breath technique for CO.
(e): White blood cell count, platelet count, blood glucose, triglycerides, and total cholesterol
(f): Apo A1 and Apo B were only performed in the ZRHM-REXA-08-US study.
(g): Oxysterol was only performed in the ZRHM-REXA-08-US study.
(h): Bio-banking samples were collected only if additional consent for bio-banking was given by the subject.
(i): Bio-banked samples of nasal epithelial and buccal samples were only collected in the ZRHM-REXA-08-US study.

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6.1.4.1.4.2 Statistical Methods

A brief description of statistical methods used is provided below. For more details please refer to individual study summaries (Section 6.1.3) and the respective clinical study reports and statistical analysis plans for each study.

The primary analysis set for the one-week studies in confinement setting (ZRHR-REXC-03-EU and ZRHR-REXC-04-JP) was the full analysis set (FAS). The FAS consisted of all the randomized subjects who had at least one post-randomization product use experience (if randomized to THS or CC) and had at least one valid BoExp measurement (THS, CC, SA arms). The FAS was selected as primary analysis set in the confined studies as, by definition, product adherence was enforced by controlled product distribution by the site staff.

For the 3-month studies in confinement and ambulatory settings (ZRHM-REXA-07-JP and ZRHM-REXA08-US), in contrast, the Per-Protocol (PP) Population was used for primary analysis and was defined as:

- Subjects that were correctly randomized and assigned study product.
- Subjects with at least one post-randomization product use (for CC and THS).
- Subjects with at least one valid post-randomization BoExp measurement.
- Subjects that were adherent with their assigned study product.
- Subjects without major protocol deviations that impacted the validity of the evaluation of the study results.

The PP Population was selected as the primary analysis for the ambulatory studies in order to assess the maximum exposure reduction achievable (optimal effect) in subjects who were using THS ad-libitum and exclusively or at least predominantly, rather than the effect in the full population representing a heterogeneous exposure (e.g., as mixed product use, or non-use of the assigned product). As the main difference between the two populations was adherence to product use, the PP Population in the ambulatory studies was comparable to the FAS (following the intent to treat principle) in the confinement studies where product distribution was controlled.

The clinical risk endpoints were analyzed based on the distribution of the data, those that were not normally distributed were log-transformed (base e) prior to analysis. A generalized linear model, an analysis of covariance [ANCOVA] (Snedecor & Cochran, 1989) was applied to the clinical risk endpoints at Day 90 with study arm as a factor adjusting for baseline value, sex, average daily CC consumption over the last 4 weeks as reported during screening. Estimates of differences from the model when data were log transformed were back-transformed to calculate LS mean ratios of the level of clinical risk endpoints in THS arm compared to CC or SA.

6.1.4.2 Selection of Disease Mechanisms

The 2010 U.S. Surgeon General's Report "How Tobacco Smoke Causes Diseases" identifies inflammation and oxidative stress, among others, as key mechanisms underlying all major smoking-related diseases (USDHHS, 2010b). There is ample evidence showing that both mechanisms are intricately linked to each other and to disease development in smokers.

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Cigarette smoking is causally linked to the development of cardiovascular disease (CVD) contributing to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation and an abnormally increased tendency toward coagulation. Evidence gathered in *in vitro* and *in vivo* studies, as well as population-based observational studies, has led to the recognition that vascular inflammatory processes are central to all stages of atherogenesis, from local endothelial dysfunction to plaque development and rupture (Harrison et al, 2003; Libby et al, 2002; Madamanchi et al, 2005; Ross, 1999) and play a central role in modifying most of the known main risk factors for CVD including hypertension, hypercholesterolemia, obesity, and diabetes (Asmat et al, 2015; Ceriello & Motz, 2004; Keaney et al, 2003; Rodriguez-Porcel et al, 2001).

Chronic obstructive pulmonary disease (COPD) is a respiratory disease characterized by progressive airflow limitation and associated with an abnormal inflammatory response of the lung to noxious particles and gases (GOLD, 2013). The development of COPD in smokers has been linked to a number of sustained cellular stress responses including endoplasmic reticulum, hypoxic stress and autophagic stress (Chen et al, 2008; Hengstermann & Müller, 2008; Hwang et al, 2010; Malhotra et al, 2009; Vasilios & Ioannis, 2008). Most of these responses are the result of oxidative insults that arise from direct exposure to cigarette smoke and cause tissue injury through oxidative stress. Concomitantly, cigarette smoke-related oxidative stress also leads to the attraction and activation of immune and inflammatory cells and the release of pro-inflammatory mediators which in turn produce a number of (endogenous) oxidative stressors capable of perpetuating an inflammatory response.

There is conclusive evidence that cigarette smoking is the major cause of lung cancer in most human populations, and an increased risk of lung cancer in smokers has been demonstrated in a number of epidemiological studies (Doll & Peto, 1981; Doll et al, 2004; Harris et al, 2004; Wakai et al, 2006; Zhang & Cai, 2003). Most of the inflammatory processes identified in smokers with COPD can also be observed in smokers with lung cancer, suggesting a shared etiology, and it has been shown that COPD is a risk factor for the development of lung cancer in smokers (Viegi et al, 2001; Watson et al, 1993). In addition to inflammatory processes promoting a microenvironment favoring tumor formation, direct damage to DNA, proteins and lipids by ROS and reactive nitrogen species (RNS) is also known to contribute to the initiation of events that ultimately lead to lung cancer (Fearon et al, 2011; Halliwell, 2007). In addition, ROS can also act as second messengers and mediate processes such as proliferation, cell survival, angiogenesis and cell transformation (Federico et al, 2007; Naugler & Karin, 2008b; Storz, 2005), thus contributing to tumor promotion and progression.

Considering the importance of oxidative stress and inflammation in the development and progression of the major smoking-related diseases, CVD, COPD and lung cancer, PMI's product assessment approach assesses mechanistic risk endpoints for oxidative stress and inflammation that are not specific to a single disease. In addition, a selection of disease-specific risk endpoints that are associated with either CVD, lung disease, or cancer have been included. These endpoints allow the assessment of the comparative risk profile, by examining a collection of logical, empirically coherent and mutually supportive data from

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multiple clinical risk components across several biological processes, physiological systems, and mechanisms within both the non-clinical and clinical setting that are integrated to create the evidentiary basis for the evaluation of an MRTP.

6.1.4.3 Justification of Biological and Functional Endpoints

6.1.4.3.1 Inflammation

Inflammation can be triggered by various insults, such as infection, tissue injury, and cellular stress. This first line defense mechanism rapidly triggers the release of pro-inflammatory substances, including cytokines, free radicals, hormones and other small molecules to combat the stressor by initiating inflammation-resolving processes such as the innate immune response (Berridge, 2012). However, a number of other cells also participate in the concerted effort to limit and repair tissue injury (Berridge, 2012; Deng et al, 2009; Shen et al, 2004), and a tightly regulated transcriptional program yielding gene products integral to the inflammatory response (and indirectly contributing to the activation of the cells involved) is executed (Deng et al, 2009). While this acute inflammatory response can lead to pathological consequences, it is in itself not a disease, but a means to maintain homeostasis; how the response is eventually executed typically depends on the original trigger. However, if resolution of the acute inflammation is not achieved, ongoing stress or chronic exposure leads to the development of smoking-related diseases (Demirovic & Rattan, 2013; Glover-Cutter et al, 2013).

6.1.4.3.1.1 Mechanistic Link between Inflammation and Cardiovascular Disease

Cardiovascular disease describes a group of diseases affecting the heart and blood vessels that can be broadly classified into coronary heart disease, cerebrovascular disease and peripheral vascular disease. The build-up of fatty deposits termed “atherosclerotic plaques” on the inner walls of the blood vessels supplying the heart or brain is the common feature of these diseases. The resulting vessel blockage, with narrowing of the vascular lumen and induction of a hypercoagulable state, leads to serious and sometimes fatal events, such as myocardial infarction or stroke (USDHHS, 2010b). Cigarette smoking is a major risk factor for CVD, and evidence to date from *in vitro*, *in vivo* and population-based studies indicate that vascular inflammatory processes are central to all stages of atherogenesis (Libby et al, 2002; Ross, 1999).

The healthy endothelium regulates vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation and vessel wall inflammation in response to various inflammatory and oxidative stimuli (Münzel et al, 2008), and the endothelium is maintained in a quiescent state through the inhibitory signaling of nitric oxide (NO), an important vasodilator (Deanfield et al, 2007). When the endothelium is injured, there is a switch from the NO-mediated silencing of cellular processes to redox signaling, resulting in the activation of transcription factors and the production of inflammatory mediators including interleukins (IL), and tumor necrosis factor alpha (TNFα) (Mudau et al, 2012; Yang et al, 2006). This triggers the production of cellular adhesion molecules such as P-selectin and integrins, attracting platelets to the site of endothelial injury. The process of

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adhesion leads to platelet activation and the release of growth factors, cytokines, chemoattractants and coagulation factors that in turn promote the recruitment of monocytes to the site of inflammation, platelet aggregation, coagulation, cell survival and proliferation (Gawaz et al, 2005). Monocytes that have been attracted to the sites of endothelial damage attach to the endothelial surface, transmigrate to the subendothelial space and differentiate into macrophages which begin to secrete pro-inflammatory mediators supporting the establishment of chronic inflammation (Facchinetti et al, 2007; Libby, 2012). Moreover, these macrophages also phagocytose oxidized lipids, which are elevated in smokers (Bloomer, 2007; Yamaguchi et al, 2005), and develop into lipid-laden foam cells, eventually forming the fatty streaks that are the first visible signs of atherosclerosis (Fearon et al, 2011). Activated endothelial cells and monocytes/ macrophages release cytokines that attract vascular smooth muscle cells which start migrating into the vessel just beneath the endothelial cell layer where they proliferate and contribute to the growth of the developing plaque. In humans, the final stage of atheroma formation is reached after years of continuous exposure to cigarette smoke (Celermajer, 1997). The natural disease progression results in plaque growth and vessel remodeling to maintain a functional lumen size. Although atherosclerotic plaques remain clinically silent for decades, they may evolve to become advanced lesions that are prone to calcification, cap thinning, hemorrhage and rupture. Platelets play a major role in the advanced stages of plaque development where neovascularization, thrombosis, plaque erosion and rupture constitute fatal complications.

6.1.4.3.1.2 Mechanistic Link between Inflammation and COPD

Pulmonary cells are also exceptionally susceptible to the harmful effects of cigarette smoke, and chronic exposure to cigarette smoke has been linked to the development and progression of a variety of lung diseases, including COPD (Barnes, 2008; Tuder et al, 2006). Although the specific sequence of events leading to the development of these lung diseases is still under active investigation, recent studies have accentuated the contribution of inflammatory responses to their pathogenesis (Chung & Adcock, 2008; Sopori, 2002; Tetley, 2005).

Upon injury or exposure to noxious agents, the healthy lung can mount an effective acute inflammatory response aimed at eliminating the insult, limiting the extent of tissue damage and protecting the host (Levy & Serhan, 2014). Inflammatory molecules are released, attracting circulating leukocytes to the injury site. Resident macrophages execute an acute phase response to the inflammatory trigger and release pro-inflammatory cytokines. These, in turn, increase endothelial permeability and enhance endothelial cell production of additional cytokines which cause rapid recruitment of neutrophils to the site of injury (Sadik et al, 2011). In addition, there is evidence that mast cells also contribute to leukocyte recruitment by producing histamine, leukotrienes and prostaglandins resulting in increased vascular permeability and permitting the rapid influx of neutrophils and other leukocytes. Through interaction with lung epithelial cells, neutrophils produce lipoxins which interact with their receptor(s) to regulate neutrophil apoptosis and promote phagocytosis of apoptotic neutrophils by monocyte-derived macrophages in order to clear the inflamed tissue (Barnig & Levy, 2015; Levy & Serhan, 2014; Serhan et al, 2007). At the same time, neutrophil-derived potent anti-inflammatory

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lipid mediators (resolvins and protectins), reduce neutrophil infiltration and enhance macrophage phagocytic capabilities, thus limiting and resolving inflammation (Kohli & Levy, 2009; Levy & Serhan, 2014).

Exposure to cigarette smoke has been reported to induce and potentiate inflammation through a variety of mechanisms involving multiple cell types (Barnes et al, 2003; Stampfli & Anderson, 2009; Thorley & Tetley, 2007). Chronic exposure to cigarette smoke can lead to increased airway cell damage and death, which further exacerbates pulmonary inflammation through the release of damage-associated molecular pattern (DAMP) molecules (Chen & Nunez, 2010; Morbini et al, 2006; Reynolds et al, 2011). Although the exact mechanisms remain to be fully elucidated, current evidence suggests that cigarette smoke can modulate and enhance pulmonary inflammation via several distinct yet partially overlapping mechanisms involving a myriad of different cell types. Over time, the persistence of these mechanisms in the lung may not only lead to the goblet cell metaplasia and hyperplasia/mucus hypersecretion, airway remodeling, and alveolar destruction phenotypes that are characteristic of COPD (Barnes, 2004; Rogers, 2007).

6.1.4.3.1.3 Mechanistic Link between Inflammation and Lung Cancer

Lung cancer in smokers exhibits most, if not all, of the inflammatory processes described to underlie the pathogenesis of COPD, suggesting a shared etiology between the two diseases. Additionally, recent research suggests that cigarette smoking-related chronic lung inflammation can also alter the pro- and anti-inflammatory activity of cells within the inflamed pulmonary environment (Curtis et al, 2007; Hodge et al, 2007; King et al, 1988; Mucida & Cheroutre, 2010; Woodruff et al, 2005). Moreover, the chronic production of ROS by immune cells can lead to DNA damage which can, if not repaired adequately, give rise to mutations and interfere with natural safeguarding processes aimed at eliminating damaged cells. Over time, these mutations can accumulate, providing a survival advantage. The resulting initiated population can then further profit from the multitude of growth-promoting soluble mediators released during chronic inflammation and facilitate tumor growth.

6.1.4.3.1.4 Assessment of Vascular Inflammation in Experimental Models

In Vitro Models

Atherosclerosis is the principal form of cardiovascular disease characterized by inflammatory processes governing the development of atherosclerotic lesions and their progression up until plaque rupture. The complex pathology makes atherogenesis difficult to recapitulate in *in vitro* models. Nevertheless, some aspects can be reasonably well modeled *in vitro* to provide some insights into the biological effects of cigarette smoke and its constituents on the pathogenesis of CVD (Fearon et al, 2013), including:

- activation of the vascular endothelium, and
- leukocyte recruitment, adhesion and transmigration.

PMP have chosen two assays to model processes occurring at an early stage in the process of atherogenesis in smokers:

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Monocyte adhesion to endothelial cells

The adhesion assay examines the rate of leukocyte-endothelial cell adhesion *in vitro* and was previously successfully employed to show increased leukocyte adhesion via upregulation of the cellular adhesion molecules ICAM-1 and VCAM-1 following treatment of endothelial cells with cigarette smoke extract (CSE) or cigarette smoke condensate (CSC) (Chen et al, 2014; Giunzioni et al, 2014; Kalra et al, 1994; Rius et al, 2013; Shen et al, 1996).

In addition, an indirect approach using conditioned cell culture medium from CSE-treated monocytes also showed that soluble inflammatory mediators such as TNFα are released from the activated leukocytes and act as potential stimuli inducing cell adhesion molecule expression on endothelial cells, thus resulting in increased leukocyte adhesion (Poussin et al, 2014; Poussin et al, 2015; Zhang et al, 2002).

Transendothelial migration (TEM assay)

The TEM assay showed increased leukocyte migration rates through an endothelial monolayer when monocytes and endothelial cells were directly treated with CSC or when endothelial cells were incubated with conditioned medium from CSC-stimulated monocytes, but not when monocytes were directly pretreated with CSC (Giunzioni et al, 2014; van der Toorn et al, 2015b). PMP therefore believes that the two *in vitro* assays may provide valuable insights into the proinflammatory effects of candidate MRTPs in the context of critical steps preceding the development of atherosclerotic plaques.

In Vivo Models

While providing significant new insights into mechanisms underlying atherogenesis in man, *in vitro* models can never fully reproduce the interplay between the different cell types in an organism and the spectrum of biological processes occurring during the development of atherosclerosis. However, many gaps in our understanding of these processes can be filled by knowledge gained from animal models, and CVD research has employed such models since the early 1900s (Leong et al, 2015).

While atherosclerosis can be observed in many species fed a high-fat diet, the mouse remains the most frequently used animal model owing to its easy maintenance and low cost (Getz & Reardon, 2012). However, mice are rather resistant to diet-induced atherogenesis. It is known that – unlike in humans – HDL cholesterol is the main lipoprotein in mouse and mice do not express cholesteryl ester transfer protein, a plasma protein facilitating exchange of esterified cholesterol and triglycerides from lipoproteins (Getz & Reardon, 2012). In addition, atherosclerotic lesions in the mouse also differ from human disease in other aspects: They preferentially form in the aortic root and typically do not develop a fibrous cap, so unstable plaques are uncharacteristic (Getz & Reardon, 2012). Most of these differences, however, are addressed by genetically modified mice such as the *Apoe* knockout (*Apoe*$^{-/-}$) model, which has become the most widely studied murine model of atherosclerosis.

Experimental evidence suggests that cigarette smoke exposure accelerates atherosclerosis in *Apoe*$^{-/-}$ mice, with increased intimal thickness, plaque size and plaque area in exposed relative to control animals (Catanzaro et al, 2007; Gairola et al, 2001; Gairola et al, 2010;

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Kunitomo et al, 2009; Schroeter et al, 2008; Tani et al, 2004; Von Holt et al, 2009). Importantly, in *Apoe*$^{-/-}$ mice that were first exposed to cigarette smoke for 3 months before being switched to air exposure for a further 3 months (mimicking smoking cessation), aortic plaque area was smaller than that in mice exposed to cigarette smoke for 6 months, with aortic arches containing approx. 50% less total cholesterol and overall decreased lipid levels (by lipid class) in plaques and plasma (Lietz et al, 2013), indicating that this mouse model also capitulates the cardiovascular benefits of smoking cessation that is seen in man. Furthermore, we and others also noted a distinct pulmonary inflammatory response to cigarette smoke in the *Apoe*$^{-/-}$ mouse, characterized by influx of leukocytes into the lungs and elevated levels of proinflammatory mediators in the bronchoalveolar lavage fluid (BALF), eventually culminating in alveolar destruction similar to that of emphysema (Arunachalam et al, 2010; Boué et al, 2013), suggesting that the *Apoe*$^{-/-}$ mouse model may also prove useful as a cigarette smoke-induced COPD model.

6.1.4.3.1.5 Assessment of Lung Inflammation in Experimental Models

In Vitro Models

Current knowledge about the role of inflammation in cigarette smoking-related lung disease is built on decades of research into the molecular underpinnings of specific inflammatory processes, including epithelial proinflammatory responses or macrophage activation following exposure of individual cell types to cigarette smoke (de Boer et al, 2000; Mio et al, 1997; Murugan & Peck, 2009; Parsanejad et al, 2008; Russell et al, 2002). There are, however, no defined *in vitro* models of COPD that are capable of recapitulating the complexity of the disease. More recently, the availability of systems-wide technologies (e.g., transcriptomics, proteomics, and metabolomics) have enabled pulmonary inflammation following cigarette smoke exposure *in vivo* or *in vitro* to be analyzed using complex, global data sets. These investigations have made significant inroads into our understanding of the temporal and cell-type specific complexities of lung inflammation, especially when applied to humans (Adair-Kirk et al, 2008; Heguy et al, 2006).

There is a large number of studies examining the effects of cigarette smoke in lung epithelial cells that are either genetically immortalized or cancerous, simply because of their legacy value and ease of maintenance. These studies have demonstrated, albeit with varying success, that lung epithelial cells respond to acute cigarette smoke exposure by producing increased amounts of proinflammatory mediators (Lee et al, 2015; Li et al, 2012; Puljic & Pahl, 2003; Richter et al, 2002; Starrett & Blake, 2011; Victoni et al, 2014). However, there is growing criticism as to the validity of continuous cell lines in disease modeling (Adamson et al, 2011), in particular since observed biological effects in response to various stimuli are now known to differ considerably from those seen in primary human airway cells (Ghio et al, 2013; Ioannidis et al, 2013; Koning et al, 2012; Lopez-Souza et al, 2004).

Moreover, some of the early effects of cigarette smoke exposure appear to be more adequately reflected in primary (rather than immortal) lung epithelial cells grown under submerged conditions *in vitro*, as evidenced by comparison to epithelial cells obtained

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from bronchial brushings performed in smokers (Dvorak et al, 2011; Martinez-Anton et al, 2013). Nevertheless, classical, submerged cultures of primary bronchial epithelial cells permit limited conclusions as several structural properties, such as the polarization of cells, are lost *in vitro* (Paquette et al, 2003). An alternative that is finding increasing use for the testing of inhaled toxicants exists: Raising a confluent layer of primary large or small airway epithelial cells – grown on a porous membrane in the presence of essential growth factors, hormones and micronutrients – to the air-liquid interface induces terminal differentiation and ultimately leads to the formation of a pseudostratified airway epithelium that closely resembles that seen in the human airways (Gray et al, 1996; Jetten, 1989; Paquette et al, 2003). Well differentiated cultures created in this fashion consist of columnar epithelial, goblet, ciliated and basal cells. They exhibit tight junctions, display functional cilia and are capable of mucus production which accumulates at the apical surface, atop a thin layer of airway surface liquid (Adamson et al, 2011; Prytherch et al, 2011). The functional and morphological similarities of these cultures to human airway epithelium, for this reason also called organotypic cultures, make them prime candidates for the *in vitro* evaluation of cigarette smoke and its fractions.

Ready-to-use bronchial organotypic cultures such as EpiAirway™ (AIR100; MatTek Corp., Ashland, MA, USA) and MucilAir™ (Epithelix SàRL, Geneva, Switzerland) are now commercially available and have been PMI's primary choice of *in vitro* system for the past few years. Using the EpiAirway™, we could demonstrate that cigarette smoke exposure causes changes in gene and microRNA expression that resembled those seen in epithelial cells collected from large and small airways of current and former smokers. These were characterized by a distinct dose- and time-dependent oxidative stress response resulting in NF-κB inhibition, a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response (Kuehn et al, 2015; Mathis et al, 2015; Mathis et al, 2013). Additional tests with the full-thickness (fibroblast-containing) bronchial and nasal MucilAir™ systems demonstrated significant increases in the levels of multiple proinflammatory cytokines and chemokines at 24 hours following repeated cigarette smoke exposure (Talikka et al, 2014). A marked increase in soluble mediators of inflammation and tissue remodeling was also seen in the basal medium of bronchial MucilAir™ cultures without fibroblasts at 48 hours following exposure (Iskandar et al, 2015). Together these data suggest that organotypic cultures are a suitable *in vitro* system with which to model acute inflammatory responses to cigarette smoke exposure in bronchial organotypic cell cultures.

In Vivo Models

Many cellular and molecular mechanisms involved in the pathogenesis of COPD in humans are also observed in murine pulmonary emphysema models (Brusselle et al, 2006), with several features shared between human early stage disease and mouse disease development which primarily recapitulates the emphysema component (Churg et al, 2011). Lesions in mice caused by chronic cigarette smoke exposure resemble mild centrilobular human emphysema, both morphologically and physiologically (Churg et al, 2008). Small airway remodeling, an important cause of airflow obstruction in cigarette

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smokers with COPD (Hogg et al, 2004), is also observed in cigarette smoke-exposed mice (Churg et al, 2008). In addition, exposure activates the innate immune response cascade in mice, leading to protease/anti-protease imbalances in lung tissue and eventual alveolar wall degradation, suggesting that murine models can also replicate these features of human COPD (Yoshida & Tuder, 2007). Finally, as observed in humans, the small airways of cigarette smoke-exposed C57BL/6 mice exhibit persistent up-regulation of type I pro-collagen and pro-fibrotic cytokine gene expression, which would be expected to contribute to airway remodeling (Churg et al, 2006).

However, murine models of COPD/emphysema have several limitations:

- First, these models only display a subset of disease phenotypes and do not fully recapitulate airflow limitation caused by chronic small airway obstruction, which defines COPD (Hogg et al, 2004).
- Second, there are considerable differences in respiratory physiology and anatomy between mice and humans making disease modeling difficult. In contrast to humans, mice are obligate nose breathers and filter tobacco smoke differently; the submucosal glands in mice are limited to the trachea and mice lack a cough reflex, (Groneberg & Chung, 2004). Importantly, pulmonary emphysema in humans is classified according to the distribution of the airspace enlargement within the acini in centrilobular emphysema, and in cigarette smokers with COPD, significant airspace enlargement occurs primarily across the three generations of respiratory bronchioles (Hogg et al, 2004). However, rodents lack clearly defined respiratory bronchioles and distinct lobular architecture, further obscuring anatomically equivalent comparisons of disease development (March et al, 2000).
- Third, important differences between mice and human exist in the innate and adaptive immune responses, which play a critical role in the initiation and maintenance of disease. These include differences in the expression of defensins, toll-like receptors, chemokine receptors and a unique repertoire of active chemokines in each species. Differences in adaptive immunity include immunoglobulin subtypes, Th1/Th2 differentiation dynamics, and the roles and ratios of $CD4^+$ and $CD8^+$ T cell populations, together with co-stimulatory molecule expression and function (Mestas & Hughes, 2004). The theory of an autoimmune component in advanced COPD contributing to the self-perpetuating nature of the disease in man could not be corroborated in mice (Taraseviciene-Stewart et al, 2005).
- Fourth, unlike in humans, cigarette smoke exposure-induced emphysema in mice does not progress after smoking cessation, and mice never develop spontaneous exacerbations.
- Finally, mice do not develop more severe COPD symptoms characteristic of the human GOLD stages III and IV.

Despite these anatomical and physiological differences, several studies have shown that chronic exposure of mice to cigarette smoke results in reduced lung function, sustained pulmonary inflammation and emphysema with features resembling those underlying

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human COPD pathophysiology (Awji et al, 2015; Churg et al, 2009; Kang et al, 2007; March et al, 2006; Phillips et al, 2015; Sato et al, 2008; Stinn et al, 2013).

6.1.4.3.1.6 Assessment of Inflammation in Clinical Studies

There are numerous inflammation biomarkers currently used in clinical research (Myers et al, 2004; Pearson et al, 2003; Vasan, 2006), yet none of them is truly specific for inflammation caused by smoking. White blood cell (WBC) count, C-reactive protein (CRP), fibrinogen and soluble cell adhesion molecules have been proposed as biomarkers of cigarette smoke-related cardiovascular harm in the context of tobacco product assessment (Stratton et al, 2001). Multiple studies suggest that these biomarkers are linked to serious cardiovascular events and may provide an insight into cardiovascular disease risk in long-term smokers (MacCallum, 2005; Upadhyay, 2015).

Similarly to its role in CVD, inflammation is also well established in lungs of smokers by, for example, increased numbers of leukocytes as well as increased concentrations of proinflammatory mediators in the BALF (1990; Burke et al, 1992; Burnham et al, 2013; Capelli et al, 1999; Karimi et al, 2012; Kuschner et al, 1996; McCrea et al, 1994; Nagai et al, 2005; Rennard et al, 1990; Rennard et al, 2002), induced sputum (Costa et al, 2008; Rovina et al, 2007) and in the circulation (Kuhlmann et al, 2013; Thorleifsson et al, 2009; Vestbo et al, 2011). Of interest, higher WBC counts in smokers than non-smokers have been correlated with decreased forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC), two indicators of lung function (Bridges et al, 1985; Fernández et al, 2012; Sparrow et al, 1984). While it is currently unclear whether any of these blood-based markers have utility in predicting the risk of COPD development arising from cigarette smoke-related chronic lung inflammation, they are easily assessable in the clinical setting and have proven useful as general indicators of the extent of systemic inflammation in smokers.

As described above, the disorders induced by smoking are complex, with multiple causal chains and the diseases develop over a period of many years, no one single clinical risk endpoint or biomarker, in and of itself, can be considered as a surrogate measure for the adverse health effects associated with smoking-related diseases. The exposure to HPHCs affects multiple organ systems, disease pathways, and mechanisms such as inflammation, oxidative stress, platelet activation and lipid metabolism, which occur simultaneously and cannot be expressed by a single endpoint. Therefore, clinical risk endpoints and intermediate effects that can be reasonably measured within the timeframe of a premarket assessment program are used to provide collective evidence about the modification of the risk profile of the candidate MRTP.

Therefore, the potential clinical risk endpoints[1] that can be tested in a controlled clinical study setting, should be:

[1] A clinical risk endpoint in this context is defined as a measure of a biological process, physiological system, and/or a mechanism of action that is associated with or known to contribute to smoking-related diseases.

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- associated with clinical events of interest,
- affected by smoking,
- affected by smoking cessation (reversible).

Many of the clinical risk endpoints included within the Smokers' Health Profile are also mentioned in the 2010 Surgeon General's Report "How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease"(USDHHS, 2010b).

The clinical risk endpoints collected in the Reduced Exposure studies are not independent from one another, but rather work together with the non-clinical results to form a system that facilitates the risk assessment of a candidate MRTP. The strength of the evidence lies in how these measures demonstrate a coherent profile that reflects the smoking cessation-induced effects and to what extent they provide mutual evidentiary support for each other.

Each clinical risk endpoint was assessed against a set of evidentiary prerequisites to ensure consistent use of current best evidence derived from published clinical and epidemiological research. These evidentiary prerequisites were:

- Epidemiological evidence suggesting a robust relationship between each clinical risk endpoint and at least one known smoking-related health outcome
- Clinical evidence linking cigarette smoking to the clinical risk endpoint (consistent with the epidemiological evidence)
- Clinical evidence linking smoking cessation to the clinical risk endpoint, and evidence indicating that the clinical risk endpoint is reversible following smoking cessation (consistent with the epidemiological evidence)

The clinical risk profile of the THS is informed by mechanisms that quantify the biological impact of exposure. Defined biological network models that represent the key mechanisms in the onset and progression of smoking-related diseases permit a systematic and quantitative mechanistically based comparison of switching to the candidate MRTP versus continuing to smoke cigarettes.

In the THS clinical assessment program, the candidate MRTP is compared to continuing to smoker cigarettes, but in order to interpret the clinical relevance of the changes the risk profile is benchmarked against the established evidence for smoking cessation and the data from the clinical studies following smoking abstinence.

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6.1.4.3.1.6.1 White Blood Cell (WBC) Count

The WBC count (the enumeration of all leukocytes in a blood sample) is a routine clinical assessment employed to aid in the diagnosis of many diseases. While the test does not allow for a conclusion to the underlying cause, an elevated WBC count is indicative of a local or systemic inflammation, triggered by an infection, injury or exposure to noxious agents such as cigarette smoke.

Since leukocytes are involved in atherogenesis, many studies have investigated the ability to use WBC counts to predict risk of cardiovascular diseases and confirmed an association between elevated WBC count and coronary artery atherosclerosis (Huang et al, 2001; Ikata et al, 2000; Muscari et al, 1994) and peripheral artery disease (PAD) (Giugliano et al, 2010). In addition, an elevated WBC count was found to be predictive of myocardial infarction (Barron et al, 2000; Friedman et al, 1974; Yarnell et al, 1991), stroke (Koren-Morag et al, 2005; Lee et al, 2001; Prentice et al, 1982) and mortality (Brown et al, 2001; Gillum et al, 1993; Held et al, 2000), independent of other known risk factors including gender, hypercholesterolemia or smoking history.

Smoking has been shown to lead to an increase in WBC count and there is a correlation between WBC count and lifetime exposure to cigarette smoke (Helman & Rubenstein, 1975; Smith et al, 2003; Van Tiel et al, 2002) and smoking intensity (Carel & Eviatar, 1985; Lakshmi et al, 2014; Van Tiel et al, 2002; Whitehead et al, 1995). More importantly, smoking cessation leads to a sharp decline in the WBC count in as little as 6-12 months following quitting (Friedman & Siegelaub, 1980; Hansen et al, 1990; Jensen et al, 1998; Smith et al, 2003), suggesting that the WBC count can be used as a clinical risk endpoint for general inflammation in smokers switching to a MRTP.

Appendix A6.1.4.1 provides a detailed literature and data review of the association of WBC count with smoking-related diseases, the relationship between WBC count and smoking, as well as the reversibility of smoking-related findings upon smoking cessation.

6.1.4.3.1.6.2 C-Reactive Protein (CRP)

C-reactive protein (CRP) is one of the most widely studied and employed inflammatory markers. This acute phase reactant is rapidly induced in the liver following a rise in circulatory IL-1β, IL-6 and TNFα levels. As a result, CRP levels markedly increase in the circulation. Whether or not CRP plays a direct role in atherogenesis is still under debate (Zimmermann et al, 2014). However, elevated CRP levels have been shown to correlate with the risk of myocardial infarction, stroke, peripheral artery disease, and recurrent cardiac events (Zakynthinos & Pappa, 2009). Using high-sensitive (hs) immunoassays, CRP levels were found to be increased in smokers compared with non-smokers in some studies (Frost-Pineda et al, 2011; McEvoy et al, 2015; Ohsawa et al, 2005; Wannamethee et al, 2005), but not in others (Fröhlich et al, 2003; Helmersson et al, 2005; Nordskog et al, 2015). There are known confounders affecting CRP measurements such as gender, ethnicity, BMI, alcohol consumption and various drugs which could, at least in part, explain these discrepancies (Albert et al, 2003; Greenfield et al, 2004; Ledue & Rifai, 2003; Macy et al, 1997; Ray et al, 2005; Ridker et al, 1999; Silvestri et al, 2003). However, the determination of CRP levels is frequently included in blood tests in the clinical setting, and a joint working group of the Centers for Disease Control and

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Prevention (CDC) and the American Heart Association (AHA) recommended its use as an "adjunct to the major risk factors to further assess absolute risk for coronary disease primary prevention" and, optionally, as a means of identifying "patients without known CVD who may be at higher absolute risk than estimated by major risk factors" (Pearson et al, 2003). Therefore, CRP may be a useful biomarker for the assessment of biological effects of an MRTP.

Since CRP levels were shown to decline over time following smoking cessation, exhibiting a trend of larger reductions with increasing time since quitting (Lowe et al, 2009; Ohsawa et al, 2005; Wannamethee et al, 2005), suggests that although CRP may be a good clinical risk endpoint, it will be more informative in studies with longer exposure and follow-up period (i.e., greater than 6-12 months).

Appendix A6.1.4.2 provides a detailed literature and data review of the association of CRP with smoking-related diseases, the relationship between CRP and smoking, as well as the reversibility of the CRP smoking-related findings upon smoking cessation.

6.1.4.3.1.6.3 Soluble Intercellular Adhesion Molecule 1 (sICAM-1)

ICAM-1 is a glycoprotein constitutively expressed on the surface of endothelial cells and leukocytes. ICAM-1 expression can be induced by pro-inflammatory cytokines which are also thought to signal its shedding from the membrane so that it can be detected in cell culture supernatants and serum or plasma (Tsakadze et al, 2004; Tsakadze et al, 2006). Its importance in inflammatory processes is evidenced by its interactions with lymphocyte function-associated antigen through which it facilitates neutrophil adhesion and transmigration in response to vascular injury (Futosi et al, 2013). ICAM-1 has also been implicated in atherogenesis, atheroma growth and plaque rupture (de Boer et al, 1999; Kitagawa et al, 2002; Lawson & Wolf, 2009; Watanabe & Fan, 1998), and increased sICAM-1 levels have been shown to independently predict carotid artery atherosclerosis and incident coronary heart disease (Hwang et al, 1997), PAD (Pradhan et al, 2002; Pradhan et al, 2008), and future cardiovascular events and their outcomes (Blankenberg et al, 2001; Mulvihill et al, 2001; Ridker et al, 1998).

Smokers have significantly higher plasma ICAM-1 levels than non-smokers, and sICAM-1 levels have been shown to decline within 4 to 6 weeks following cessation (Bermudez et al, 2002; Blann et al, 1997; Levitzky et al, 2008; Palmer et al, 2002; Scott et al, 2000). Although care needs to be taken when comparing results, as the most frequently employed immunoassays depend on antibodies incapable of recognizing all population variants of the adhesion molecule (Register et al, 2004), sICAM-1 is considered to be a useful and reliable clinical risk endpoint in the assessment of vascular inflammation in smokers switching to an MRTP.

Appendix A6.1.4.3 provides a detailed literature and data review of the association of sICAM-1 with smoking-related diseases, the relationship between sICAM-1 and smoking, as well as the reversibility of the sICAM-1 smoking-related findings upon smoking cessation.

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6.1.4.3.1.6.4 Fibrinogen

Like CRP, fibrinogen is an acute phase protein produced in the liver and increases quickly and markedly in the circulation in response to vascular injury, infection and inflammation. Fibrinogen plays an essential role in coagulation, mediating fibrin assembly and platelet recruitment to form a platelet plug thus limiting bleeding and promoting wound healing (Mosesson, 2005). Fibrinogen also interacts with ICAM-1 resulting in increased endothelin-1 (ET-1) production in activated endothelial cells thereby contributing to vasoconstriction and endothelial cell survival (Pluskota & D'Souza, 2000; Sen et al, 2009). However, it is its ability to interact with platelets via P-selectin and the Mac-1 integrin receptor on leukocytes, predominantly monocytes, that provides a connection between the coagulation cascade and the inflammatory response, ultimately resulting in the production of pro-inflammatory mediators (Davalos & Akassoglou, 2012). In addition, there is evidence for a direct link between fibrinogen and vascular disease (Davalos & Akassoglou, 2012), and fibrinogen levels have been shown to be predictive of the development of peripheral arterial disease (PAD) and future cardiovascular events including myocardial infarction and stroke (Davalos & Akassoglou, 2012; Engström et al, 2009; Feher et al, 1990; Heinrich et al, 1994; Maresca et al, 1999; Rothwell et al, 2004; Wilhelmsen et al, 1984).

Smoking has been associated with higher plasma fibrinogen levels and smoking cessation has been shown to result decline of circulatory fibrinogen over time (Bakhru & Erlinger, 2005; Dobson et al, 1991; Haustein et al, 2004; Heinrich et al, 1994; Lam et al, 1999; Meade et al, 1987; Sinha et al, 2005). However, the differences in the levels of fibrinogen between smokers and non-smokers is rather small and have not demonstrated a strong dose-repose relationship, making fibrinogen a potential, but not strong clinical risk endpoint for MRTP assessment in clinical studies. However, the role of fibrinogen in the pathophysiology of major smoking-related diseases leads to considering its use in large epidemiological studies.

Appendix A6.1.4.4 provides a detailed literature and data review of the association of fibrinogen with smoking-related diseases, the relationship between fibrinogen and smoking, as well as the reversibility of the fibrinogen smoking-related findings upon smoking cessation.

6.1.4.3.2 Oxidative Stress

Cigarette smoke is a complex and dynamic mixture consisting of more than 8,000 unique chemical compounds (Perfetti, 2011). It is very likely that the biological effects exerted by cigarette smoking are related to the complex interaction of these compounds and their metabolites with each other and with various cells, including those of the respiratory tract and the cardiovascular system where smoke exposure elicits a series of adaptive cellular stress responses including oxidative stress responses (Müller & Gebel, 2006).

Oxidative stress results from an imbalance between the levels of ROS and antioxidants. ROS and other reactive species act directly on cellular macromolecules damaging lipids, proteins and DNA. In general, cells respond to oxidative stress by activation of the transcription factor Nrf-2 (encoded by the nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) gene), a key regulator of detoxification and antioxidant genes (Ishii et al,

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2002). When expressed, the products of these genes then either act as quenchers to eliminate the oxidative insult or contribute to the regulation of downstream effectors that determine cell fate based on the extent of damage. For example, oxidative damage is also known to elicit a number of other cellular responses triggering various protein kinase signaling cascades, most notably those of the mitogen-activated protein kinase (MAPK) family, resulting in proliferation, cell growth arrest, senescence or cell death, typically depending on the type of stressor and the duration and extent of stress (Martindale & Holbrook, 2002).

6.1.4.3.2.1 Mechanistic Link between Oxidative Stress and Cardiovascular Disease

Cigarette smoking is causally linked to the development of CVD by contributing to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation and an abnormally increased tendency toward coagulation. While sustained unfolded protein response (UPR), endoplasmic reticulum (ER) and hypoxic stress have been reported to contribute to all of these disease-related processes, most of the evidence to date focuses on the roles of oxidative stress and inflammation in the development and progression of CVD (Austin, 2009; Kaufman, 2002; Minamino & Kitakaze, 2010; Minamino et al, 2010; Tabas, 2010; Wang & Kaufman, 2012). Both gas and particulate (tar) phase of cigarette smoke contain high levels of oxidants, with an estimated 10^{15} radicals per puff and 10^{17} radicals per gram of tar (Pryor, 1992; Pryor, 1997). Many of these free radicals, but also endogenously generated ROS and RNS, are the prime mediators of endothelial dysfunction in smokers, a key event in the development of atherosclerosis (Salahuddin et al, 2012).

The healthy endothelium produces a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation and vessel wall inflammation in response to various inflammatory and oxidative stimuli (Münzel et al, 2008), and the endothelium is maintained in a quiescent state through the inhibitory signaling of nitric oxide (NO) (Deanfield et al, 2007). When the endothelium is injured, a host response is evoked which involves a switch from the NO-mediated silencing of cellular processes to redox signaling (Mudau et al, 2012; Yang et al, 2006). Endothelial nitric oxide synthase (eNOS), the source of NO under physiological conditions, becomes uncoupled and produces superoxide O_2^- which can interact with NO, thus reducing its bioavailability, while forming the highly reactive peroxynitrite $ONOO^-$ and adding to the overall oxidant burden (Nedeljkovic et al, 2003; Versari et al, 2009). The activated endothelium expresses surface molecules that attract platelets and mediate platelet rolling and adhesion, leading to platelet activation and the subsequent release of soluble mediators that in turn promote the recruitment of monocytes to the site of inflammation, platelet aggregation, coagulation, cell survival and proliferation (Gawaz et al, 2005). In this complex cascade of events, oxidative stress has been identified as a contributing factor to each one of these steps (Cai & Harrison, 2000; Förstermann, 2010; Schulz et al, 2004; Thomas et al, 2008).

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6.1.4.3.2.2 Mechanistic Link between Oxidative Stress and COPD

The development of COPD in smokers has been linked to a number of sustained cellular stress responses, such as the exposure of lung epithelial cells to cigarette smoke *in vitro* causing an increase in intracellular levels of ubiquitin-protein conjugates suggesting extensive protein damage. At the same time, and although nascent protein synthesis decreases, a decrease in proteosomal activities leads to accumulation of proteins in the ER and ER stress ensues (Malhotra et al, 2009; Somborac-Bačura et al, 2013). Moreover, unresolved ER stress triggers several components of the UPR and results in the activation of anti-apoptotic, metabolic and oxidative stress effector pathways (Harding et al, 2000; Harding et al, 2003; Hengstermann & Müller, 2008; Jiang & Wek, 2005; Jorgensen et al, 2008; Teske et al, 2011). However, the fate of cigarette smoke-exposed lung epithelial cells in COPD smokers can also be determined by DNA damage responses as a consequence of a rise in oxidative DNA and RNA lesions.

As a result, DNA repair pathways that utilize a specific set of enzymes able to recognize the type and extent of damage are initiated to re-establish DNA duplex continuity. However, if DNA damage is too severe, programmed cell death mechanisms are executed (Aoshiba et al, 2012; Hu et al, 2009; Zeng et al, 2012), and more recent studies suggest that autophagic cell death also plays an important role in COPD (Chen et al, 2008; Hwang et al, 2010; Kim et al, 2008). Moreover, cellular senescence induced by exposure to oxidants from cigarette smoke may contribute to the enhancement of COPD by augmenting inflammatory processes and alveolar tissue degradation (Nyunoya et al, 2013; Nyunoya et al, 2006; Tsuji et al, 2006). Most if not all of these stress responses are executed as a response to oxidative insults that arise from direct exposure to cigarette smoke and cause tissue injury through oxidative stress. At the same time, cigarette smoke-related oxidative stress also leads to the attraction and activation of immune cells and the release of pro-inflammatory mediators from these cells which in turn produce a number of (endogenous) oxidative stressors capable of perpetuating an inflammatory response.

6.1.4.3.2.3 Mechanistic Link between Oxidative Stress and Lung Cancer

Direct damage to DNA by ROS and RNS is also an accepted explanation for the initiation of events that ultimately lead to lung cancer (Halliwell, 2007). It is well known that the interaction of DNA with the hydroxyl radical leads to the formation of mutagenic purines and pyrimidines such as 8-OHdG, one of the most abundant and readily-formed oxidized DNA bases and able to induce G:T transversions, which are among the most frequent mutations in human cancers (Pilger & Rüdiger, 2006; Valavanidis et al, 2009). Oxidative damage to proteins can also promote mutagenesis, in particular when proteins involved in DNA replication or repair or signal transducers regulating cell cycle and proliferation are affected. Finally, ROS also act on lipids causing their peroxidation, in the process generating a number of highly reactive intermediary carbonyl compounds that form DNA-adducts and hence give rise to mutagenic lesions (Fayolle et al, 2007; Fearon et al, 2011; Kinoshita et al, 2007; Matés et al, 2008).

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In addition to the deleterious effects on cellular macromolecules, ROS can also act as second messengers through the regulation of transcription factors such as NF-κB and AP-1, ultimately governing cell proliferation, angiogenesis and cell transformation (Federico et al, 2007; Naugler & Karin, 2008a; Storz, 2005). Furthermore, ROS and RNS can trigger a variety of cell stress signaling cascades by activating members of the MAPK family such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERKs), thus modulating responses to apoptotic cues (Benhar et al, 2002; Cuda et al, 2002; Shen & Liu, 2006; Siebel et al, 2013). Finally, the oxidative stress exerted by exposure to cigarette smoke also elicits a strong Nrf-2-mediated response which may, in a pro-tumor environment, further enhance cell survival and proliferation, thus contributing to tumor formation and progression (Müller & Hengstermann, 2012).

6.1.4.3.2.4 Assessment of Oxidative Stress in Experimental Models

6.1.4.3.2.4.1 In Vitro Models

While ROS can be directly and reliably measured by electron spin resonance or radical trapping, *in vitro* assays traditionally rely on their ability to readily bind to or react with a reporter molecule yielding stable, often chromogenic, chemiluminescent or fluorescent products to determine the presence and extent of oxidative stress (Fearon & Faux, 2009; Kohen & Nyska, 2002). Alternatively, formation of oxidative DNA lesions, lipid peroxidation products or protein modifications resulting from interaction of ROS with intracellular macromolecules can be utilized to assess oxidative stress *in vitro* and *in vivo*. There are also a large number of toxicity assays measuring various endpoints including the differential regulation of specific cellular pathways. Rapid development, in particular in the course of drug discovery and testing, has made these assays amenable to high-throughput platforms, and it is envisaged that a number of such assays together with sophisticated computational tools will shape the future of toxicity testing (NRC, 2007). Discussing this concept in the context of the cellular stress response, Simmons and colleagues propose an ensemble approach that could provide useful information about pathway activation patterns giving insights into specific stress signatures that are linked to cell fate and mechanisms of toxicity (Simmons et al, 2009).

There are several *in vitro* studies utilizing fluorescing indicator dyes such as dihydroethidium (DHE), dichlorofluorescin diacetate (DCFDA) and dihydrorhodamine (DHR) for the determination of oxidative stress in live cells. These assays are based on the probes' interaction with ROS resulting in their oxidation to yield a bright fluorescent product that can be measured using flow cytometry, fluorometry or fluorescence microscopy. Using DCDFA with fluorescence microscopy or flow cytometry, oxidative stress was shown to increase in human bronchial epithelial cells (Kode et al, 2008; Lin et al, 2014; Pace et al, 2013; Tagawa et al, 2008; Tan et al, 2014; Wu et al, 2014), nasal epithelial cells (Pace et al, 2014), lung fibroblasts (Carnevali et al, 2003), cardiac myocytes (Yamada et al, 2009) and vascular endothelial cells (Edirisinghe et al, 2008; Michaud et al, 2006; Milara et al, 2010) following acute (short-term) treatment with CSE. Oxidative stress also increased in a dose- and time-dependent manner in lung epithelial cells treated with cigarette smoke total particulate matter (TPM) (Faux et al, 2009), and in bronchial epithelial cells cultured at the air-liquid interphase (ALI) and exposed to whole smoke (Scheffler et al, 2015). Recent technical advances have made high-throughput

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screening with indicator probes easy, fast and cost-effective, allowing for the examination of intracellular ROS loads in simple monolayer cell cultures under various conditions, and PMP has chosen this approach for their non-clinical assessment studies.

In addition, PMP has also selected an additional assay, the determination of intracellular glutathione (GSH), using a similar high-throughput technology, to provide a complementary readout for oxidative stress *in vitro*. Glutathione is an essential intracellular tripeptide with potent free radical scavenging and detoxifying capabilities which derive from its cysteine thiol moiety. GSH exists in cells in its reduced form, making up more than 95% of the total GSH pool. Interaction with ROS oxidizes GSH to GSH disulfide (GSSG), which can be recycled to GSH by the action of glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), and therefore, the ratio of GSH to GSSG can be considered as a good indicator of oxidative stress (Owen & Butterfield, 2010). Decreased cellular GSH content and GSH:GSSG ratio, implying a strong oxidative stress response, has been observed in human lung epithelial cells, alveolar macrophages and lung fibroblasts treated with CSE or CSC (Carnevali et al, 2003; Hodge et al, 2011; Lau et al, 2011; Luppi et al, 2005; Rahman et al, 1995; van der Toorn et al, 2007).

Furthermore, assays indicative of oxidative DNA damage can be employed to elucidate the differential effects of exposures to novel tobacco products as compared with conventional products. One common example is the *in vitro* γH2AX assay. This assay, using flow cytometry or high-content analysis, was previously proposed as a valuable tool to assess DNA damage *in vitro*, in particular in the context of assessing cigarette smoke genotoxicity (Garcia-Canton et al, 2012). In response to DNA damage, a complex series of events known as the DDR becomes activated. The function of the DDR is to arrest cell cycle progression and division to prevent transfer of DNA damage to progeny cells, to engage the DNA damage repair mechanisms and to activate the apoptotic pathway in order to eliminate cells with excessive DNA damage that cannot be repaired (Zhao et al, 2009). One of the earliest DDR events is the activation of H2AX as a result of DNA double-strand breaks (DSBs) (Rogakou et al, 1998). This is quickly followed by establishment of a signal amplification loop to ensure DDR is executed efficiently at the appropriate location (Nakamura et al, 2010; Paull et al, 2000). Since the formation of γH2AX is rapid and its amount correlates with the amount of DSBs (Nakamura et al, 2010), it has become increasingly attractive as an indicator of DNA damage. Many groups using the *in vitro* γH2AX assay on a high-content platform have shown that exposure of human lung epithelial cells to CS caused an increase in γH2AX in a dose- and time-dependent manner (Albino et al, 2004; Albino et al, 2006; Garcia-Canton et al, 2014; Jorgensen et al, 2010; Tanaka et al, 2007), and similar observations were made for select cigarette smoke constituents when tested individually in primary human bronchial epithelial cells (Garcia-Canton et al, 2013; Gonzalez-Suarez et al, 2014), leading PMP to believe in the value of this assay in non-clinical product assessment.

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6.1.4.3.2.4.2 In Vivo Models

Similar to the assessment of oxidative stress in humans, biomarkers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and F2-isoprostanes can also be measured in animal models to estimate the oxidative damage to DNA and lipids as has been shown in models of acute inflammation or oxidative stress (Basu, 2008). In C57Bl/6 mice acute, one-hour cigarette smoke exposure resulted in a significant increase in BALF levels of 8-OHdG at 1 hour post-exposure, declining to undetectable levels at 24 hours post-exposure. Oxidative lung damage was further confirmed by positive immunostaining for 8-OHdG and 4-hydroxy-2-nonenal (4-HNE), a lipid peroxidation marker, in the bronchiolar and alveolar epithelium of exposed mice (Aoshiba et al, 2003). Increases in lung 8-OHdG levels were further noted in A/J mice exposed to sidestream smoke for 2, 4 and 6 weeks, peaking at ca. 3 hours post-exposure and decreasing over time but never reaching levels observed in sham animals (Thaiparambil et al, 2007). In addition, 8-isoprostane F2 alpha (8-iso-PGF2α) levels were shown to increase in urine of male *Apoe*$^{-/-}$ mice following cigarette smoke exposure for 4 days (Catanzaro et al, 2007), in urine and serum of male Sprague-Dawley rats exposed to mainstream cigarette smoke for 56 days (Chan et al, 2009) or sidestream smoke for 4 days (Visioli et al, 2000), and in serum of female Hartley guinea pigs following a 6-month cigarette smoke exposure (Churg et al, 2012). Furthermore, one study showed not only elevated 8-OHdG and 8-iso-PGF2α BALF levels, but also an increase in BALF 3-nitrotyrosine (3-NT), a marker of oxidative protein damage, when female Balb/c mice were exposed to cigarette smoke for 5 days (Guan et al, 2013). There was also a significant induction of lung catalase and reduction of lung glutathione reductase (GR) activities, suggesting a considerable oxidative burden in the respiratory tract upon cigarette smoke exposure (Guan et al, 2013). Although limited in number, these studies indicate that 8-OHdG and F2-isoprostane can be successfully employed in animal models as indicators of oxidative stress.

6.1.4.3.2.5 Assessment of Oxidative Stress in Clinical Studies

To our knowledge, there is no single biomarker capable of reflecting the complexity of cellular stress responses to exogenous stressors such as cigarette smoke in man. Therefore, in order to be able to monitor cellular stress responses in smokers, a combination of multiple biomarkers is required, and this combination will most likely consist of markers traditionally employed to measure e.g., oxidative stress and multi-molecule signatures or profiles derived from large scale data sets such as those provided by transcriptomics or proteomics.

In the context of tobacco product assessment, the 2001 IOM report "Clearing the Smoke" provides a starting point for the selection of biomarkers that are reflective of cigarette smoke-induced harm and might be indicative of heightened disease risk in smokers (Stratton et al, 2001). Among the listed markers are 8-OHdG and F2-isoprostanes for the assessment of oxidative damage to DNA and lipids, respectively. In addition, the report also proposes transcriptomics, proteomics, and methylation analysis as useful tools for the assessment of novel tobacco products. While we do not routinely assess 8-OHdG in non-clinical or clinical studies, we did include the measurement of 8-iso-PGF2α as a measure of oxidative damage in smokers and consumers switching to the MRTP. In

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addition, we also included measurements of thromboxane metabolites as an indicator of oxidative stress in platelets.

6.1.4.3.2.5.1 8-iso-F2-isoprostane-alpha (8-epi-PGF2α)

The peroxidation of arachidonic acid gives rise to isoprostanes, prostaglandin-like compounds produced in tissues by non-enzymatic reactions that are catalyzed by free radicals. A broad variety of isoprostane molecules is generated by oxidative damage *in vivo*, and the various molecules exhibit a broad range of biological activities (Janssen, 2001). The most widely studied group of isoprostanes is the family of F2-isoprostanes with its most well-known member 8-iso-prostaglandin F2 alpha (8-iso-PGF2α, also known as 8-epi-PGF2α), a potent pulmonary and renal vasoconstrictor (Jourdan et al, 1997; Takahashi et al, 1992) and mediator of platelet aggregation (Minuz et al, 1998; Takahashi et al, 1992). Numerous studies have demonstrated that the quantification of F2-isoprostanes by mass spectrometry is the most accurate measure of oxidative stress *in vitro* and in animal models of oxidant injury (Basu, 2008; Milne et al, 2005; Roberts & Morrow, 2000; Visioli et al, 2000). In smokers, plasma and urinary free and esterified F2-isoprostanes were found to be elevated relative to non-smokers (Helmersson et al, 2005; Morrow et al, 1995; Reilly et al, 1996) and rapidly declined upon smoking cessation in some studies (Chehne et al, 2001; Pilz et al, 2000; Reilly et al, 1996), while others reported no changes relative to current smokers (Lowe et al, 2009; Mah et al, 2013). Despite these inconsistencies in findings PMI consider 8-epi-PGF2α to be a useful indicator of smoking-related oxidative stress in clinical studies aimed at the assessment of oxidative stress responses in smokers willing to switch to an MRTP.

Appendix A6.1.4.5 provides a detailed literature and data review of the association of 8-epi-PGF2α with smoking-related diseases, the relationship between 8-epi-PGF2α and smoking as well as the reversibility of the 8-epi-PGF2α smoking-related findings upon smoking cessation.

6.1.4.3.2.5.2 Thromboxane Metabolites

Thromboxanes are members of the eicosanoid family of molecules and are of interest in the context of cigarette smoke-induced alterations of platelet biology and function. Upon activation, platelets release arachidonic acid from the plasma membrane which is then converted into thromboxane A2 (TXA2). TXA2 promotes platelet aggregation, vasoconstriction, and smooth muscle proliferation (Needleman et al, 1976), though it is highly unstable and rapidly converted into the more stable thromboxane B2 (TXB2). TXB2 is released in large quantities from aggregating platelets and is metabolized in the circulation to 11-dehydro TXB2. Levels of 11-dehydro TXB2 in plasma or urine are thought to be reflective of systemic TXA2 production and can be easily assessed by mass spectrometry (MS) analysis (Catella et al, 1986; Lawson et al, 1986). Using this method, significantly higher urinary levels of 11-dehydro TXB2 were measured in smokers than in non-smokers (Calapai et al, 2009; Frost-Pineda et al, 2011; Lowe et al, 2009; Rångemark et al, 1992; Uedelhoven et al, 1991; Wennmalm et al, 1991), and these levels rapidly declined in smokers ceasing to smoke (Rångemark et al, 1993; Saareks et al, 2001; Wennmalm et al, 1993). This responsiveness to changes in cigarette smoke exposure together with the ease of its detection and quantification makes the

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thromboxane metabolite a suitable biomarker candidate for the monitoring of oxidative stress-induced platelet activation.

Appendix A6.1.4.6 provides a detailed literature and data review of the association of DTX8-epi-PGF2α with smoking-related diseases, the relationship between 8-epi-PGF2α and smoking as well as the reversibility of the 8-epi-PGF2α smoking-related findings upon smoking cessation.

6.1.4.3.3 Cardiovascular Disease Risk Endpoints

Cigarette smoking is a well-established, independent risk factor for CVD, but is also known to affect and interact with other cardiovascular risk factors such as altered lipid metabolism, elevated blood pressure, and increased blood glucose (USDHHS, 2010a). The 2001 IOM report also mentions other CVD risk markers including a broad range of hemodynamic parameters and circulating molecules among others as useful indicators of altered endothelial and platelet function in smokers. In addition, the report also proposes transcriptomics, proteomics, and methylation analysis as useful tools for the assessment of novel tobacco products (Stratton et al, 2001).

6.1.4.3.3.1 Assessment of Cardiovascular Disease Risk Endpoints in Clinical Studies

6.1.4.3.3.1.1 Carboxyhemoglobin (COHb)

Carbon monoxide (CO) is produced by the incomplete combustion of organic material, including tobacco. The binding of CO to hemoglobin leads to the formation of carboxyhemoglobin (COHb) and results in less oxygen binding capacity of hemoglobin and therefore reduced transport of oxygen to peripheral tissues. CO-induced adverse effects on the cardiovascular system have been shown in a series of controlled human exposure studies of individuals with CVD. Moreover, studies of chronic environmental exposure to CO are consistent with the results of the controlled human exposure studies, reporting positive associations between ambient CO exposure and hospital admissions for CVD, including ischemic heart disease and congestive heart failure (Silkoff et al, 2006).

Cigarette smoking is known to elevate both respiratory and blood CO levels, and the levels of COHb correlate with CVD risk in non-smokers and smokers (Hedblad et al, 2006; Hedblad et al, 2005; Lind et al, 2004). Following smoking cessation, the levels of COHb decline quickly (Frost-Pineda et al, 2008; Kambam et al, 1986; Roethig et al, 2007), making it a useful endpoint for the assessment of novel tobacco products.

Appendix A6.1.4.7 provides a detailed literature and data review of the association of COHb with smoking-related diseases, the relationship between COHb and smoking as well as the reversibility of the COHb smoking-related findings upon smoking cessation.

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6.1.4.3.3.1.2 Lipid Profile and Oxysterols

Lipids are essential for a number of cellular processes including synthesis of cellular membranes, formation of lipoprotein particles, signal transduction and energy storage among others, and the tight regulation of their metabolism contributes to the maintenance of cellular homeostasis (Liu et al, 2011). Circulating lipoproteins such as very low density lipoprotein (VLDL) and high density lipoprotein (HDL) are particles that transport triglycerides and cholesterol between the liver and peripheral tissues and cells. Various factors interfere with regular lipid shuttling and metabolism leading to an accumulation of lipids in cells, an increase in circulating lipoproteins or their modification.

Cigarette smoking has been linked to high LDL-cholesterol, VLDL-cholesterol and triglyceride and decreased HDL-cholesterol levels. Abnormal lipid profiles have a strong positive correlation with endothelial dysfunction and CVD risk (Craig et al, 1989; Khurana et al, 2000; Mammas et al, 2003; Rao & Subash, 2013; Tang et al, 1997b; Willett et al, 1983). Furthermore, smoking cessation has been associated with a positive effect on the lipid profile, with HDL-cholesterol increasing and LDL-cholesterol and triglyceride levels decreasing in as little as 2-8 weeks following quitting (Allen et al, 1994; Eliasson et al, 2001; Komiyama et al, 2013; Moffatt, 1988; Noh et al, 2012; Tang et al, 1997a).

In addition to triglycerides and cholesterol levels, advanced lipid testing methods providing particle size and number and apolipoprotein (Apo) concentrations are now available to clinicians. ApoA1, a major protein component of HDL-cholesterol, plays a crucial role in cellular cholesterol efflux and transport, a capability that has been characterized as a main contributor to the anti-atherogenic effects of HDL-cholesterol (Ansell et al, 2005). Non-HDL-cholesterol (i.e., VLDL- and LDL-cholesterol) predominantly contains ApoB which mediates its interaction with the vascular endothelium via engagement of the LDL receptor (Olofsson et al, 2007). Since ApoB associates with LDL-cholesterol at a ratio of 1:1, its measurement can provide an insight into the number of circulating LDL particles and hence the level of pro-atherogenic cholesterol levels (Andrikoula & McDowell, 2008). This was also, at least in part, the basis for proposing the ApoB/ApoA1 ratio as an alternative to the LDL/HDL indices (Walldius & Jungner, 2006), and multiple studies have shown strong associations between the ApoB/ApoA1 ratio and risk of coronary artery/heart disease (Boekholdt et al, 2006; Lu et al, 2011; Simons et al, 2009), myocardial infarction (Dunder et al, 2004; Yusuf et al, 2004), heart failure (Ingelsson et al, 2005), and stroke (Kostapanos et al, 2010; O'Donnell et al, 2010). It was also shown to correlate well with early atherosclerosis as indicated by intima-media thickness (Panayiotou et al, 2008) and to perform better than the traditional lipid profile in predicting the risk of CVD and major cardiovascular events (Boekholdt et al, 2006; Ingelsson et al, 2005; Pan et al, 2014; Yusuf et al, 2004). In addition, cigarette smoking has been linked to increased ApoB and decreased ApoA1 levels (Jensen et al, 1995; Meenakshisundaram et al, 2010; Slagter et al, 2013), and successful quitters exhibited higher ApoA1 serum levels than current smokers in as little as 12 weeks (Iwaoka et al, 2014; Richard et al, 1997).

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The initiating event of endothelial injury lies in the accumulation of oxidized or otherwise modified lipids (Siegel-Axel et al, 2008). Oxidatively modified cholesterol species or oxysterols that arise endogenously from the catabolism of cholesterol or are taken up from exogenous sources such as diet are known to augment endothelial cell activation and monocyte/macrophage inflammatory responses, thereby contributing to progression of atherosclerosis (Poli et al, 2013). Some of the most biologically active oxysterols are also among the most abundant serum oxysterols, e.g. 27-hydroxycholesterol, 24(R)-hydroxycholesterol, 4β-hydroxycholesterol and 7α-hydroxycholesterol (Poli et al, 2013; Schroepfer, 2000). Levels of these and other (less abundant) proatherogenic oxysterols such as 25-hydroxycholesterol, 6α-hydroxy-5α-cholestane, 5α,6α-epoxycholestanol, 7-ketocholesterol, 7β-hydroxycholesterol, 22(R)-hydroxycholesterol, and 5β,6β-epoxycholestanol have been shown to be associated with obesity and metabolic syndrome, progression and severity of atherosclerosis, and PAD (Brown & Jessup, 1999; Brown et al, 1997; Chang et al, 1997; Dyer et al, 1997; Schroepfer, 2000; Virginio et al, 2015). How cigarette smoking affects circulating oxysterol is, however, not very well described. While some studies have found elevated levels of cholesterol oxidation products in smokers relative to non-smokers (Mol et al, 1997; Seet et al, 2011), others reported no differences (van Poppel et al, 1997; Yasunobu et al, 2001).

Taken together, we believe that the measurement of lipids in the circulation provides a useful insight into the extent of cigarette smoking-induced oxidative alterations to circulating lipids and oxidative stress in the cardiovascular system. Measurements of selected oxysterols have been incorporated in the clinical assessment program for further evaluation of this endpoint's utility in the assessment of CVD risk associated with the use of MRTPs.

Appendices A6.1.4.8-11 provide detailed literature and data reviews of the association of HDL-C (Appendix A6.1.4.8), LDL-C (Appendix A6.1.4.9), apolipoproteins (Appendix A6.1.4.10) and oxysterols (Appendix A6.1.4.11) with smoking-related diseases, the relationship between these endpoints and smoking as well as the reversibility of endpoints upon smoking cessation.

6.1.4.3.3.1.3 HbA1c, Insulin and Insulin Resistance

There is evidence that CVD is interrelated with insulin homeostasis, a relationship that is complex owing to the fact that both insulin resistance and chronic hyperglycemia can contribute to atherogenesis and progression of atherosclerotic lesions (Bornfeldt & Tabas, 2011; Monnier et al, 2013). Insulin resistance, a loss of sensitivity towards insulin in liver, skeletal muscle, and white adipose tissue, develops in states of over nutrition such as obesity and is accompanied by chronic low-grade inflammation, gluco- and lipotoxicity leading to hyperinsulinemia, hyperglycemia, and hyperlipidemia (Shulman, 2000). Smoking is linked to elevated insulin levels and has been shown to increase insulin resistance (Chiolero et al, 2008; Eliasson et al, 1994; Facchini et al, 1992; Houston et al, 2006; Morimoto et al, 2013), with some studies indicating that smoking cessation may be beneficial in restoring normal insulin secretion (Morimoto et al, 2013; Rönnemaa et al, 1996).

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The glycation product of hemoglobin, HbA1c, which is indicative of average blood glucose concentrations over a period of weeks or months, serves as a commonly used indicator of glycemic control (D'Alessandro et al, 2013; Woodward et al, 2006) in the diabetic population. This is primarily related to its predictive value for developing diabetes-related complications, although its relationship to CVD risk and that of related events in non-diabetic patients has been debated for some time (Chamnan et al, 2013; Di Angelantonio et al, 2014; Eeg-Olofsson et al, 2010; Goto et al, 2015; Ikeda et al, 2013; Orchard et al, 1998; Pradhan et al, 2007; Simmons et al, 2008; Singer et al, 1992; van't Riet et al, 2012).

It is nevertheless of interest in MRTP assessment studies since cigarette smoking has also been associated with elevated HbA1c levels (Hong et al, 2015; Nilsson et al, 2004; Soulimane et al, 2014). Moreover, smoking cessation was found to have a positive impact on blood HbA1c levels, showing a significant downward trend that increases with years since quitting (Ohkuma et al, 2015). Owing to this long time frame for reversibility, HbA1c as a clinical risk endpoint may be of most interest in longer term studies and observational or post-market studies that may include diabetic participants.

Appendix A6.1.4.12 provides a detailed literature and data review of the association of HbA1c with smoking-related diseases, the relationship between HbA1c and smoking as well as the reversibility of the HbA1c smoking-related findings upon smoking cessation.

6.1.4.3.3.1.4 Blood Pressure

Pathologically elevated blood pressure or hypertension affects millions of people globally and constitutes a major independent risk factor for CVD and CVD-related mortality (Antonakoudis et al, 2007; Kannel, 1996; Whelton et al, 2002). Cigarette smoking in turn is a risk factor for hypertension, and an ever-growing amount of evidence supports a synergism between hypertension and smoking that modifies CVD risk even further (Ambrose & Barua, 2004; Kannel & Higgins, 1990; Talukder et al, 2011; Virdis et al, 2010). Blood pressure measurements, often together with the evaluation of other risk endpoints, have traditionally been used to assess CVD risk and are an accepted outcome measure in drug trials (Tardif et al, 2006).

The nicotine exposure from smoking causes an acute increase in blood pressure lasting for many minutes, however smoking has not been conclusively proven to cause high blood pressure (USDHHS, 2010b).

As blood pressure is collected routinely in clinical studies and is an endpoint that is related to CVD, it is considered to be of interest for evaluation, although not expected to be informative on its own, especially in short term studies of one year or less.

6.1.4.3.4 Lung Function Endpoints

6.1.4.3.4.1 Assessment of Lung Function in Experimental Models

Lung function measurements in experimental models are a useful test for delineating effects of inhalation exposure on pulmonary function. They also form an essential part of preclinical safety pharmacology (FDA, 2001). Generally, a distinction between non-invasive and invasive tests is made, and while there is no standardized method, both plethysmography (non-invasive) and forced maneuvers (invasive) have been used in the

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past to study the effects of pharmacological intervention or toxicant exposures in small rodents (Hoymann, 2012; Vanoirbeek et al, 2010).

Plethysmography can be performed on conscious animals without restraint and allows for the determination of respiratory rate, minute and tidal volumes, and peak inspiratory and expiratory flows. Plethysmography parameters reflect physiological breathing patterns of the animal, and changes in these parameters may be indicative of exposure-related irritation and airflow limitation (Mauderly, 1989). Several studies demonstrated a decrease in respiratory frequency and tidal volume in rats following cigarette smoke exposure which was considered indicative of respiratory irritation (Ayres et al, 2001; Dorman et al, 2012; Heck et al, 2002; Higuchi et al, 2004; Kinsler et al, 2003; Terpstra et al, 2003; Tsuji et al, 2011; Vanscheeuwijck et al, 2002). While this method does not permit a conclusion toward pulmonary mechanics, head-out plethysmography is recommended in the context of safety assessment (Glaab et al, 2007; Hoymann, 2012). Therefore, PMP has selected this method for the evaluation of inhalation exposure effects on the respiratory system in its rat OECD studies.

Invasive approaches are considered the "gold standard" to assess lung function in mice accurately and to gain an insight into pulmonary mechanics (Glaab et al, 2007). Forced oscillation techniques in anesthetized animals involve the generation of a sinusoidal pressure waveform on the airway in order to produce measurements of pressure, volume and flow at a single defined frequency. These single compartment measurements provide a means to calculate pulmonary resistance, elastance and compliance using a variety of mathematical models. Forced oscillation at low frequency covering a wide range of frequencies yields respiratory system input impedance which, analyzed with the constant phase model, may provide further insight into the affected lung compartment (SCIREQ). Pressure-volume (PV) loops reflect respiratory mechanics of inspiration and expiration and derive quasi-static elastance and compliance as a marker of lung deformation during breathing (Harris, 2005). Together, these lung function parameters are useful in understanding mechanisms underlying airflow limitation (Glaab et al, 2007; Hoymann, 2012), and they have been used, albeit inconsistently, as endpoints for several mouse studies. Increased tissue resistance and decreased tissue elasticity and compliance were seen in C57BL/6 mice exposed to cigarette smoke for 4 weeks (Seys et al, 2015). Airway resistance was increased in C57BL/6 mice exposed to cigarette smoke for 2 months (Biselli et al, 2011). Conversely, transpulmonary and airway-specific resistance were decreased and dynamic compliance was increased in Balb/c mice exposed to cigarette smoke for 8 weeks (Beckett et al, 2013). Similarly, decreased dynamic resistance and increased compliance were observed in C57BL/6 mice treated with elastase or a combination of elastase and LPS to mimic emphysema and COPD exacerbations, respectively (Sajjan et al, 2009), and in cigarette smoke-exposed C57BL/6 mice (Nakanishi et al, 2009). However, compliance was not different between cigarette smoke-exposed A/J and CBA/J x C57BL/6 mice compared with sham mice (Foronjy et al, 2005). An earlier PMP study also explored lung function in C57BL/6 mice following exposure to 3R4F cigarette smoke or to aerosol from a potential MRTP (pMRTP) with a carbon-based heat source. Single compartment measurements indicated lower dynamic resistance and elastance and higher dynamic compliance in 3R4F cigarette smoke-

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exposed mice relative to sham-exposed animals. This indicated a loss of elastic recoil in the smoke-exposed lungs which became evident after 1 month of exposure. No significant differences were noted in the single compartment parameters in the pMRTP aerosol-exposed mice at any time point. Moreover, when mice were exposed to cigarette smoke for 2 months and then switched to air or to pMRTP aerosol to mimic cessation and switching, respectively, dynamic resistance, elastance and compliance began to improve, differing significantly from 3R4F cigarette smoke-exposed animals as soon as 1 month after cessation or switching. Similarly, exposure to 3R4F cigarette smoke resulted in a leftward shift of the P–V loops for both the inflation and deflation phase at the 2-, 3-, and 7-month time points, suggesting that lung volumes at maximum pressure in 3R4F cigarette smoke-exposed mice were larger than in sham and pMRTP-exposed mice. These differences persisted throughout the study. After discontinuation of exposure to 3R4F cigarette smoke or switching to pMRTP aerosol, quasi-static parameters also improved (Phillips et al, 2015).

6.1.4.3.4.2 Assessment of Lung Function in Clinical Studies

Lung or pulmonary function tests evaluate how well an individual's lungs operate. Spirometry is often employed as a screening tool to assess general lung health, measuring inhalation and expiration of air as a function of time (Miller et al, 2005). The most common parameters measured in spirometry are the Forced Expiratory Volume (FEV), the volume exhaled during a forced expiratory maneuver started from the level of Total Lung Capacity (TLC), at timed intervals of 0.5, 1.0 (FEV_1), 2.0, and 3.0 seconds as well as Forced Vital Capacity (FVC), the maximum amount of air that can be exhaled during a forced maneuver. Other measured parameters include Forced Expiratory Flow 25–75% ($FEF_{25–75\%}$) or maximal (mid) expiratory flow (MMEF or MEF). FEV_1 values are presented as raw data (liters or liters per second) or as percent predicted (FEV_1 % predicted) in which the test result is presented as the percentage of the predicted value for individuals of similar characteristics including height, age, sex, race and weight. Lung function is considered normal if the FEV_1 is within 80% to 120% of predicted values and the FEV_1/FVC ratio between 70 and 80%. FEV_1 is known to decline with age, but smoking has been shown to accelerate this decline dramatically (Vestbo & Lange, 2016). For each smoking history, however, there is considerable heterogeneity in FEV_1 response, with a high variability in FEV_1 measurements (Vestbo & Lange, 2016). In addition, cessation has been shown to be beneficial in slowing down the decline in FEV_1 in some smokers, but not in others (Burchfiel et al, 1995; Camilli et al, 1987; Lange et al, 1989; Willemse et al, 2004; Xu et al, 1994). For the detection of COPD, spirometry and FEV_1 in particular are considered the gold standard ((GOLD), 2013), even though spirometry alone may not be sufficiently sensitive to account for early lung damage in the distal airways that remains asymptomatic, particularly in the younger smoker (Celli et al, 2003). Other lung function parameters such as $FEF_{25–75\%}$ and single breath carbon monoxide (CO) transfer factor (also known as diffusing capacity for CO, DLCO) and exhaled NO may provide additional or complementary information.

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The forced expiratory flow at 25-75% of the pulmonary volume $FEF_{25–75\%}$ or MEF indicates the average expiratory flow over the middle half of the FVC and has been proposed as an indicator of airway obstruction, in particular in the small airways in the past (Pellegrino et al, 2005). While findings across studies are not consistent, some data suggest that $FEF_{25–75\%}$ together with FEV_1 can support both large airway obstruction and small airway disease (Burdon & Pain, 1980; Hansen et al, 2006; Lunn & Sheller, 1995; Quanjer et al, 2014). In addition, some studies showing that $FEF_{25–75\%}$ changes favorably upon smoking cessation within a relative short period of time support the evaluation of this lung function index in product assessment studies (Buist et al, 1976; Wilhelmsen, 1967).

DLCO is a measurement that permits an insight into the uptake of CO and gas exchange across the alveolar-endothelial barrier which is impaired in certain pulmonary and extrapulmonary pathological conditions (Macintyre et al, 2005; Pellegrino et al, 2005). DLCO is a good indicator of the alveolar-capillary destruction seen in emphysema, and there is increasing evidence that smokers with normal lung function and reduced DLCO develop emphysema within a shorter timeframe than subjects with normal FEV_1/FVC and normal DLCO (Harvey et al, 2015; Osmanliev et al, 2005; Salit et al, 2012; Strulovici-Barel et al, 2014; Viegi et al, 2001). More importantly, smoking cessation rapidly improves DLCO (Sansores et al, 1992; Verbanck et al, 2006; Watson et al, 1993), suggesting that gas transfer assessment, complementing spirometry, may prove beneficial for the evaluation of the effects elicited by novel tobacco products on lung health and the risk of developing COPD.

Measurements of fractional exhaled NO have become commonplace in the diagnosis and monitoring of asthma, although its utility as a diagnostic aid for other respiratory diseases is still debated (Silkoff et al, 2006). Some studies report a good correlation between exhaled NO and both FEV_1 and FEV_1/FVC ratio (Brindicci et al, 2009; Kharitonov et al, 2003). It is well-known that cigarette smoking causes oxidative and nitrosative stress in the airways (Rytila et al, 2006), and it has been reported that exhaled NO may be a useful indicator of airway inflammation and airway obstruction (Bazeghi et al, 2011; Malerba et al, 2014).

Appendix A6.1.4.13 provides a detailed literature and data review of the association of lung function measures with smoking-related diseases, the relationship between lung function measurements and smoking as well as the reversibility of the lung function measures upon smoking cessation.

6.1.4.3.5 Genotoxicity Endpoints

6.1.4.3.5.1 Assessment of Genotoxicity in Experimental Models

Traditionally, risk to human health has been addressed by performing a number of *in vitro* assays and *in vivo* tests belonging to the standard battery of tests to assess both cytotoxicity and genotoxicity of chemicals. The latter include, for example, the bacterial mutagenicity or Ames assay and the mouse lymphoma assay (MLA) which permit assessment of risk pertaining to DNA damage and its fixation and, hence, provide a means for predicting carcinogenicity (FDA, 2012b). Similarly, animal studies are an essential part of risk assessment approaches in toxicology. The FDA MRTP Application

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Guidance (FDA, 2012a) recommends to include *in vivo* studies in the evidence package for the evaluation of tobacco products with potential for risk reduction. PMP routinely includes both *in vitro* tests and *in vivo* inhalation studies in the nonclinical assessment of candidate MRTPs and further details on these type of studies for THS are provided in section 6.1.2 in this dossier.

6.1.4.3.5.2 Assessment of Genotoxicity in Clinical Studies

Proof of reduced exposure to HPHC in subjects switching from smoking to the use of THS was achieved by the measurement of biomarkers of exposure (BoExp) (for further details see section 6.1.3). Quantitation of selected BoExp, however, can also serve the purpose of demonstrating reduced genotoxicity and cancer risk, in particular if the endpoints of interest reflect the uptake and metabolism of HPHC that are categorized as IARC class I and class II carcinogens (Hatsukami et al, 2003; Hatsukami et al, 2006; Stratton et al, 2001). Within PMI's exposure assessment program the following IARC class I and II HPHCs were evaluated (Table 5).

Table 5 List of IARC Class I and II Carcinogens Assessed in Clinical Studies

HPHC	BoExp	IARC Class	FDA Toxicity Classes				
			CA	CT	RT	RDT	AD
1,3-butadiene	MHBMA	I	●	●	-	-	-
Acrylonitrile	CEMA	IIb	●	-	●	-	-
ethylene oxide	HEMA	I	●	-	●	●	-
Benzene	S-PMA	I	●	●	-	●	-
2-Aminonaphthalene	2-NA	I	●	-	-	-	-
4-Aminobiphenyl	4-ABP	I	●	-	-	-	-
o-toluidine	o-tol	I	●	-	-	-	-
NNK	Total NNAL	I	●	-	-	-	-
NNN	Total NNN	I	●	-	-	-	-
Benzo[a]pyrene	B[a]P	I	●	-	-	-	-

In addition to the measurement of these BoExp, the mutagenicity of smokers' urine in *Salmonella typhimurium* (Ames test) is informative with respect to exposure to genotoxic agents in cigarette smoke and aerosols of novel tobacco products. This has been

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confirmed in several studies, including studies conducted by PMP, comparing the effects of conventional cigarettes relative to heat-not-burn tobacco products (Bowman et al, 2002; deBethizy et al, 1990; Gregg et al, 2013; Smith et al, 1996; Tricker et al, 2012a; Tricker et al, 2012b; Tricker et al, 2012c).

The clinical assessment of THS also included cytochrome P450 (CYP) enzymes, a superfamily of heme-containing mono-oxygenases with cytochrome 1A2 (CYP1A2) constitutively expressed at higher levels in the liver, representing about 4–16% of the total hepatic P450 pool (Yuan et al, 2002). Besides the importance of CYP1A2 for the elimination of a large number of drugs (Zanger & Schwab, 2013), the metabolism of endogenous substrates (Gunes & Dahl, 2008), and the elimination of environmental xenobiotics, CYP1A2 activates pre-carcinogens, such as heterocyclic amines, mycotoxins, TSNAs, and aryl amines, all of them HPHCs contained cigarette smoke (Eaton et al, 1995). Heterocyclic and aromatic amines are initially N-oxidized by the action of cytochrome CYP1A2 in the liver (MacLeod et al, 1997). Reactive N-acetoxy intermediates can react with DNA to form covalent heterocyclic amine-DNA adducts (MacLeod et al, 1997). The formation of DNA adducts and the extent of tumor induction is dependent on the amount of aromatic or heterocyclic amines which are converted to the reactive metabolites.

Induction of CYP1A2 by smoking is well established and has been shown in *in vivo* studies (Dobrinas et al, 2011; Ghotbi et al, 2007). Components of cigarette smoke, like polycyclic aromatic hydrocarbons, induce CYP1A2 activity to a different extent in various smokers (Butler et al, 1992). CYP1A2 activity was shown to decrease by more than 30% compared to baseline after three weeks of smoking cessation (MacLeod et al, 1997), indicating a potential reduction in DNA adducts formation and tumor induction, leading to reduced genotoxicity. PMI considers CYP1A2 activity as a valuable marker in the context of genotoxicity assessment of MRTPs.

6.1.4.4 Results

6.1.4.4.1 Assessment of Inflammation and Oxidative Stress in Experimental Models

6.1.4.4.1.1 In Vitro Models

####### *6.1.4.4.1.1.1 Adhesion and Transendothelial Migration Assays*

The indirect and direct effects of extracts derived from 3R4F cigarette smoke (CSE) and THS aerosol on adhesion of monocytes to endothelial cells was investigated in the adhesion assay as previously described (Poussin et al, 2014). With an indirect approach, monocyte-endothelial cell adhesion was promoted by treatment with low concentrations of CSE or THS aerosol extract. Compared with CSE, 10 and 20 times higher concentrations of THS aerosol extract were necessary for indirect and fresh direct treatments, respectively, to induce comparable monocytic MonoMac 6 (MM6) cell adhesion to endothelial cells. In addition, the highest concentrations of both 3R4F CSE and THS aerosol extract, using the fresh direct approach, significantly decreased the number of HCAECs. The other treatment conditions did not affect HCAEC numbers. In contrast, when HCAECs were exposed to a high concentration of previously frozen

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(direct approach) or freshly prepared (fresh direct approach) CSE, MM6 cell adhesion to endothelial cells was not observed (Poussin et al, 2016).

In addition, results of the transendothelial migration (TEM) assay indicated that pretreatment of THP-1 cells with CSE or THP aerosol extract resulted in a significant concentration-dependent inhibition of CXCL12-stimulated chemotaxis. This was accompanied by a significant and concentration-dependent decrease in TEM rate, independent of the type of extract tested. However, the THS aerosol was approximately 50 times less effective than CSE at inhibiting chemotaxis and about 18 times less effective at slowing TEM. Moreover, both extracts caused a rapid concentration-dependent disruption of the endothelial barrier in HCAECs, with CSE nearly 16 times more potent than THS aerosol extract at decreasing impedance (van der Toorn et al, 2015a).

Taken together, these results indicated that aqueous extracts from 3R4F reference cigarettes or THS aerosol induced concentration-dependent responses in endothelial barrier function, monocyte adhesion, monocyte chemotaxis and TEM. The effects of the THS aerosol extract were considerably weaker than those of the 3R4F CSE.

6.1.4.4.1.1.2 Fraction Impact Assessment Study

The respective impacts of 3R4F cigarette smoke (CS) and THS aerosol fractions on submerged primary human bronchial epithelial cells (NHBECs) were evaluated using real-time impedance and high-content screening (Gonzalez-Suarez et al, 2016). A dose-dependent increase in DHE fluorescence and depletion of reduced glutathione (GSH), both indicative of an increased oxidative burden, was observed in NHBECs treated with 3R4F gas vapor phase (GVP) for 4 or 24 hours. As this was accompanied by significant cell death, it is very likely that the results did not reflect actual oxidative stress. ROS loads were also increased in cells treated with high doses of 3R4F cigarette smoke extract (CSE) for 24 hours, again coinciding with increased cell death. In contrast, there was no indication of ROS formation in cells exposed to THS aerosol extract and GVP. THS GVP caused a dose-dependent decrease in GSH, detected at 4 hours. This effect was not observed at a later time point, suggesting that intracellular antioxidant pools had recovered. Oxidative stress following 3R4F total particulate matter (TPM) exposure could not be assessed because of marked interference with the DHE signal by this smoke fraction. However, there was a dose-dependent decrease in GSH levels in NHBECs treated with 3R4F or THS TPM for 4 or 24 hours. Compared with the extent of GSH depletion in NHBECs treated with 3R4F TPM, THS TPM caused a much smaller decrease in intracellular GSH.

In additional experiments, exposure to 3R4F GVP or CSE at high, but not low, doses resulted in increased γH2AX signals, reflecting DNA damage and, hence, genotoxicity. TPM from reference cigarettes increased DSBs in a dose-dependent manner. After longer treatments, there was a dramatic reduction in cell viability, indicating cytotoxicity-induced genotoxicity. With THS TPM exposures, only doses far exceeding those of the comparable 3R4F fraction caused dose-dependent DNA damage at both 4 and 24 hours. Neither THS GVP nor aerosol increased the number of γH2AX-positive cells.

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In summary, these results indicated that fractions of 3R4F CS have a much greater potential to induce cytotoxicity, as well as oxidative and genotoxic stresses, in NHBECs, compared with fractions derived from THS aerosol.

6.1.4.4.1.1.3 Organotypic Oral Study

To compare the biological impact of THS aerosol with 3R4F CS on physiologically relevant systems representative of the human upper airway, human organotypic tissue cultures of the oral epithelium (EpiOral™, MatTek Corp., Ashland, MA, USA) were exposed for 28 minutes to air (control) and increasing doses of 3R4F CS and THS aerosol. 3R4F CS was applied at dilutions of 15% and 24% (v/v in air), equivalent to target nicotine concentrations of 0.32 and 0.54 µg/L respectively. THS aerosol was applied at dilutions of 25%, 34% and 69% (v/v in air), corresponding to target nicotine concentrations of 0.31, 0.46 and 1.09 µg/L respectively. Cytotoxicity and gene expression responses were assessed 4, 24, 48 and 72 hours after the exposure. Several inflammatory mediators released into the culture medium were quantified at 24, 48 and 72 hours and tissue integrity was evaluated at 48 and 72 hours after the exposure.

Exposure of EpiOral™ tissues to 3R4F CS resulted in tissue injury, as evidenced by keratinization and desquamation, which was most prominent at the higher concentration and later time point. In contrast, THS aerosol-exposed cultures showed a strongly attenuated response. The cytotoxicity assay gave comparable results. Moreover, cultures exposed to 3R4F CS secreted higher levels of inflammatory mediators than those exposed to air. For example, at comparable nicotine concentrations, THS aerosol exposure resulted in significantly lower MMP1 (-45.8%) and IL-1β (-88.8%) levels than 3R4F Cs exposure. Far fewer and less pronounced changes in secretion of these mediators were observed in oral cultures exposed to THS aerosol at comparable or even higher nicotine exposure levels. Together, these results indicated that exposure to THS aerosol caused less cytotoxicity, tissue damage and inflammation in oral organotypic models than exposure to 3R4F CS (1743_Organotypic_Buccal_SR, (Zanetti et al, 2016)).

6.1.4.4.1.1.4 Organotypic Nasal Study

The impact of exposure to 3R4F CS and THS aerosol were also studied in human organotypic tissue cultures of the nasal epithelium (Nasal MucilAir™, Epithelix Sarl, Geneva, Switzerland). In this study, the cultures were exposed for 28 minutes to air (control); 8% or 15% (v/v in air) 3R4F CS, equivalent to target nicotine concentrations of 0.15 and 0.25 µg/L respectively; or to 15%, 24% and 31% (v/v in air) aerosol from THS, corresponding to target nicotine concentrations of 0.15, 0.27 and 0.44 µg/L respectively. Cytotoxicity and tissue integrity, as well as gene expression responses, were assessed 4, 24, 48 and 72 hours following exposure. Inflammatory mediators released into the culture supernatants were measured at 24, 48 and 72 hours post-exposure.

While exposure to 8% 3R4F CS induced little to no cytotoxicity at any time point, exposure to 15% 3R4F CS elicited substantial cytotoxicity at 24, 48 and 72 hours post-exposure. However, exposure to various concentrations of THS aerosol did not induce any cytotoxicity. Histological examination confirmed that exposure to 3R4F CS resulted in more damage than did exposure to THS aerosol, with tissues exhibiting less damage at 48 than at 72 hours post-exposure. THS aerosol exposure with a nicotine concentration at

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least four times higher than that of 3R4F CS was needed to elicit similar morphological changes and cytotoxicity in the nasal cultures. Multi-analyte profiling revealed that cultures exposed to THS aerosol secreted lower levels of various inflammatory mediators, including CXCL-8 (-52.9%) and sICAM-1 (-80.6%), at 24 hours after exposure than those exposed to 3R4F CS. In contrast, nasal tissue cultures exposed to THS aerosol at nicotine levels comparable to those in the CS exposures, showed no significant increase in secretion of inflammatory mediators. (1722_Organotypic_Nasal_SR; (Iskandar et al, 2016)).

In conclusion, these data demonstrated that exposure of human organotypic nasal epithelial tissues to whole smoke generated from 3R4F reference cigarettes resulted in time- and dose-dependent cytotoxicity, tissue damage and increased secretion of inflammatory mediators. In contrast, exposure of the cultures to the corresponding concentrations of THS aerosol (matched by nicotine levels) had effects more comparable to those of fresh air exposures, with negligible cytotoxicity or tissue damage.

6.1.4.4.1.1.5 Organotypic Bronchial Study

Similar studies were conducted in human organotypic tissue cultures of the bronchial epithelium (bronchial MucilAir™, Epithelix Sarl, Geneva, Switzerland or bronchial EpiAirway™, MatTek Corporation, Ashland, MA, USA). In these studies, the cultures were exposed under the same conditions as the human nasal epithelium cultures under Section 6.1.4.4.1.1.4. These cultures were then examined for exposure-related effects at 4, 24, 48 and 72 hours post-exposure.

At the earliest time point, no cytotoxicity was noted, independent of the type of exposure. However, cultures exposed to 3R4F CS at 0.25 mg/mL nicotine showed approximately 40% cytotoxicity at 48 and 72 hours post-exposure, while THS aerosol exposure did not cause cytotoxicity in bronchial tissues, independent of dose or post-exposure time point. These results were confirmed by histological evaluation, highlighting that the morphology of THS aerosol-exposed cultures resembled that of those exposed to air. 3R4F CS exposure induced a strong inflammatory response in bronchial organotypic cultures, as indicated by significantly higher levels of soluble mediators released into the cell culture medium, in particular at the higher CS dose. With exposure to THS aerosol, however, this response was much lower. For example, secretion of CXCL-8 and sICAM-1 was significantly reduced (-56.5% and -91.2%, respectively) in the basolateral medium of the THS aerosol-exposed bronchial tissue cultures than in those of the 3R4F CS-exposed cultures at 24 hours after the exposure.

Together, these results demonstrated that exposure to THS aerosol, in contrast to 3R4F CS, caused little to no cytotoxicity, tissue damage or inflammation in organotypic bronchial epithelium tissue cultures (1709_Organotypic_Bronch_SR; 1709_Organotypic_Bronch _Pub).

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6.1.4.4.1.2 In Vivo Models

6.1.4.4.1.2.1 90-day sub-chronic inhalation studies in rats

Two independent 90-day inhalation studies were conducted with THS (Rat-THS-TG413) and THS Menthol (Rat-THSM-TG413) according to OECD guideline TG413 (OECD, 2009), comparing the toxicity of CS from 3R4F to the aerosol from THS and THS Menthol. For this purpose, male and female Sprague-Dawley rats were nose-only exposed to filtered fresh air (sham) or 3 target nicotine concentrations of 3R4F CS (8, 15 or 23 µg/L) or aerosol from THS/THS menthol (15, 23 or 50 µg/L), respectively, for 6 h/day, 5 days/week for 13 weeks. Additional groups of animals were included for exposure to THS aerosol at the highest nicotine concentration for 13 weeks, followed by a 42-day recovery period, to investigate the reversibility or persistence of test substance-related effects. In addition to the OECD endpoints, pulmonary inflammation was assessed by BALF cell counts and quantification of BALF inflammatory mediators at the end of exposure (90 days) and following the post-exposure period (90+42 days). Furthermore, potential molecular changes as a consequence of exposure were evaluated by employing transcriptomics, proteomics and lipidomics analyses.

The results of the OECD TG413 toxicological assessments in rats were described in another section of this dossier (Section 6.1.2). This part of the document will focus on the results of the inflammation- and oxidative stress-related endpoints obtained from the additional integrative systems toxicological evaluation of samples from the OECD inhalation studies (the "OECD Plus" part).

6.1.4.4.1.2.1.1 Rat-THS-TG413

Evidence for stress-related changes in the airway epithelium were noted upon histological evaluation of the respiratory tract organs—nose, larynx, trachea and lungs—of the exposed rats. For example, exposure to 3R4F CS over a 90-day period resulted in the development of moderate to marked reserve cell hyperplasia, marked squamous metaplasia with and without cornification, particularly in animals exposed to higher concentrations, and loss of goblet cells in the rat respiratory nasal epithelium. In addition, slight infiltration by neutrophilic granulocytes was observed. Similarly, reserve cell hyperplasia and squamous metaplasia were also observed in rats exposed to THS aerosols. However, lesions in these animals showed remarkably less cornification and loss of goblet cells than those seen in the 3R4F groups. There were no differences in incidence and severity of these nasal lesions between the two genders, independent of exposure. Moreover, with the exception of squamous metaplasia, severity of lesions decreased following a 42-day post-exposure period, suggesting that the observed changes represented a local adaptation to irritant inhalation.

Lung inflammation following exposure to 3R4F CS was further confirmed by an increase in the total free cell number recovered in the BALF. This was primarily driven by increased macrophage and neutrophil numbers in both female and male animals. In contrast, exposure to THS aerosol did not affect the total number of free lung cells in BALF, even at the highest nicotine concentration.

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In addition to the analysis of free lung cells, BALF inflammatory mediators were also quantified. The BALF levels of a number of cytokines and growth factors were increased following the 90-day inhalation period in rats exposed to 3R4F CS. The levels of 10 inflammatory mediators were was statistically significantly lower in BALF samples from male and female rats exposed to THS aerosol at the medium concentration. For example, M-CSF-1 and MCP-1 BALF levels were 57.3% and 97.2%, respectively, lower in male rats exposed to THS aerosol (23 µg nicotine /L) compared with rats exposed to comparable concentrations of 3R4F CS. Following the 42-day recovery period for sham animals and those exposed to the high nicotine concentration, a few of the mediators that had been increased at the end of exposure to 3R4F CS returned to levels at or below those seen in sham animals. The majority, however, remained elevated or even rose to higher levels by completion of the 90-day inhalation in 3R4F CS-exposed rats. Such a phenomenon was not observed in rats exposed to THS aerosol.

Systems toxicological evaluation of the nasal epithelium and lung from 3R4F CS-exposed male and female rats illustrated extensive perturbations in nearly all analyzed networks, with a major impact on the Inflammatory Process Network (IPN), which captures processes related to epithelial proinflammatory signaling and epithelial–immune cell interactions as well as the resulting epithelial tissue damage. In rats exposed to THS aerosol, the same networks were affected, albeit to a lesser extent than with CS exposure. Relative biological impact factors (RBIF) for THS aerosol exposure in the lung were reduced by 94% and 96% in male and female rats, respectively, compared with 3R4F smoke exposure at the higher comparable concentration, and at the 2-fold higher THS aerosol concentration, the reduction was still by 93 and 94% for males and females, respectively.

Untargeted proteomics of lungs from exposed rats revealed that differential protein expression was much more extensive following exposure to CS than following THS aerosol exposure. There was a significantly lower number of differentially abundant proteins (compared with the sham controls) in THS aerosol exposed rats: 0 and 32 proteins for male and female rats, respectively, as opposed to 273 and 240 in 3R4F CS-exposed males and females, respectively at the higher comparable concentration (23 µg nicotine/L). Of note, after the 42-day recovery period, proteome changes, though decreasing in number, persisted in the noses of animals that had been exposed to 3R4F CS, while the effects of THS menthol aerosol exposure largely disappeared. A similar pattern of changes in protein abundances was also observed in the lungs of exposed animals (Section 7.5).

6.1.4.4.1.2.1.2 Rat-THSM-TG413

In this second 90-day inhalation study with a mentholated version of THS (THSM), rats were exposed to filtered fresh air (sham), to 3R4F CS, and to two mentholated reference cigarettes (MRC), all corresponding to 23 µg/L nicotine, or to a low, medium, or high concentration of the aerosol from THSM corresponding to 15, 23, and 50 µg nicotine/L, respectively. MRC low menthol (LM) and MRC high menthol (HM) were designed to meet the tar, nicotine, carbon monoxide, and cigarette diameter specifications of 3R4F reference cigarettes and to produce approximately 1.2 mg and 2.4 mg menthol in smoke, respectively, when smoked according to International Organization for Standardization

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(ISO) standard 3308:2012. As in the previous OECD study, respiratory tract organs from animals of all exposure groups were subjected to histopathological evaluation following the 90-day inhalation, and pulmonary inflammation was assessed at the end of exposure (90 days) and following the 42-day post-exposure period (90 + 42 days). Furthermore, potential molecular changes as a consequence of exposure were evaluated using transcriptomics, proteomics, and lipidomics analyses.

Histopathological changes, including inflammation seen in the respiratory tract organs of 3R4F or MRC CS-exposed animals, were adaptive and degenerative in nature and similar to findings from previously conducted nose only CS smoke inhalation studies on rats. Those changes were either not observed or significantly lower in severity in the THSM aerosol-exposed rats. Most changes induced by aerosol exposure in the respiratory tract organs showed reversal of effects at the end of the post-inhalation (recovery) period.

Lungs of CS-exposed rats exhibited mild-to-moderate inflammation, as evidenced by increased numbers of unpigmented and pigmented macrophages and the presence of macrophage nests, which were not seen in THSM aerosol-exposed animals. Lung inflammation in CS-exposed rats was further reflected in increased counts of total free cells in BALF, which, at least in part, declined after post-exposure recovery. In contrast, total free lung cell counts in THSM aerosol-exposed animals were not markedly elevated above those seen in sham animals. In addition, the secretion of 15 inflammatory mediators was statistically significantly lower in the BALF from male rats exposed to the medium concentration (23 µg nicotine/L) of THSM aerosol compared with rats exposed to the comparable concentration of smoke from the MRC LM. When the THSM aerosol concentration was doubled, eight inflammatory mediators were statistically significantly reduced by 47.0% (for VCAM-1) up to 96.5% (for MCP-1) compared with rats exposed to MRC HM CS, and very similar results were seen when compared with rats exposed to 3R4F CS. Lipidomics revealed significant changes in 20 lipid species (most of them being sphingolipids) in the lung following exposure of male rats to MRC HM CS for 90 days, and only one change, a decrease in 8,9-DHET, in the lungs from rats exposed to the comparable concentration (23 µg nicotine/L) of THSM aerosol; for exposure to the two-fold higher THSM aerosol concentration, one additional lipid was found in a higher abundance. For female rats, the exposure effects on the lipidome were qualitatively similar but weaker.

Network analysis of the transcriptomics data showed that, in both respiratory nasal epithelia and lung tissue from both sexes, exposure to MRC or 3R4F CS caused perturbations in several biological processes, mainly inflammatory, cell stress, cell fate, and tissue repair and angiogenesis. The RBIF values for exposure to CS from both MRC (LM and HM) at 23 µg nicotine/L were 100% and 81%, respectively, in male rat lungs, whereas THSM aerosol exposure at the comparable nicotine level elicited an RBIF of 1%, and at the 2-fold higher THSM aerosol concentration, the RBIF was 6%. For female rats, the RBIF for both MRC CS exposures were 100% and 73%, respectively, and 7% for the matching THSM aerosol concentration, while it was 8% for the high THSM aerosol concentration.

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6.1.4.4.1.2.2 Switching Study in Apoe$^{-/-}$ Mice

The development and progression of COPD and CVD were evaluated in *Apoe*$^{-/-}$ mice (Lo Sasso et al, 2016a) following chronic exposure to mainstream CS or to an aerosol from THS (Lo Sasso et al, 2016b; Phillips et al, 2016). Female *Apoe* $^{-/-}$ mice were exposed to CS from 3R4F reference cigarettes at a target nicotine concentration of 29.9 µg/L, to aerosol from THS at a matched nicotine concentration, or to filtered fresh air for 3 hours per day, 5 days per week, for up to 8 months. After 2 months exposure to 3R4F CS, designated animals were switched to THS aerosol exposure (switching group) or to filtered air (cessation group) for up to 6 months, following the same daily exposure regimen. At designated time points (months 1, 2, 3, 6 and 8 after commencing exposure), animals were examined for multiple parameters to comprehensively assess the development and progression of COPD or CVD indicators. These parameters included hematology and clinical chemistry assessments, pulmonary inflammation measurements, assessment of pulmonary function, lung histopathology and morphometry, evaluation of atherosclerotic plaque formation in the aortic arch and descending aorta and examination of molecular changes by transcriptomics, proteomics and lipidomics in various tissues.

6.1.4.4.1.2.2.1 Inflammation

There were no significant differences in WBC counts among exposure groups.

Histopathological evaluation of respiratory tract organs illustrated typical CS exposure-related effects in animals from the 3R4F CS-exposed group. These included adaptive changes of the nasal, laryngeal and tracheal epithelia and infiltration of inflammatory cells into the alveolar and perivascular spaces of the lung. These effects were present from month 1 onward. Of note, the degree of inflammatory cell infiltration diminished markedly after cessation and switching to THS aerosol. At the 8-month time point, only the extent and severity of pigmented macrophage nests in these two groups remained significantly higher than in sham animals. Importantly, with respect to these inflammatory endpoints, there were no significant differences between the sham controls and THS aerosol-exposed mice at any time point. Histopathological and morphometric assessment of the lungs also confirmed the presence of emphysematous lesions in animals exposed to 3R4F CS, but not in those exposed to THS aerosol. Although very mild age-dependent emphysematous lesions were noted in all animals, both cessation and switching contributed noticeably to lung tissue regeneration and repair.

Relative to values in sham mice, the total number of free lung cells in BALF was much higher after 1 month of 3R4F CS exposure and remained elevated throughout the study. This was primarily due to a significant increase in BALF neutrophil counts. In contrast, prolonged exposure to the THS aerosol had no effect on total free lung cell counts. Both cessation and switching resulted in a rapid decline in the total number of free lung cells, nearly reaching levels seen in sham or THS aerosol-exposed mice after only 1 month of CS discontinuation and returning to these control levels by month 8 (ca. 85% decrease in total free lung cells).

Analysis of 63 inflammatory mediators in the BALF indicated a rapid increase in levels of most analytes following 1 month of 3R4F CS exposure, and in general, those elevated levels persisted over the 8-month exposure period. However, inflammatory mediator

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concentrations in the BALF of THS aerosol-exposed mice were very similar to those in BALF samples obtained from sham animals. Moreover, both switching and cessation resulted in a decline of BALF protein levels towards those seen in sham or THS aerosol-exposed animals in most, though not all, cases.

Using the network model approach, the biological mechanisms most perturbed in the lungs following 3R4F CS exposure belonged to the IPN, with lesser perturbations of all other analyzed networks. In contrast, lungs of THS aerosol-exposed animals exhibited little or no perturbations. In the cessation and switching groups, strongly reduced perturbation patterns were seen after cessation and switching, indicating an almost complete resolution of CS- related perturbations after 6 months of cessation or switching to THS aerosol. RBIF was 1% of the reference (6 months of 3R4F CS exposure) in the lungs of mice exposed to THS aerosol for 6 months and ~9 and ~8 % in the lungs of mice in the cessation and switching groups, respectively, at the same time point.

Lung proteomics analysis revealed time-dependent effects of 3R4F CS exposure, with the abundance of more than 700 proteins significantly altered after 8 months of exposure. In contrast, exposure to THS aerosol had no significant effect on protein abundance during the entire 8-month exposure period. Both cessation and switching to THS aerosol resulted in a comparable and rapid attenuation of the 3R4F CS exposure effect; at the 3-month time point (1 month after switching/the start of cessation), differential abundance was still detectable for 10 and eight proteins in the cessation and switching groups, respectively. However, at 8 months, lung proteomes of animals from the cessation and switching arms were matching those of the sham group, with metallothionein-1 being the only remaining upregulated protein in the switching group.

6.1.4.4.1.2.2.2 Oxidative Stress

For the analysis of biomarkers of exposure and oxidative stress, urine was collected from selected *Apoe*$^{-/-}$ mice from each study arm in the THS Switching Study. CS exposure resulted in elevated 8-OHdG levels compared with sham animals, though this increase was not statistically significant. In addition, there were no changes in urinary 8-OHdG levels following continuous THS exposure or with switching or cessation. Both MDA and 4-HNE, markers of lipid peroxidation, were detected at significantly higher levels in mice exposed to 3R4F CS than in sham animals at month 8 (p<0.05). Again, no differences were noted in levels of these markers in urine from THS aerosol-exposed mice and mice subjected to cessation or switching protocols compared to sham controls. Of note, urinary MDA and 4-HNE concentrations were significantly lower in *Apoe*$^{-/-}$ mice exposed to THS aerosol than to 3R4F CS for 6 or 8 months.

6.1.4.4.1.2.2.3 CVD Risk Endpoints

Several translational CVD risk endpoints were evaluated in the *Apoe*$^{-/-}$ THS switching Study, including lipid profiles in plasma and aortic arch tissue.

Analysis of plasma samples from non-fasted animals revealed a statistically significant increase in total cholesterol (TC) in the CS-exposed group at months 1 and 3, with a decrease to sham levels after 1 month of either cessation or switching to THS aerosol. Accompanying the increase in TC was a similar increase in HDL-cholesterol (HDL-C), though both TC and HDL-C levels approached sham levels by month 8. Similarly, while

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triglyceride levels were higher in 3R4F CS-exposed animals during the first 3 months of the study, by months 6 and 8 there were no differences between any of the exposure and sham groups. There were also no evident differences in LDL levels among the exposed groups relative to the sham controls, at any time point.

Mass-spectrometric lipidomics analyses, performed on samples from the 8-month time point, demonstrated small changes in the abundance of a few glycerolipids in plasma in mice exposed to 3R4F CS. A similar trend was observed in the THS aerosol-exposed group though, in general, the plasma lipidome of mice from the THS and switching groups did not exhibit as strong a change as that seen in the mice from the 3R4F CS-exposed group. In aortic arch tissue from 3R4F CS-exposed mice, levels of glycerophospholipids, sphingolipids, and sterols were significantly higher than in sham animals, while only small differences in two glycerophospholipids were seen in the tissues from the THS group. Small increases, relative to sham levels, in a few sphingolipids and glycerophospholipids were detected in the cessation and switching groups.

Image analysis of the plaque area formed in the aortic arch revealed a progressive increase in plaque burden in all groups over the 8-month study period, which was expected in this mouse strain. From month 2 onwards, a significantly increased aortic plaque area was observed in *Apoe*$^{-/-}$ mice exposed to 3R4F CS compared with either sham or THS aerosol- exposed animals. In contrast, exposure to THS aerosols resulted in plaque areas that did not differ significantly from those seen in sham animals, except at month 6 when the plaque areas were larger than in the sham mice, albeit less so than in those exposed to 3R4F CS. In animals that were switched to either air or THS aerosol, the plaque area was significantly smaller than in the mice exposed to 3R4F CS at months 3 and 8, though the plaques remained slightly, but not significantly, larger than those in sham or long-term THS aerosol-exposed mice. These findings were confirmed by additional quantitative investigation, using micro-computed tomography, at the 7-month time point.

6.1.4.4.1.2.2.4 Lung Function

Lung function was evaluated in anesthetized animals by performing a series of perturbations with the flexiVent rodent ventilator set (Scireq, Montreal, Canada). After 2 months, lung function in *Apoe*$^{-/-}$ mice worsened markedly in mice exposed to 3R4F CS relative to sham animals. This was indicated by significantly lower resistance and elastance and significantly higher lung compliance in the snapshot perturbation. Exposure of *Apoe*$^{-/-}$ mice to 3R4F CS also resulted in a leftward shift of the pressure-volume (P–V) loops for both the inflation and deflation phases of the maneuver, with an increased lung volume at the maximum pressure in mice exposed to 3R4F CS compared with the sham group. Exposure of the *Apoe*$^{-/-}$ mice to the THS aerosol, however, did not affect any of these lung function parameters in comparison with the sham group. In addition, neither switching nor cessation affected the snapshot perturbation parameters, with elastance, resistance and compliance remaining similar to those at the time point of switching exposures. It is worth noting that an age-related decline in lung elastance and resistance and an increase in compliance in the mice may have masked potential beneficial effects of switching and/or cessation on lung function. In contrast, P-V loops indicated

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"improved" elastance upon cessation or switching to THS aerosol and this lung function parameter became significantly different in these groups than in the 3R4F CS-exposed group at month 6.

In conclusion, *Apoe*$^{-/-}$ mice were assessed for physiological, histopathological and molecular evidence of lung inflammation, as well as the extent of atherosclerosis, following exposure to CS or THS aerosol. The results presented here collectively showed that cigarette smoke exposure accelerated development of atherosclerotic plaques and emphysema. While the 2-month exposure of mice to CS resulted in early manifestations of emphysema and atherosclerosis endpoints, both switching and cessation were beneficial, as evidenced by partial (lung function, plaque area and lung morphometry) or even complete (pulmonary inflammation) recovery to levels seen in sham controls. Most importantly, chronic exposure of *Apoe*$^{-/-}$ mice to THS aerosol (at nicotine concentrations matched to those in the CS) had minimal biological impact, reflecting the potential of THS to positively modify the risk of CVD and COPD in an animal model (15015_CVD_Resp_ApoE_SW_SR; 15015_CVD_Resp_ApoE_SW_SR_AA; (Lo Sasso et al, 2016a; Lo Sasso et al, 2016b; Phillips et al, 2016)).

6.1.4.4.1.2.3 A/J Study

To assess the impact of lifetime exposure to THS aerosol, compared with 3R4F CS, on development of emphysema and on lung tumor incidence and multiplicity, female A/J mice were exposed to fresh air (sham), 3R4F mainstream CS corresponding to 13.4 µg nicotine/L, or aerosol from THS aerosol equivalent to nicotine concentrations of 6.7 (Low), 13.4 (Medium) or 26.8 µg/L (High). Mice were exposed for 6 hours per day, 5 days per week, for up to 18 months following OECD Test Guideline 453 (OECD, 2009). In addition, two groups of male A/J mice were also exposed to fresh air (sham) and THS aerosol at 26 µg nicotine/L. Histopathological evaluation of respiratory tract organs and muscle tissues were performed at interim dissections of female mice, following 1, 5 and 10 months of exposure. The mice were also assessed at these time points for hematological and clinical chemistry parameters, lung function and pulmonary inflammation (the latter at months 1 and 5 only). Samples of nasal epithelium and lungs were collected from exposed female animals for transcriptomics and proteomics analyses.

Since this study's exposure phase has been completed recently, only the available clinical endpoints, histopathological findings and the transcriptomics data from nasal respiratory epithelial and trachea samples collected during the interim dissections at months 1, 5 and 10 are included here. Whether lifetime exposure to THS aerosol affects lung tumor incidences and multiplicities or causes pathologies in other than the respiratory organs will require analysis of samples obtained at terminal dissection (month 18).

6.1.4.4.1.2.3.1 Inflammation

3R4F CS-exposed female animals had lower WBC counts than sham animals, with higher absolute and relative neutrophil counts in the 3R4F CS-exposed A/J mice at month 10 compared with the sham mice and THS aerosol-exposed mice. No changes in WBC count were seen in mice exposed to THS aerosol.

Nasal respiratory and olfactory epithelium displayed morphological changes, identified as reserve cell and goblet cell hyperplasia and squamous metaplasia, following exposure to

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3R4F CS and THS aerosol. However, the incidence and severity of these adapative lesions were generally lower in the THS aerosol-exposed mice. Similarly, reserve cell hyperplasia and squamous metaplasia in the larynx were prominent histopathological findings in 3R4F CS-exposed animals and occurred less frequently and were less extensive in those exposed to THS aerosol, independent of concentration. In addition, typical CS exposure-related epithelial changes, including epithelial hyperplasia and squamous metaplasia, were found at the carina of bifurcation at months 5 and 10, while tracheas from A/J mice exposed to THS aerosol did not exhibit any remarkable exposure-related effects.

The lungs of 3R4F CS-exposed animals exhibited inflammatory cell infiltrates from month 1 onward, and these were absent in the animals from the THS and sham study groups. Pigmented and unpigmented macrophages were more frequent in A/J mice exposed to 3R4F CS from month 5, while this effect was not found in those exposed to THS aerosol. Furthermore, histopathological evaluation confirmed the presence of mild to moderate emphysematous lesions in mice exposed to 3R4F CS for 5 and 10 months. In contrast, lungs of THS aerosol-exposed mice showed no signs of the alveolar destruction typical of emphysema.

Analysis of BALF confirmed lung inflammation in 3R4F CS-exposed A/J mice, with higher total free lung cell counts at months 1 and 5 relative to sham animals. By comparison, neither total free lung cell count nor differential were different from sham in the THS aerosol-exposed animals. In addition, of 54 soluble mediators analyzed in BALF, 39 analytes including inflammatory, chemotactic, and growth factors were increased in samples of 3R4F CS-exposed compared with sham animals at month 1. Similarly, 38 of 53 analytes were increased in BALF samples of 3R4F CS-exposed compared with sham animals at month 5. Myoglobin was the only analyte that showed a concentration-dependent increase in the BALF of THS aerosol-exposed A/J mice at month 5.

To date, transcriptomics analysis of nasal respiratory and tracheal epithelia from female animals has been performed to gain insight into the mechanisms that may be involved in mediating exposure effects in this mouse strain. Systems toxicological evaluation of nasal epithelia revealed wide-ranging effects on inflammatory processes following exposure to 3R4F CS from month 1 onward. Exposure to a matching (by nicotine) THS aerosol concentration did not cause comparable network perturbations, while THS aerosol at the highest concentration had a more widespread impact, albeit of smaller amplitude than 3R4F CS. Of note, prolonged exposure to THS aerosol, independent of concentration, resulted in far fewer and less extensive network perturbations than were initially seen at the earlier time point. While most networks affected by THS aerosol exposure for 5 month suggested involvement of inflammatory processes, tissue repair and cell stress responses appeared to be more significantly affected at month 10.

6.1.4.4.1.2.3.2 Oxidative Stress

Biomarkers of exposure and oxidative stress were quantified in 24-hour urine collected from selected A/J mice from each study arm at defined intervals (months2, 6, and 10).

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Contrary to expectation, the levels of urinary 8-OHdG were lower in the 3R4F CS-exposed compared with the sham animals at all 3 time points. The levels of urinary 8-OHdG were also lower in female THS Medium and THS High group animals relative to the Sham group at month 10 and in the male THS High group animals relative to sham animals at months 6 and 10. No difference in urinary 8-OHdG levels was observed between the 3R4F and THS Medium (nicotine concentration-matched) groups.

6.1.4.4.1.2.3.3 Lung Function

Similar to the *Apoe*$^{-/-}$ Switching Study, lung function in exposed A/J mice was evaluated at months 1 and 5 with the flexiVent rodent ventilator set (Scireq, Montreal, Canada).

In the single-compartment model (single frequency forced oscillation), higher compliance and lower elastance were seen in the lungs of 3R4F CS-exposed mice compared with sham and THS aerosol-exposed mice at both time points. Exposure of the animals to THS aerosol did not have any obvious effect on any of these parameters relative to the Sham group. P-V loops in 3R4F CS-exposed A/J mice exhibited an upward and leftward shift for both the inflation and deflation phases of the maneuver relative to air-exposed animals. The maneuver also demonstrated an increase in lung volume at maximum pressure (30 cm H_2O) in 3R4F CS-exposed compared with sham and THS aerosol-exposed animals, while no obvious changes were noted for the THS group mice. Together, these results indicate decreased elastic recoil and increased compliance in animals exposed to 3R4F CS, which are suggestive of emphysematous changes in the lungs.

In summary, the data generated from the chronic inhalation study on mice show that exposure to aerosol from THS, produces less inflammation and adaptive changes than mainstream smoke from 3R4F, even at double the nicotine concentration in the test atmosphere. Thus the chronic toxicity of THS aerosol is much lower than that elicited by 3R4F smoke after 10 months of exposure. In addition, for a significant number of endpoints, the outcome of the exposure to THS aerosol resembles fresh air (Sham) exposure, however, a number of systemic changes require follow up at the 18-month necropsy time point (15020_AJ LC_QA-checked Interim Report).

6.1.4.4.2 Assessment of Inflammation in Clinical Studies

WBC counts were assessed in both a non-clinical setting (*Apoe*$^{-/-}$ study) and in two ambulatory Reduced Exposure studies, ZRHM-REXA-07-JP and ZRHM-REXA-08-US, conducted in Japan and the US, respectively. In the *Apoe*$^{-/-}$ study, there were no discernable trends or differences in WBC counts within or among any of the exposure groups. In both ambulatory Reduced Exposure studies, there was a reduction in WBC counts over the course of the study. The reductions were generally largest in the smoking abstinence group, but there were consistent reductions approaching these levels in the groups of smokers that had switched to THS. In the Japanese study, THS exposure resulted in reduced WBC counts, as early as Day 30, to levels similar to those seen with smoking abstinence. In the US study, THS exposure resulted in WBC counts that were lower than for continued smoking (CC group), at each time point except for at the end of study (Day 90).

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There was a lower evaluable sample size in the US study, due to non-adherence with product allocation (as compared with the Japanese study) and the timeframe for these studies was shorter than optimal (WBC reductions following smoking cessation are optimally detected after 6–12 months [Appendix A6.1.4.1]). Nonetheless, both studies showed that, in smokers who switched to THS, there was a decline in WBC counts, in the same direction as with smoking cessation, over the course of the study (Table 6).

Table 6 WBC Counts at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
WBC Count	THS (n=71)	5.57 GI/L (5.28, 5.86)	THS (n=47)	7.26 GI/L (6.84, 7.68)
	CC (n=41)	6.14 GI/L (5.76, 6.52)	CC (n=32)	7.09 GI/L (6.59, 7.59)
	SA (n=37)	5.73 GI/L (5.34, 6.13)	SA (n=9)	6.15 GI/L (5.19, 7.11)
	Diff THS-CC	-0.57 GI/L (-1.04, -0.10)	Diff THS-CC	0.17 GI/L (-0.47, 0.81)

WBC count = white blood cell count; THS = Tobacco Heating System; CC = conventional cigarettes; SA=smoking abstinence; Diff THS-CC = the difference between THS and CC; GI/L = gills/liter

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors.

Soluble ICAM (sICAM-1) was included as a clinical risk endpoint in the ambulatory Reduced Exposure studies. Reports in the literature strongly supported a role for sICAM as a clinical risk marker for inflammation. Levels of sICAM were strongly associated with smoking and showed reversibility upon smoking cessation, usually within 3 months (Appendix A6.1.4.2). In both studies, smokers who switched to THS had lower sICAM-1 levels than did subjects who continued to smoke, after adjusting for baseline sICAM-1 levels, sex and baseline CC consumption. These sICAM-1 levels (approximately 8.5–10.5% reduced) agreed with those in subjects who abstained from smoking. These changes were generally seen within the first 30 days of exposure and were maintained throughout the ambulatory period (Table 7).

Baseline C Reactive Protein (CRP) levels were similar among all subjects in each study. Following the 3-month exposure period, changes in CRP levels were very small, with no significant differences among the exposure groups. From a review of the literature (Appendix A6.1.4.3), the reversal of CRP levels following smoking cessation takes

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longer than a year. Therefore, the lack of differences among groups in this study was expected. Interestingly, though consistent with known ethnic differences (Azuma, 2013), differences between the study populations (i.e., Japanese vs. American) were larger than differences observed among groups within either study.

Fibrinogen assessments gave very similar results in the two studies. Though the subjects who switched to THS had consistently lower fibrinogen levels than those who continued to use CC, there were very little differences among all the exposure groups. As described in the literature review (Appendix A6.1.4.4), fibrinogen may be a better clinical risk endpoint in large epidemiological studies, than in smaller studies like these, because of its role in the pathophysiology of smoking-related diseases. This is despite the lack of a strong dose-response relationship between fibrinogen levels and smoking.

Table 7 Additional Inflammation-Related Clinical Risk Markers Measured at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
CRP	THS (n=70)	0.24 mg/L (0.18, 0.32)	THS (n=47)	1.23 mg/L (0.96, 1.56)
	CC (n=41)	0.26 mg/L (0.18, 0.36)	CC (n=32)	1.46 mg/L (1.09, 1.95)
	SA (n=37)	0.22 mg/L (0.15, 0.31)	SA (n=9)	1.26 mg/L (0.72, 2.18)
	THS:CC % Reduction	6.41% ↓ (-40.75, 37.77)	THS:CC % Reduction	16.23% ↓ (-21.69, 42.33)

(table continues)

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Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
sICAM-1	THS (n=70)	181.23 ng/mL (173.63, 189.17)	THS (n=45)	229.84 ng/mL (219.48, 240.68)
	CC (n=41)	198.55 ng/mL (187.71, 210.01)	CC (n=32)	257.07 ng/mL (243.58, 271.30)
	SA (n=37)	176.97 ng/mL (166.87, 187.68)	SA (n=9)	231.60 ng/mL (208.89, 256.79)
	THS:CC % Reduction	8.72% ↓ (2.05, 14.94)	THS:CC % Reduction	10.59% ↓ (4.03, 16.71)
Fibrinogen	THS (n=70)	274.62 mg/dL (262.33, 287.50)	THS (n=47)	305.0 mg/dL (289.9, 320.9)
	CC (n=41)	290.36 mg/dL (273.91, 307.81)	CC (n=32)	310.1 mg/dL (291.7, 329.6)
	SA (n=37)	276.30 mg/dL (256.37, 294.34)	SA (n=9)	316.0 mg/dL (281.6, 354.6)
	THS:CC % Reduction	5.42% ↓ (-1.80, 12.13)	THS:CC % Reduction	1.63% ↓ (-6.42, 9.08)

CRP = C Reactive Protein; sICAM-1 = soluble intercellular adhesion molecule 1; THS = Tobacco Heating System; CC = conventional cigarettes; SA=smoking abstinence

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors.

The nonclinical data for general or systemic inflammation endpoints presented above demonstrated a consistent improvement in inflammation related measurements following the switch from smoking to THS or cessation. These non-clinical findings were further supported by the results obtained in the ambulatory Reduced Exposure studies for WBC count and sICAM-1 levels, which showed initial improvements in smokers who switched to THS as compared with smokers who continued to smoke. In general, all the clinical risk endpoints changed in the same direction, as expected, following cessation and as seen in smokers who abstained from smoking during these studies.

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6.1.4.4.3 Assessment of Oxidative Stress in Clinical Studies

Based on literature reviews, both 8-epi-PGF2α and 11-DTX-B2 were regarded as clinical risk markers potentially informative about changes in oxidative stress within the timeframe of the ambulatory Reduced Exposure studies (Appendices A6.1.4.5 and A6.1.4.6).

The 8-epi-PGF2α levels were consistent across the two studies, with the 8-epi-PGF2α levels in smokers that switched to THS showing consistent reductions in the same direction as those observed with smoking abstinence. The smokers that switched to THS showed a more than 12% reduction in 8-epi-PGF2α levels compared with smokers who continued to smoke. This effect was consistent with results reported in the literature review in subjects following smoking cessation (Table 8).

Though shifts in the 11-DTX-B2 levels for smokers who switched to THS were in the same direction as those seen with smoking abstinence, the magnitude of the change was smaller than expected, especially in the US study (ZRHM-REXA-08-US). In the literature review (Appendix A6.1.4.6) variable results were observed for 11-DTX-B2, with the most relevant and consistent results seen in heavier smokers. The findings in the US study were further confounded by non-adherence with product allocation (in both the THS and SA arms), as compared with in the Japanese study. This resulted in a reduced sample size and a variability that was greater than expected in the 11-DTX-B2 results.

Table 8 Oxidative Stress Clinical Risk Markers Measured at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
8-epi-PGF2α	THS (n=70)	193.56 pg/mg CRE (181.00, 206.99)	THS (n=45)	264.61pg/mg CRE (244.14, 286.79)
	CC (n=41)	221.74 pg/mg CRE (203.07, 242.12)	CC (n=32)	305.75 pg/mg CRE (277.98, 336.31)
	SA (n=37)	208.62 pg/mg CRE (190.24, 228.78)	SA (n=9)	279.73 pg/mg CRE (233.63, 334.92)
	THS:CC % Reduction	12.71% ↓ (2.55, 21.81)	THS:CC % Reduction	13.46% ↓ (-1.95, 23.61)

(table continues)

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Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
11-DTX-B2	THS (n=70)	500.52 pg/mg CRE (464.53, 539.29)	THS (n=45)	428.66 pg/mg CRE (365.22, 503.13)
	CC (n=41)	549.90 pg/mg CRE (498.48, 606.63)	CC (n=32)	444.47 pg/mg CRE (368.57, 536.00)
	SA (n=37)	443.37 pg/mg CRE (399.99, 491.45)	SA (n=9)	412.67 pg/mg CRE (288.89, 589.47)
	THS:CC % Reduction	5.42% ↓ (-1.80, 12.13)	THS:CC % Reduction	3.56% ↓ (-23.31, 24.57)

8-epi-PGF2α = prostaglandin F2 alpha; 11-DTX-B2 = 11-dehydro-thromboxane B2; THS = Tobacco Heating System; CC = conventional cigarettes; SA=smoking abstinence; CRE=creatinine (results were adjusted for creatinine)

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors.

Overall the 8-epi-PGF2α data supported the existence of changes in oxidative stress for smokers who switch to either THS or to smoking abstinence. The 11-DTX-B2 data were encouraging because they consistently trended in the right direction (similar to the data for cessation). However, the reductions were not large enough to be conclusive, especially with the high variability seen in the Reduced Exposure studies.

Several translational CVD risk endpoints were evaluated in the *Apoe*$^{-/-}$ THS Switching Study and the ambulatory Reduced Exposure studies.

6.1.4.4.4 Assessment of CVD Risk Endpoints in Clinical Studies

Cholesterol and Triglycerides

Similar to what was seen in the *Apoe*$^{-/-}$ THS Switching Study, in the ambulatory Reduced Exposure studies, smokers who switched to THS had higher HDL-C levels as compared to smokers who continued to smoke. In the Japanese study (ZRHM-REXA-07-JP), the HDL-C levels in smokers following the switch to THS were similar to those following smoking abstinence, after adjusting for baseline HDL-C levels, sex and cigarette consumption at baseline. Though the levels in smokers switching to THS were greater than those in smokers that continued to smoke, the results for the smokers switching to smoking abstinence were not replicated in the US study (ZRHM-REXA-08-US); the HDL-C levels in smokers continuing to smoke CC were very similar to those in smokers who switched to smoking abstinence. In the US ambulatory Reduced Exposure study, only 9 of the 40 subjects randomized to the smoking abstinence group reported adherence

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with smoking abstinence during the 3-month follow-up period, thereby limiting the ability to interpret the smoking abstinence findings in that study (Table 9).

The other clinical risk endpoints measured in the ambulatory Reduced Exposure studies (LDL-C, total cholesterol, triglycerides, ApoA1 and ApoB) did not change and results were inconsistent within and between product exposures (even with smoking abstinence) within or between the two studies (Table 9). This was not surprising given the healthy nature of the study population, the small sample size, the level of variability among measurements of each marker and the limited exposure follow-up in these studies.

Table 9 Cholesterol and Triglyceride Clinical Risk Markers of CVD - Results Measured at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
HDL-C	THS (n=70)	61.62 mg/dL (59.58, 63.65)	THS (n=47)	54.7 mg/dL (52.3, 57.0)
	CC (n=41)	57.09 mg/dL (54.42, 59.77)	CC (n=32)	53.3 mg/dL (50.5, 56.1)
	SA (n=37)	63.45 mg/dL (60.66, 66.25)	SA (n=9)	53.3 mg/dL (48.1, 58.6)
	Diff THS-CC	4.53 mg/dL (1.17, 7.88)	Diff THS-CC	1.4 mg/dL (-2.3, 5.0)
LDL-C	THS (n=70)	112.05 mg/dL (107.52, 116.58)	THS (n=47)	111.0 mg/dL (105.4, 116.6)
	CC (n=41)	111.17 mg/dL (105.24, 117.11)	CC (n=32)	114.3 mg/dL (107.6, 121.0)
	SA (n=37)	116.79 mg/dL (110.52, 123.06)	SA (n=9)	116.1 mg/dL (103.5, 128.8)
	Diff THS-CC	0.87 mg/dL (-6.55, 8.30)	Diff THS-CC	-3.3 mg/dL (-12.0, 5.4)

(table continues)

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Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
Total Cholesterol	THS (n=70)	189.61 mg/dL (184.33, 194.90)	THS (n=47)	186.3.mg/dL (180.3, 192.2)
	CC (n=41)	187.62 mg/dL (180.67, 194.56)	CC (n=32)	190.3 mg/dL (183.2, 197.5)
	SA (n=37)	197.91 mg/dL (190.56, 205.27)	SA (n=9)	187.7 mg/dL (174.1, 201.2)
	Diff THS-CC	2.00 mg/dL (-6.68, 10.67)	Diff THS-CC	-4.0 mg/dL (-13.3, 5.2)
Triglycerides	THS (n=70)	130.07 mg/dL (120.96, 139.19)	THS (n=47)	136.0 mg/dL (127.3, 144.7)
	CC (n=41)	136.32 mg/dL (124.42, 148.23)	CC (n=32)	135.1 mg/dL (124.6, 145.6)
	SA (n=37)	148.77 mg/dL (136.06, 161.47)	SA (n=9)	124.4 mg/dL (104.4, 144.3)
	Diff THS-CC	-6.25 mg/dL (-21.20, 8.69)	Diff THS-CC	0.9 mg/dL (-12.8, 14.6)
Apo A1	NA(1)		THS (n=47)	151.5 mg/dL (146.6, 156.4)
			CC (n=32)	148.5 mg/dL (142.5, 154.4)
			SA (n=9)	147.8 mg/dL (136.7, 158.9)
			Diff THS-CC	3.1 mg/dL (-4.6, 10. 7)

(table continues)

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Clinical Risk Endpoint	ZRHR-REXA-07-JP	ZRHM-REXA-08-US	
Apo B	NA[(1)]	THS (n=47)	85.00 mg/dL (81.39, 88.62)
		CC (n=32)	86.61 mg/dL (82.26, 90.95)
		SA (n=9)	88.18 mg/dL (79.89, 96.48)
		Diff THS-CC	-1.6 mg/dL (-7.24, 4.03)

HDL-C = high density lipoprotein-cholesterol; LDL-C = low density lipoprotein-cholesterol Apo A1 = apolipoprotein A1; Apo B = apolipoprotein B THS = Tobacco Heating System; CC = conventional cigarettes; SA=smoking abstinence; Diff THS-CC = the difference between THS and CC

(1) ApoA1 and ApoB were not measured in the ZRHM-REX-07-JP study. NA, not available.

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors.

In addition to the findings presented in Table 9, a panel of oxysterols (*i.e.*, oxidatively modified cholesterol species) were assessed descriptively in the US ambulatory Reduced Exposure study (Table 10) as an exploratory endpoint to investigate if the reduction observed in animal models of disease upon smoking cessation and switching to THS would also be observed in humans.

Table 10 Panel of Oxidatively Modified Cholesterol Species Collected in the ZRHM-REXA-08-US Study

6α-hydroxy-5α-cholestane	24(R)-hydroxycholesterol
7α-hydroxycholesterol	25-hydroxycholesterol
5α,6α-epoxycholestanol	27-hydroxycholesterol
7-ketocholesterol	22(R)-hydroxycholesterol
7β-hydroxycholesterol	4ß-hydroxycholesterol
5β,6β-epoxycholestanol	

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Levels of oxysterols measured throughout the exposure period, in all exposure groups, were comparable to baseline levels.

Blood Pressure

In the ambulatory Reduced Exposure studies, blood pressure was measured as part of the safety procedures and no significant changes were expected as a result of changes in product exposure for only 3 months. However, because of the accepted link between changes in blood pressure and reduced cardiovascular risk, the blood pressure measurements were included in the clinical risk assessment. In these studies, a small, consistent, but not clinically relevant (<1 mmHg) change in systolic blood pressure was observed after 3 months exposure to THS as compared with continued CC smoking. This may be interesting to further investigate in longer-term clinical studies or in post-market studies in populations with increased cardiovascular risk (Table 11).

Table 11 Blood Pressure Clinical Risk Markers Measured at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
Systolic Blood Pressure	THS (n=70)	103.17 mmHg (101.21, 105.12)	THS (n=45)	116.5 mmHg (114.0, 118.9)
	CC (n=41)	103.76 mmHg (101.19, 106.33)	CC (n=32)	117.2 mmHg (114.2, 120.1)
	SA (n=37)	103.92 mmHg (101.15, 106.69)	SA (n=9)	114.8 mmHg (109.2, 120.4)
	Diff THS-CC	-0.59 mmHg (-3.80, 2.62)	Diff THS-CC	-0.7 mmHg (-4.5, 3.1)

(table continues)

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Clinical Risk Endpoint	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
Diastolic Blood Pressure	THS (n=70)	62.25 mmHg (60.81, 63.69)	THS (n=45)	70.4 mmHg (67.9, 72.9)
	CC (n=41)	62.93 mmHg (61.04, 64.82)	CC (n=32)	70.2 mmHg (67.2, 73.2)
	SA (n=37)	63.93 mmHg (61.92, 65.94)	SA (n=9)	68.0 mmHg (62.4, 73.6)
	Diff THS-CC	-0.68 mmHg (-3.04, 1.69)	Diff THS-CC	0.2 mmHg (-3.7, 4.0)

THS = Tobacco Heating System; CC = conventional cigarettes; SA=smoking abstinence; Diff THS-CC = the difference between THS and CC

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors.

The non-clinical results, together with the clinical results for the CVD clinical risk endpoints, show a fairly consistent trend in a favorable direction. This trend was consistent with results reported in the literature following smoking cessation.

6.1.4.4.5 Assessment of Lung Function in Clinical Studies

The ambulatory Reduced Exposure studies, similar to the other clinical assessment studies conducted for THS, included spirometry measurements primarily as a measure of safety. However, because of the interest in understanding the impacts of THS and smoking cessation on lung function in mid- to long-term follow-up studies, including the ambulatory Reduced Exposure studies, these results were also used to assess changes in lung function. The US Study (ZRHM-REXA-08-US) further expanded the assessment of lung function beyond spirometry, also assessing gas transfer parameters (DLCO and KCO), and lung volume parameters (FRV, VC, TLC and IC). In addition to the different lung function parameters that were collected in the two studies, there was also a difference in administration of the spirometry testing. In the Japanese study (ZRHM-REXA-07-JP), post-screening spirometry testing was conducted prior to administering a bronchodilator, while in the US study (ZRHM-REXA-08-US) the post-screening spirometry testing was performed after administering a bronchodilator.

In the Japanese study (ZRHM-REXA-07-JP), smokers who switched to THS had an increase of 1.91 percent of predicted value (%Pred) in their FEV_1 as compared with smokers who continued to smoke CC, with no notable differences between those switching to THS and with smoking abstinence. These data were promising but studies of

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longer duration (at least 6 to 12 months) will be required to fully assess the impact of the exposures on FEV_1.

In the US study (ZRHM-REXA-08-US), vital capacity was the only lung function test where a notable difference (0.10 L) was observed between smokers that switched to THS and those who continued to smoke. The difference in FEV_1 values between smokers who switched to THS and those who continued to smoke was smaller in magnitude in this study, as compared with in the Japanese study. Nonetheless, the results were consistent and trended in the expected direction following smoking abstinence. There were no other notable differences in gas transfer, lung volume or spirometry parameters between smokers that switched to THS and those who continued to smoke CC (Table 12).

Table 12 Lung Function Clinical Risk Markers Measured at the Day 90 Visit in the Reduced Exposure - Ambulatory Studies

Clinical Risk Endpoint [1]	ZRHR-REXA-07-JP		ZRHM-REXA-08-US	
FEV_1	THS (n=70)	95.36 %Pred (94.11, 96.62)	THS (n=47)	91.18 %Pred (89.08, 93.27) 3.45 L (3.38, 3.52)
	CC (n=41)	93.45 %Pred (91.81, 95.09)	CC (n=30)	90.64 %Pred (88.05, 93.24) 3.41 L (3.32, 3.49)
	SA (n=37)	95.38 %Pred (93.66, 97.11)	SA (n=9)	92.64 % Pred (87.87, 97.41) 3.43 L (3.28, 3.58)
	Diff THS-CC	1.91 %Pred (-0.14, 3.97)	Diff THS-CC	0.53 % Pred (-2.09, 3.00) 0.05 L (-0.06, 0.15)

(table continues)

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Clinical Risk Endpoint [1]	ZRHR-REXA-07-JP	ZRHM-REXA-08-US	
FEV_1/FVC	NA	THS (n=47)	0.79 (0.77, 0.81)
		CC (n=30)	0.79 (0.77, 0.81)
		SA (n=9)	0.80 (0.77, 0.83)
		Diff THS-CC	0.00 (-0.02, 0.02)
MEF 25-75 (L/s)	NA	THS (n=47)	5.21 (1.62, 8.81)
		CC (n=30)	5.88 (1.45, 10.31)
		SA (n=9)	3.99 (-4.20, 12.17)
		Diff THS-CC	-0.67 (-6.33, 4.99)
DLCO (mL/min/mmHg)	NA	THS (n=45)	24.56 (23.64, 25.48)
		CC (n=30)	24.25 (23.17, 25.32)
		SA (n=8)	22.61 (20.50, 24.73)
		Diff THS-CC	0.31 (-1.09, 1.72)

(table continues)

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Clinical Risk Endpoint [1]	ZRHR-REXA-07-JP	ZRHM-REXA-08-US	
KCO (mmol/min/kPa/L)	NA	THS (n=45)	1.41 (1.36, 1.46)
		CC (n=30)	1.36 (1.30, 1.42)
		SA (n=7)	1.38 (1.26, 1.49)
		Diff THS-CC	0.05 (-0.02, 0.12)
TLC (L)	NA	THS (n=47)	5.78 (5.56, 5.99)
		CC (n=30)	5.69 (5.43, 5.95)
		SA (n=9)	5.38 (4.90, 5.87)
		Diff THS-CC	0.09 (-0.25, 0.43)
FRV (L)	NA	THS (n=47)	2.80 (2.66, 2.94)
		CC (n=30)	2.89 (2.71, 3.06)
		SA (n=9)	3.21 (2.90, 3.53)
		Diff THS-CC	-0.09 (-0.31, 0.13)

(table continues)

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Clinical Risk Endpoint (1)	ZRHR-REXA-07-JP	ZRHM-REXA-08-US	
IC (L)	NA	THS (n=47)	3.01 (2.82, 3.19)
		CC (n=30)	2.79 (2.57, 3.02)
		SA (n=9)	2.13 (1.72, 2.54)
		Diff THS-CC	0.21 (-0.08, 0.51)
VC (L)	NA	THS (n=47)	4.43 (4.36, 4.50)
		CC (n=30)	4.33 (4.25, 4.41)
		SA (n=9)	4.38 (4.23, 4.52)
		Diff THS-CC	0.10 (0.00, 0.21)

THS = Tobacco Heating System; **CC** = conventional cigarettes; **SA**=smoking abstinence; **%Pred** = percent of predicted value (%Pred)

DLCO= diffusion capacity for lung CO; **FEV_1**= forced expiratory volume in 1 second; **FRV**=functional residual volume; **FVC**=forced vital capacity **IC**= inspiratory capacity; **KCO**= rate constant of CO; **MEF**= mid expiratory flow; **TLC**= total lung capacity; **VC** = vital capacity

(1) Spirometry measures in the ZRHM-REXA-07-JP study (FEV_1) were conducted pre-bronchodilator while those the in the ZRHM-REXA-08-US study (FEV_1, FEV_1/FVC and MEV 25-75) were conducted post-bronchodilator

Note: Values are the adjusted least square means and 95% confidence intervals from the ANCOVA model conducted with baseline value, product exposure, sex and baseline CC consumption as fixed effect factors. NA, not available.

The lung function results from the non-clinical assessment, together with those from the clinical assessment programs, showed consistent trends in a favorable direction that were consistent with results reported in the literature following smoking cessation.

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6.1.4.4.6 Assessment of Genotoxicity in Clinical Studies

The clinical assessment program was not designed to directly assess cancer through any cancer-specific clinical risk endpoints. However, as described in Section 6.1.4.3, there were many indirect measurements collected within the ambulatory and confinement Reduced Exposure studies that supported a potential reduction in cancer risk including:

- Reduced Exposure to IARC Class I and II Compounds
- Reduced CYP1A2 Activity
- Reduced Urine Mutagenicity

The reduction in exposure to carcinogenic HPHCs was demonstrated unequivocally in the THS assessment program with exposure reductions in every carcinogenic HPHC measured within each of the studies. There were reductions of up to 85% in smokers who switched to THS compared with those continuing to smoke CC. Equally important, the reductions observed in these studies were very close to those observed in subjects who stopped smoking for the duration of the study. A similar finding was observed in comparisons of THS-exposed to sham-exposed animals in the non-clinical studies.

The observed reductions in exposure in the THS study arms in the clinical assessment program translated into a reduction in CYP1A2 activity and reduced urine mutagenicity, similar to those observed in subjects who abstained from smoking. CYP1A2 is involved in activation of carcinogenic heterocyclic and aromatic amines, strong carcinogens associated with colon and bladder cancers (Surgeon General's Report 2010) and is induced to a large extent by polycyclic aromatic hydrocarbons (PAH) (Libby et al, 2002). Levels of PAH were largely reduced in smokers who switched to THS compared with those who continued to smoke CC (see sections 6.1.1 and 6.1.3).

In summary, the results presented in this dossier indicated indirectly, but consistently, that switching from smoking cigarettes to THS will reduce a smoker's exposure to many of the HPHCs related to cancer in cigarette smokers in a manner similar to smoking cessation. These results are similar with respect to both the rate of reduction and the levels obtained, even in an ambulatory setting.

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