7.5 Executive Summary: Mechanistic and Systems Toxicology Studies

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7.5.1 Introduction to systems toxicology

Evidence is steadily accumulating that better and more informed decisions can be made using systems data alongside conventional histopathology and clinical chemistry data than can be made using traditional safety assessment methods. The U.S. National Research Council, commissioned by the U.S. Environmental Protection Agency, developed a vision for toxicity testing in the 21st century (called Tox 21-c). It was postulated that there will be a shift from traditional toxicity testing toward efforts focused on exploring and improving our understanding of the signaling pathways that are perturbed by biologically active substances or their metabolites, potentially leading to adverse health effects in humans (Hartung, 2010; Krewski et al, 2010).

A systems toxicology approach is broader than just an attempt to understand the effects of exposure at the pathway level. It is a highly interdisciplinary approach using advanced analytical and computational tools to integrate classical toxicology and quantitative analysis of large networks of molecular and functional changes occurring across multiple levels of biological organization. Systems toxicology depends on data produced using rapidly developing -omics technologies, such as transcriptomics, metabolomics, and proteomics (Hoeng et al, 2014a). Systems toxicology enables the integration of quantitative systems wide molecular changes in the context of chemical exposure measurements and a causal succession of molecular events linking exposures with toxicity and eventual disease outcome (Sturla et al, 2014). The objective is to generate a comprehensive overview of the effects of exposure and to gain an understanding of the data by combining complex biological network models with quantitative measurements of affected pathways at all levels of biochemical and biological organization (Hoeng et al, 2012; Sturla et al, 2014). The systems toxicology approach has been used successfully in *in vitro* and *in vivo* studies to assess prototypic modified-risk tobacco products (Ansari et al, 2016; Hoeng et al, 2014b; Kogel et al, 2014a; Kogel et al, 2015; Phillips et al, 2015b).

A systems toxicology assessment of the THS 2.2 in various *in vitro* and *in vivo* exposure studies is summarized in this section. The overall outcome shows that biological systems are affected less severely by THS 2.2 aerosol than by reference cigarette smoke (3R4F).

7.5.2 Mechanistic and systems toxicology studies performed to assess the biological impacts of exposure to THS 2.2 aerosol

Smoking-related diseases have a complex etiology. Broadly accepted mechanisms underlying many smoking-related diseases are related to impaired organ function from progression of pathological changes and comorbidity. Exposure to cigarette smoke induces molecular changes in the exposed organism and disrupts various biological processes. This in turn causes alterations at the cell and tissue level that result in physiological changes which eventually manifest themselves as diseases (Figure 1).

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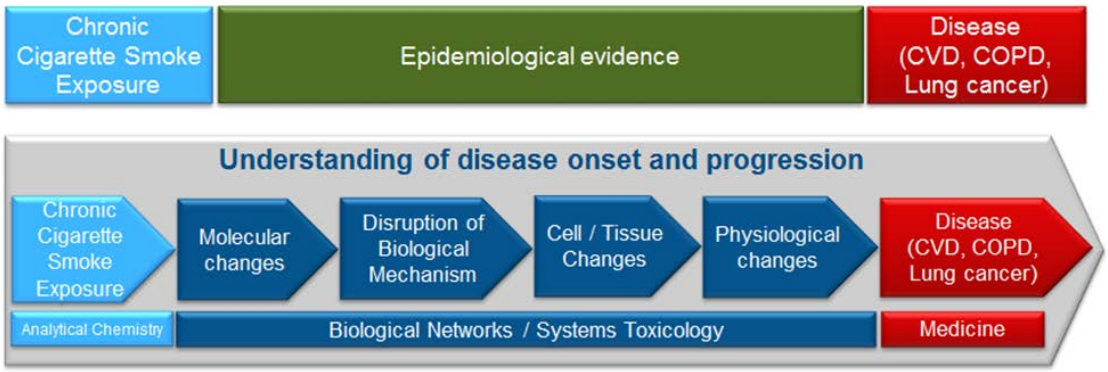


Figure 1. A schematic describing the granularity that systems toxicology provides to the understanding of the hazards resulting from cigarette smoke exposure.

A number of non-clinical studies using laboratory animals and *in vitro* tests were conducted to identify potential hazards and characterize mechanisms underlying tobacco-related diseases associated with the use of THS 2.2 (Table 1).

Table 1. Studies Performed to Measure Biological and Functional Changes

Study	Type	Design	Reference
Clinical	*Clinical*	Randomized, three-arm (CC, THS 2.2, and SA) parallel study	REXC03EU_1762_SysTox_Pub REXA07JP_REXC04JP_1760_1761_SysTox_SR
Fraction impact assessment study	*In vitro*		167321_High_Content_Screening_ SysTox _Pub
Organotypic oral	*In vitro*	Parallel-group air, RC[a], and THS 2.2 aerosols	1743_Organotypic_Buccal_SP 1743_Organotypic_Buccal_SR 1743_Organotypic_Buccal_Pub
Organotypic nasal	*In vitro*	Parallel-group air, RC[a], and THS 2.2 aerosols	1722_Organotypic_Nasal_SP 1722_Organotypic_Nasal_SR 1722_Organotypic_Nasal_Pub
Organotypic bronchial	*In vitro*	Parallel-group air, RC[a], and THS 2.2 aerosols	1709_Organotypic_Bronch_SP 1709_Organotypic_Bronch_SR 1709_Organotypic_Bronch_Pub
Adhesion assay	*In vitro*	Parallel-group air, RC[a], and THS 2.2 aerosol extracts	1604_CVD_Adhesion_Assay_Pub

(table continues)

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Study	Type	Design	Reference
Transendothelial migration assay	*In vitro*	Parallel-group air, RC[a], and THS 2.2 aerosol extracts	1739_CVD_Migration_Assay_Pub
Rat OECD THS 2.2	*In vivo*	TG413 (air (sham), RC[a], and THS 2.2)	15006_150082 THS SP 15006_150083 THS SP 15006_150082_150083 THS SR 15006_150082_150083 THS PUB
Rat OECD THS 2.2M	*In vivo*	TG413 (air (sham), RC[b], THE2.2, and THS 2.2M)	15025_176500 THS SP 15025_176500 THS SR 15025_176500 THS PUB
ApoE$^{-/-}$ THS 2.2	*In vivo*	Parallel-group, switching (air (sham), RC[a], THS 2.2, cessation, and switching)	15015_CVD_Resp_ApoE_SW_SP 15015_CVD_Resp_ApoE_SW_SR_Part1 15015_CVD_Resp_ApoE_SW_SR_Part2 15015_CVD_Resp_ApoE_SW_SR_Part3 15015_CVD_Resp_ApoE_SW_SR_Part4 15015_CVD_Resp_ApoE_SW_SR_Part5 15015_CVD_Resp_ApoE_SW_SR_Part6 15015_CVD_Resp_ApoE_SW_SR_Part7 15015_CVD_Resp_ApoE_SW_SR_Part8 15015_CVD_Resp_ApoE_SW_SR_Part9 15015_CVD_Resp_ApoE_SW_SR_Part10 15015_CVD_Resp_ApoE_SW_Pub_Main 15015_CVD_Resp_ApoE_SW_Pub_Liver 15015_CVD_Resp_ApoE_SW_Pub_Model_Justif
Transversal docs			DOC_Xenobiotic_Metabolism DOC_Inflammation DOC_Oxidative_stress DOC_Lipid_Biology
Independent verification	*In vivo and in vitro*	External review panels	DOC_ApoE_Ext_Verif_ Histo_Panel DOC_ApoE_Ext_Verif_ Mixed_Panel DOC_ApoE_Organo_Ext_Verif_ Mixed_Panel

CC = commercial cigarettes, RC = reference cigarettes (3R4F), SA = smoking abstinence, THS 2.2 = tobacco heating system version 2.2, [a] regular, [b] mentholated

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7.5.2.1 Using systems pharmacology to identify exposure-response markers in peripheral blood after switching to THS 2.2 from using conventional cigarettes

If the primary tissue (e.g., the lung) in humans is not readily accessible, a surrogate tissue, such as whole blood, may be used to monitor the effects of exposure to cigarette smoke and the discontinuation of such exposure. Changes in whole-blood gene-expression profiles in response to smoke exposure can be complex and difficult to interpret, but the behaviors of sets of genes can be used as classifiers to separate subject groups. A gene signature that has previously been derived from whole blood can be used to distinguish between current smokers, non-smokers, and former smokers with high levels of specificity and sensitivity (Martin et al, 2015). The signature consists of the genes *LRRN3*, *SASH1*, *PALLD*, *RGL1*, *TNFRSF17*, *CDKN1C*, *IGJ*, *RRM2*, *ID3*, *SERPING1*, and *FUCA1*. The signature was used to assess the blood gene expression profiles produced in two clinical studies that were conducted to assess and compare responses to continuously smoking CCs, smoking abstinence, and switching to THS 2.2 (Haziza et al, 2016). The signature performed remarkably well, and showed that the response to exposure had significantly decreased just 5 d after a subject had switched to using THS 2.2. The response was also a significantly decreased just 5 d after smoking abstinence. Consistent decreases in the signature scores were found in two 5 d cessation confinement studies. Similar decreases were found for both smoking abstinence and switching to mentholated THS 2.2 in a study in which a 5 d confinement period was followed by an 85 d ambulatory period (REXC03EU_1762_SysTox_Pub and REXA07JP_REXC04JP_1760_1761_SysTox_SR).

7.5.2.2 *In vitro* systems toxicology assessment of exposure to THS 2.2 aerosol in a simple airway-cell-culture system

The biological impacts of the THS 2.2 aerosol and the 3R4F reference cigarette smoke were compared using primary normal human bronchial epithelial cells (Gonzalez-Suarez et al, 2016). Multiparametric indicators of cellular toxicity were measured using real-time cellular analysis and a high-content screening method. Whole transcriptome analysis was also performed on the human bronchial epithelial cells, and systems toxicology approaches were used to identify and quantify perturbed molecular pathways (Martin et al, 2014). Exposing cells to 3R4F cigarette smoke resulted in dose-dependent toxicity. Moreover, significant perturbations in multiple biological pathways, particularly those related to cellular stress, were found. Exposure to THS 2.2 aerosol resulted in a lower overall biological impact, and no toxic effects were found when THS 2.2 aerosol doses, which matched the 3R4F smoke doses, were used. Exposure to THS 2.2 aerosol led to toxic responses in some functional endpoints, only when the THS 2.2 dose was between three and 15 times higher than the 3R4F smoke doses. The biological networks were perturbed less by exposure to THS 2.2 aerosol than by exposure to 3R4F smoke. These data suggested that THS 2.2 aerosol is less toxic than combustible cigarette smoke, and,

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therefore, that the risks of smoking-related diseases occurring could be lower using a THS 2.2 than smoking conventional cigarettes (Gonzalez-Suarez et al, 2016).

7.5.2.3 *In vitro* exposure of organotypic tissue cultures to whole aerosol

Human organotypic tissue culture models have been increasingly used to study the effects of inhaled toxicants. A better recapitulation of the *in vivo* situation can be achieved using recently developed three-dimensional tissue culture models than using two-dimensional monolayer cultures. These three-dimensional models are composed of differentiated human primary epithelial cells that form a pseudostratified epithelium exhibiting polarity and tight junctions (Iskandar et al, 2013; Iskandar et al, 2015; Mathis et al, 2013; Schlage et al, 2014; Talikka et al, 2014). Such models can be exposed to whole aerosols or smoke at the air–liquid interface (Kuczaj et al, 2016; Majeed et al, 2014).

In vitro *assessment of the acute exposure of organotypic bronchial human tissue cultures to THS 2.2 aerosol*

A human organotypic bronchial epithelial tissue culture is composed of basal cells, mucus-releasing cells, and ciliated cells. Such a culture can secrete inflammatory markers and undergo adaptive morphological changes when a challenge is presented (Iskandar et al, 2013; Iskandar et al, 2015; Kuehn et al, 2015; Mathis et al, 2013; Talikka et al, 2014). This tissue model is an appropriate *in vitro* system for studying the effects of inhaled toxicants, such as whole cigarette smoke, using the Vitrocell® whole-aerosol exposure system (Kuczaj et al, 2016; Majeed et al, 2014). The objective of the study was to compare the impact of an acute exposure to 3R4F reference cigarette smoke and the THS 2.2 aerosol. Various concentrations of THS 2.2 aerosol were applied to the air–liquid interfaces of human organotypic bronchial inserts for 28 min, and the results were compared with the results for cultures exposed to either fresh air or 3R4F cigarette smoke (1709_Organotypic_Bronch_SP). The experiment was repeated multiple times using different batches of bronchial organotypic epithelia from two different adult non-smoking donors to ensure that robust data was generated. Overall, the effects of THS 2.2 aerosol exposure were comparable to the effects of exposure to air and significantly different from exposure to 3R4F smoke at comparable nicotine concentrations. This was observed for endpoints such as cytotoxicity, the release of pro-inflammatory mediators, and tissue histology. For example, the higher concentration of 3R4F smoke (0.25 mg nicotine/L) elicited approximately 40% cytotoxicity at 48 h and 72 h post-exposure, while no cytotoxicity was observed for THS 2.2 aerosol exposure at the comparable concentration and also at a 1.7-fold higher concentration. Likewise, the enhanced secretion of five inflammatory mediators was statistically significantly reduced by 56.5% (for CXCL8) up to 91.2% (for sICAM) in cultures exposed to THS 2.2 aerosol compared with 3R4F smoke-exposed cultures (both at 0.25 mg nicotine/L) at the 24 h post-exposure time-point. We compared the molecular perturbations that occurred in response to exposure to 3R4F smoke and THS 2.2 aerosol using systems-toxicology-based risk assessment approaches in which computable biological network models and gene expression changes were

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combined. The numbers of differentially expressed genes in the 3R4F smoke exposed cultures at 48 h and 72 h post-exposure were 5912 and 4736 respectively, while no differentially expressed genes were seen for THS 2.2 aerosol exposure at the comparable concentration. Only for the 3-fold higher THS 2.2 aerosol concentration, 99 and 84 genes were differentially regulated at these time-points. These transcriptomic changes induced after 3R4F smoke exposure indicated significant perturbations in several cellular networks at all post-exposure time points. Exposure to THS 2.2 aerosol at a comparable nicotine concentration to that in 3R4F smoke perturbed almost the same networks but with a smaller amplitude than when the cultures were exposed to 3R4F smoke. For example, the RBIF values for 3R4F smoke exposure at 48 h and 72 h post-exposure time-points were 30% and 23%, respectively, of the maximum impact at 4 h post-exposure (set as 100%), whereas THS 2.2 aerosol at the comparable exposure level elicited an RBIF of 2% and 0.1%, respectively, at these time-points. Even for the 1.7-fold and 3-fold higher THS 2.2 concentrations, the RBIF values were as low as 3% and 20% at 48 h and 0.8% and 0.7% at the 72-h time-point. This analysis also demonstrates that the responses to THS 2.2 aerosol were mainly transient: at 4 h post-exposure, the RBIF for the comparable THS 2.2 aerosol concentration was 8%, and for the two higher concentrations: 28% and 50%, respectively. The overall assessment results support the conclusion that acute exposure to THS 2.2 aerosol affects human bronchial epithelial tissue cultures less than acute exposure to combustible cigarette smoke (1709_Organotypic_Bronch_SR and 1709_Organotypic_Bronch_Pub).

In vitro *assessment of acute exposure of organotypic oral human tissue cultures to THS 2.2 aerosol*

Besides its well-known effects increasing predisposition to oral cancer, cigarette smoke exposure is an important risk factor for many conditions including periodontal diseases, gingivitis and other benign mucosal disorders. Human organotypic oral epithelial tissue cultures can be used to study the effects of inhaled contaminants using the Vitrocell® whole-aerosol exposure system (Kuczaj et al, 2016; Majeed et al, 2014; Schlage et al, 2014). A study was performed in which the effects of exposure to THS 2.2 aerosol were compared with the effects of exposure to 3R4F smoke. Human organotypic oral epithelial tissue cultures were exposed to 3R4F smoke or THS 2.2 aerosol, both diluted with air to give two comparable nicotine concentrations, for 28 min. A 2-fold higher THS 2.2 aerosol concentration (1.09 mg nicotine/L) was also tested. Comprehensive analyses were performed, including cytotoxicity assay buccal epithelial transcriptomic (messenger RNA and microRNA (miRNA) analysis, secreted pro-inflammatory marker measurements, and histopathological analysis (1743_Organotypic_Buccal_SP). All three THS 2.2 aerosol concentrations that were tested resulted in no cytotoxicity while exposure to 3R4F smoke caused weak but statistically significant cytotoxicity. Morphological alterations in the THS 2.2 aerosol-exposed cultures were confined to increased desquamation at the middle and high concentrations and the 72-h time-point, while this and additional structural changes, such as detachment above the basal level, were observed in cultures exposed to 3R4F smoke at both concentrations (comparable to the low and middle THS aerosol

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concentration) at 48 h and 72 h. The secretion of inflammatory mediators within 24 h following THS 2.2 aerosol exposure at the higher comparable concentration was significantly lower for MMP-1 (- 45.8%) and IL-1β (-88.8%) than for the 3R4F smoke exposure, and similar reductions were seen at the 2-fold higher THS 2.2 aerosol concentration. In contrast to the bronchial and nasal organotypic cultures, some other analytes were secreted at higher levels following THS 2.2 aerosol exposure compared with 3R4F smoke exposure, e.g., CXCL10 by 58.8% and IL-6 by 146.2%. It should be noted that the biological functions and inflammatory responses in the squamous oral epithelia differ considerably from those in the ciliated pseudostratified epithelia from the nasal and bronchial airways. The numbers of differentially expressed genes in the 3R4F smoke exposed cultures at the two comparable concentrations at 72 h post-exposure (the maximum response time-point in buccal cultures) were 10007 and 9911 respectively, while only one upregulated gene was seen for THS 2.2 aerosol exposure at each of the comparable concentrations. Only for the 2-fold higher THS 2.2 aerosol concentration, 3178 genes were differentially regulated at this time-point. The RBIF values for exposure to both 3R4F smoke concentrations at 72 h post-exposure were 100% and 92%, respectively, whereas THS 2.2 aerosol at the comparable exposure levels elicited RBIF values of 0.1% and 0.5%, respectively, at this time-point. Even for the 2-fold higher THS 2.2 concentration, the RBIF values was as low as 6% at the 72-h time-point, while the highest RBIF values for the three THS 2.2 aerosol concentrations were seen at 4 h post-exposure: 7%, 8%, and 24%. Further mechanistic analysis of the transcriptomic changes in the oral cultures exposed to 3R4F smoke showed significant perturbations in cellular networks, including apoptosis, necroptosis, senescence, xenobiotic metabolism, oxidative stress, and NFE2L2 signaling. The stress responses after exposure to THS 2.2 aerosol were markedly weaker than the responses after exposure to 3R4F cigarette smoke, and more complete recovery occurred in cultures exposed to THS 2.2 aerosol than to 3R4F cigarette smoke (1743_Organotypic_Buccal_SR and 1743_Organotypic_Buccal_Pub).

In vitro *assessment of acute exposure of organotypic nasal human tissue cultures to THS 2.2 aerosol*

Cells in the nasal cavity are some of the first targets of pathogens, allergens, and inhaled toxic insults, such as cigarette smoke. Exposure to cigarette smoke is associated with nasal inflammation, making the nasal epithelium relevant for evaluating the pathophysiological impact of exposure to cigarette smoke (Benninger, 1999; Nicola et al, 2014).

The objectives of this study were to assess the effects of exposure to smoke generated from a reference cigarette 3R4F and aerosol generated from THS 2.2 on human organotypic nasal epithelial cultures exposed to 3R4F smoke or THS 2.2 aerosol at the air–liquid interface for 28 min. A series of experimental repetitions was conducted allowing a more reliable understanding of the cellular and molecular changes following exposure (1722_Organotypic_Nasal_SP). Exposure to THS 2.2 aerosol caused less cytotoxicity and smaller effects on various biological endpoints than did exposure to 3R4F smoke at equivalent concentrations (0.15 and 0.27 mg nicotine/L). The histological

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evaluations showed that THS 2.2 aerosol with a nicotine concentration at least four times higher than the nicotine concentration in the 3R4F smoke was needed to elicit similar morphological changes and cytotoxicity in the nasal cultures. The levels of five secreted inflammatory mediators were significantly lower in the basolateral media of cultures exposed to THS 2.2 aerosol compared with 3R4F smoke exposed cultures: at 24 h following exposure to the higher comparable concentration, THS 2.2 aerosol elicited, e.g., 52.9% lower secretion of CXCL-8 and 80.6% lower levels of sICAM1, being close to the reductions seen in the bronchial organotypic model, while other reduced mediators like EGF (-88.2%) were not measured in the bronchial model. The numbers of differentially expressed genes in the 3R4F smoke exposed cultures at 48 h and 72 h post-exposure were 3309 and 1781 respectively, while no differentially expressed genes were seen for THS 2.2 aerosol exposure at the comparable concentration at 48 h and 5 genes at 72h. The maximum responses in buccal tissue were seen at the 4h time-point, with 4978 genes following 3R4F smoke exposure and 551 genes following THS 2.2 aerosol exposure. Network-based gene expression data analysis revealed that various biological networks were perturbed less by exposure to THS 2.2 aerosol than by exposure to 3R4F smoke. The RBIF values for 3R4F smoke exposure at 48 h and 72 h post-exposure time-points were 14% and 11%, respectively, of the maximum impact at 4 h post-exposure (set as 100%), whereas THS 2.2 aerosol at the comparable exposure level elicited an RBIF of 0% and 0.1%, respectively, at these time-points. Even for the 1.7-fold and 3-fold higher THS 2.2 concentrations, the RBIF values were as low as 0.3% and 1% at 48 h and 2% and 1% at the 72-h time-point, while the highest RBIF values for the three THS 2.2 aerosol concentrations were seen at 4 h post-exposure: 11%, 36%, and 49% or the response to 3R4F smoke exposure. The overall results of the study support the conclusion that exposure to THS 2.2 aerosol has smaller biological impacts on nasal cultures than does exposure to 3R4F smoke at a comparable nicotine concentration (Iskandar et al, 2016) (1722_Organotypic_Nasal_SR and 1722_Organotypic_Nasal_Pub).

7.5.2.4 *In vitro* mechanistic assays

In vitro *assessment of the effects of exposure to THS 2.2 aerosol and exposure to conventional cigarette smoke on chemotaxis and transendothelial migration*

The effects of exposure to THS 2.2 aerosol and 3R4F reference cigarette smoke on the migratory behavior of monocytes were investigated (1739_CVD_Migration_Assay_Pub) (van der Toorn et al, 2015). Chemotaxis and transendothelial migration (TEM) in a monocytic cell line (THP-1) and in human coronary arterial endothelial cells (HCAECs) were analyzed. The effects of exposure to a THS 2.2 aerosol extract and a 3R4F smoke extract on toxicity and inflammation were assessed using flow cytometry and ELISA assays. The results showed that treating THP-1 cells with 3R4F smoke or THS 2.2 aerosol extracts induced concentration-dependent increases in cytotoxicity and inflammation. The inhibitory effects of the THS 2.2 aerosol extract on chemotaxis and TEM were ~18 times less than the inhibitory effects of the 3R4F smoke extract. The 3R4F smoke and THS 2.2 aerosol extracts induced concentration-dependent decreases in the integrity of the HCAEC monolayer. The 3R4F smoke extract showed more than one

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order of magnitude stronger effects than did the THS 2.2 aerosol extract on all of the endpoints that were examined. This data indicated that the aerosol of THS 2.2 could pose less risk of cardiovascular disease occurring than 3R4F smoke (1739_CVD_Migration_Assay_Pub) (van der Toorn et al, 2015).

In vitro *assessment of the effects of THS 2.2 aerosol and conventional cigarette smoke on monocyte adhesion to endothelial cells*

Alterations in the adhesive properties of endothelial cells caused by cigarette smoke can favor the progressive development of atherosclerosis, which may cause cardiovascular disorders. A systems toxicology approach combined with a functional *in vitro* adhesion assay was used to assess the effects of THS 2.2 aerosol and 3R4F reference cigarette smoke on the adhesion of monocytic cells to HCAECs. HCAECs were treated for 4 h with conditioned media from human monocytic Mono Mac 6 cells that had been incubated with low and high concentrations of aqueous extracts of THS 2.2 aerosol and 3R4F smoke for 2 h (indirect treatment), unconditioned media (direct treatment), and fresh aqueous extracts of THS 2.2 aerosol and 3R4F smoke extracts (fresh direct treatment) (1604_CVD_Adhesion_Assay_Pub). Functional and molecular investigations revealed that the aqueous 3R4F smoke extracts promoted the adhesion of Mono Mac 6 cells to HCAECs through distinct direct and indirect concentration-dependent mechanisms. Using the same approach, we found that aqueous THS 2.2 aerosol extracts had significantly smaller effects on the adhesion of Mono Mac 6 cells to HCAECs and less pronounced molecular changes in the endothelial and monocytic cells. An aqueous THS 2.2 aerosol extract concentration 10 times higher than the 3R4F smoke extract concentration in fresh direct mode, or 20 times higher than the 3R4F smoke extract concentration in indirect mode, was required to give similar effects to the 3R4F smoke extract. Our systems toxicology study, using the adhesion of monocytic cells to human coronary endothelial cells as a surrogate for pathophysiologically relevant events in atherogenesis, showed that an aqueous extract of THS 2.2 aerosol had weaker effects than did an aqueous extract of 3R4F smoke (1604_CVD_Adhesion_Assay_Pub) (Poussin et al, 2016).

7.5.2.5 *In vivo* non clinical inhalation studies

In vivo systems toxicology studies aimed at identifying hazards and characterizing disease mechanisms were conducted on rats based on the 90 d inhalation design described in the OECD TG413 method (OECD, 2009), and on mice based on an eight month chronic inhalation study design with additional cessation and switching arms. Determining classical functional and toxicological endpoints for samples from the same study and performing additional tissue analyses related to systems toxicology allowed direct correlations to be drawn between toxicity endpoints and the molecular mechanisms derived from the results of high-throughput analyses, such as transcriptomics, proteomics, and lipidomics (Kogel et al, 2014b; Phillips et al, 2015b). This combinatory

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approach (called "OECD plus" later) decreased the amount of animal testing required compared with performing separate standard toxicity studies and mechanistic studies.

Biological impacts of exposure to THS 2.2 aerosol in a 90 d rat inhalation study (OECD plus)

A 90 d rat inhalation study was conducted on rats exposed to 3R4F smoke or THS 2.2 aerosol with a 42 d post-inhalation period (15006_150082 THS SP and 15006_150083 THS SP). The standard endpoints were complemented by molecular analyses of the lung tissue, plus respiratory nasal epithelia and liver tissue in certain groups. Analysis of the inflammatory mediators in the bronchoalveolar lavage fluid (BALF) of the lung indicated that the secretion of 10 inflammatory mediators was statistically significantly lower (e.g., 57.3% less Macrophage Colony Stimulating Factor_1 and 97.2% less Monocyte Chemotactic Protein 1 in the BALF from male rats exposed to the medium concentration (23 µg nicotine/L) of THS 2.2 aerosol compared with rats exposed to the comparable concentration of smoke from the reference cigarette 3R4F, and also 10 mediators were reduced in the BALF of female rats. Proteomics analysis of the lung tissue revealed a significantly lower number of differentially abundant proteins (compared with the sham controls) in THS 2.2 aerosol exposed rats: 0 and 32 proteins for male and female rats, respectively, as opposed to 273 and 240 in 3R4F smoke-exposed males and females, respectively at the higher comparable concentration (23 µg nicotine/L). Differential gene expression (compared with the sham controls) at the high 3R4Fconcentration (23 µg nicotine/L) in the lung tissue was observed in male rats for 574 upregulated and 214 downregulated genes and in female rats for 695 genes (up) and 607 genes (down), respectively. In the lung tissue of rats exposed to THS 2.2 aerosol at the comparable concentration, no genes were differentially expressed in the male rats and 1 gene was upregulated in the female rats, and at the two-fold higher THS 2.2 aerosol concentration, also no differential gene expression was seen in males, while in female rats, 7 genes were statistically significantly upregulated and 10 genes were downregulated. Also in the liver, the numbers of differentially expressed genes at the higher comparable concentration were lower for THS 2.2 aerosol exposure: 2 genes in male and 93 genes in female rats compared with 36 genes in male and 167 genes in female rats following 3R4F smoke exposure. Network analysis of the transcriptomics data showed that, in both respiratory nasal epithelia and lung tissue from both sexes, exposure to 3R4F smoke caused perturbations in several biological processes, including inflammatory, cell stress, cell fate, tissue repair and angiogenesis, and cellular proliferation processes. Perturbations in these processes were also found after exposure to THS 2.2 aerosol, but the perturbation amplitudes at matched nicotine concentrations were lower in response to exposure to THS 2.2 aerosol than in response to exposure to 3R4F smoke: Relative biological impact factors (RBIF) for THS 2.2 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. (15006_150082_150083 THS SR).

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A major focus of the molecular analysis within this study was miRNA profiling. miRNAs are noncoding regulatory RNA molecules that bind to target messenger RNAs and destabilize them or suppress the translation of the messenger RNAs into proteins (Krol et al, 2010). miRNA levels are affected by several known toxicants, oxidative stress, and other forms of cellular stress, suggesting that miRNAs play important roles in toxicology (Hong & Cho, 2015). miRNA concentrations in the lungs of rats exposed to THS 2.2 aerosol in a 90 d OECD TG-413 inhalation study were examined as part of the systems toxicology assessment. The aim was to compare the miRNA response to THS 2.2 aerosol and to 3R4F cigarette smoke. Exposure to cigarette smoke but not to THS 2.2 aerosol caused global miRNA downregulation, which may be explained by constituents of the cigarette smoke interfering with the miRNA processing machinery. The upregulation of specific miRNA species, such as miR-146a/b and miR-182, indicated that the miRNA species play roles in causing the inflammatory response in lungs exposed to cigarette smoke. These miRNA species were upregulated less after exposure to THS 2.2 aerosol than after exposure to cigarette smoke. Transcriptomic data were transformed into protein activities from the corresponding downstream gene expression, and this allowed us to identify potential miR-146a/b and miR-182 mechanisms that were activated by cigarette smoke but not by THS 2.2 aerosol, and possibly involved in the regulation of those miRNAs. Including miRNA profiling in systems toxicology approaches therefore improves our understanding of the mechanisms involved in complex responses to exposure and supports the reduced impact of THS 2.2 aerosol compared with the 3R4F reference cigarette smoke (15006_150082_150083 THS PUB).

Biological effects of exposure to mentholated THS 2.2 aerosol in a 90 d rat inhalation study (OECD plus)

Systems toxicology approaches were used to assess the respiratory effects of mentholated THS 2.2 (THS 2.2M) aerosol in a 90 d rat inhalation study (following OECD test guideline 413). The standard endpoints were complemented with transcriptomics and quantitative proteomics analyses of respiratory nasal epithelium and lung tissue and by lipidomics analysis of lung tissues (15025_176500 THS SP). The adaptive response of the respiratory nasal epithelium to 3R4F smoke or to the smoke of the mentholated reference cigarette, DDA3, included squamous cell metaplasia and an inflammatory response, with high correspondence between the molecular and histopathological results.

In the lung, exposure to the smoke from mentholated reference cigarettes or the 3R4F cigarette induced an inflammatory response, seen as increased numbers of inflammatory cells in the BALF and in histological sections of the lung, as well as higher concentrations of inflammatory mediators in the BALF (see below). THS 2.2M low (15 µg nicotine/L) and THS 2.2M medium (23 µg nicotine/L) exposure-related histopathological effects were comparable to sham-exposed animals at most levels of the respiratory tract, and significant THS 2.2M-related changes compared with sham controls were mostly limited to the highest THS 2.2M aerosol nicotine concentration (50 µg nicotine/L) in female rats. The secretion of 15 inflammatory mediators was statistically significantly lower in the bronchoalveolar lavage fluid from male rats exposed to the

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medium concentration (23 µg nicotine/L) of THS 2.2M aerosol compared with rats exposed to the comparable concentration of smoke from the mentholated reference cigarette, DDA3_1xMIS. When the THS 2.2M aerosol concentration was doubled, eight inflammatory mediators were statistically significantly reduced by 47.0% (for Vascular Cell Adhesion Molecule-1) up to 96.5% (for Monocyte Chemotactic Protein 1) compared with rats exposed to the mentholated reference cigarette, DDA3_2xMIS, and very similar results were seen when compared with rats exposed to 3R4F smoke. Lipidomics revealed significant changes in 20 lipid species (most of them being sphingolipids) in the lung following exposure of male rats to DDA3_2xMIS smoke for 90 d, 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 THS 2.2 M aerosol; for the two-fold higher THS 2.2M aerosol exposure, one additional lipid was found in a higher abundance. For female rats, the exposure effects were qualitatively similar but weaker.

Transcriptomics results demonstrated between 1,525 and 1,788 differentially expressed genes in the lung tissue of male rats (between 1,245 and 2,865 genes in female rats) exposed to smoke from the reference cigarettes 3R4F, DDA3 1xMIS, and DDA3 2xMIS for 90 days. In contrast, only one gene was differentially expressed following exposure to the high THS 2.2M aerosol concentration in male rats (45 genes in female rats), and no differentially expressed genes were found after THS 2.2M exposure with the medium concentration (23 µg nicotine/L) in male rats (6 genes in female rats). Network analysis of the transcriptomics data showed that, in both respiratory nasal epithelia and lung tissue from both sexes, exposure to DDA3 or 3R4F smoke caused perturbations in several biological processes, mainly inflammatory, cell stress, cell fate, and tissue repair and angiogenesis. The RBIF values for exposure to the smoke from both DDA3 menthol reference cigarettes (1xMIS and 2xMIS) at 23 µg nicotine/L were 100% and 81%, respectively, in male rat lungs, whereas THS 2.2M aerosol at the comparable exposure level elicited an RBIF of 1%, and at the 2-fold higher THS 2.2M concentration, the RBIF was 6%. For female rats, the RBIF for both DDA3 reference cigarette smoke exposures were 100% and 73%, respectively, and 7% for the matching THS 2.2M aerosol concentration as well as 8% for the high THS 2.2M aerosol concentration.

Overall, this systems toxicology analysis complemented and reconfirmed the results of the classical toxicological endpoint analyses and suggested, again, that THS 2.2M aerosol may pose less risk to health than 3R4F cigarette smoke (15025_176500 THS PUB).

Eight month inhalation/cessation study in ApoE$^{-/-}$ *mice, performed to investigate cardiovascular and respiratory effects of exposure to THS 2.2 aerosol and conventional cigarette smoke*

The apolipoprotein E-deficient (*ApoE*$^{-/-}$) mouse is a suitable model for studying cardiovascular and respiratory diseases induced by cigarette smoke (15015_CVD_Resp_ApoE_SW_Pub_Model_Justif). The goals of this study were to investigate chronic obstructive pulmonary disease and cardiovascular disease hallmarks and general exposure effects over eight months in *ApoE*$^{-/-}$ mice exposed to conventional

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cigarette smoke and THS 2.2 aerosol, and to assess the consequences of smoking cessation or switching to THS 2.2 after two months of exposure to cigarette smoke. Female *ApoE*$^{-/-}$ mice were randomized into five groups, which were exposed to air (sham), 3R4F cigarette smoke, or THS 2.2 aerosol (at nicotine concentrations matching the 3R4F smoke nicotine concentrations). The effects of smoking cessation and switching to THS 2.2 were studied by first exposing groups of mice to 3R4F cigarette smoke for two months and then exposing the mice to air or THS 2.2 aerosol for up to six months (15015_CVD_Resp_ApoE_SW_SP). The atmosphere in the aerosol exposure chambers, monitored as has been described previously, showed that the levels of the HPHCs, CO, formaldehyde, acrolein, and acetaldehyde were approximately 98%, 83%, 90%, and 77% lower, respectively, than in the 3R4F CS test atmosphere (Phillips et al, 2015a). The aerosol and smoke were reproducibly taken up by the animals, indicated by the carboxyhemoglobin concentrations found in blood, urinary nicotine metabolites and the concentrations of metabolites of selected aerosol constituents in urine and blood.

A systems toxicology approach was used, with exposure effects being investigated using conventional toxicology endpoints, such as physiological and histological parameters, combined with transcriptomics, proteomics, and lipidomics. The results confirmed that the exposure of the *ApoE*$^{-/-}$ mice to cigarette smoke induced typical physiological and histopathological effects that have been described in previous cigarette smoke studies (Arunachalam et al, 2010; Boué et al, 2013; Lietz et al, 2013). Exposure of the *ApoE*$^{-/-}$ mice to THS 2.2 aerosol had no effect on body weight, did not induce lung inflammation or emphysema, and did not consistently change the lipid profile or increase the plaque area (Phillips et al, 2015a). Cessation and switching to THS 2.2 aerosol caused less inflammatory responses, e.g. ~ 85 % reduction in the total number of free lung inflammatory cells, and halted the progression of the initial emphysematous changes and increases in aortic plaque area that had occurred in mice exposed continuously to 3R4F smoke. The highest number, 3731, of differentially expressed genes was observed in the lungs of mice exposed to 3R4F for 8 months and there were no differentially expressed genes in the THS 2.2, cessation, or switching groups. The network approach of the transcriptomics analysis revealed that biological processes, including inflammation, proliferation, and senescence, were significantly affected by cigarette smoke but not by THS 2.2 aerosol. RBIF was 1% of the reference (6 months of 3R4F exposure) in the lungs of mice exposed to THS 2.2 for 6 months and ~9 and ~8 % in the lungs of mice in the cessation and switching groups, respectively, at the same time point. Likewise, lipidomics analysis showed negative changes in plasma, aortic arch, and lung lipids in mice exposed to 3R4F smoke, but these changes were not detected in the other groups (Phillips et al, 2015a). We conclude that this mouse model indicated that cessation or switching to a THS 2.2 retarded the progression of atherosclerotic and emphysematous changes and that exposure only to THS 2.2 aerosol had no obvious adverse effects. The results of this study are described in detail in several reports and publications (15015_CVD_Resp_ApoE_SW_SR_Part1, 15015_CVD_Resp_ApoE_SW_SR_Part2, 15015_CVD_Resp_ApoE_SW_SR_Part3, 15015_CVD_Resp_ApoE_SW_SR_Part4, 15015_CVD_Resp_ApoE_SW_SR_Part5, 15015_CVD_Resp_ApoE_SW_SR_Part6, 15015_CVD_Resp_ApoE_SW_SR_Part7, 15015_CVD_Resp_ApoE_SW_SR_Part8,

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15015_CVD_Resp_ApoE_SW_SR_Part9, 15015_CVD_Resp_ApoE_SW_SR_Part10, and 15015_CVD_Resp_ApoE_SW_Pub_Main, and 15015_CVD_Resp_ApoE_SW_Pub_Liver).

7.5.2.6 Independent verification of scientific conclusion - Systems Toxicology THS2.2 studies

In addition to internal data analysis and interpretation, PMI hired SciPinion LLC (https://scipinion.com/) to recruit external key opinion leaders to assess the validity of conclusions that have been made on THS 2.2 based on the *in vitro* and ApoE$^{-/-}$ THS 2.2 studies. In total, 26 key opinion leaders (KOLs) in toxicology as well as molecular and systems biology were recruited to review 5 pre-clinical studies and ~ 100 assays addressing THS 2.2 effects. The KOLs came from 7 countries, totaling 611 person-year experience. At no stage was PMI aware of the identity of the individuals that participated in the review. In the format of a data package, the reviewers had access to all publications as well as raw data from the studies via a specific web portal designed for the external review process. The reviewers were asked multiple questions regarding study design, methods, quality of data, and interpretation of results to judge the validity of the conclusions regarding harm associated with THS 2.2. The draft questions were provided by PMI, after which an independent editor verified that the questions were clearly written, could be answered based on the data package, and were not biased or leading the review panel. An independent auditor was also recruited, and his/her job was to ensure that the peer reviewers were qualified to conduct the review and to audit the review process independently of PMI and SciPinion.

A majority of the reviewers evaluating the approach used for histopathogical evaluation and morphometry used in the ApoE−/− THS 2.2 study found it a "state of the art" scientific technique and that results were correctly interpreted by the study authors. (DOC_ApoE_Ext_Verif_ Histo_Panel). A separate group of KOLs concluded that the aerosol was sufficiently characterized and that the computational analyses of the transcriptomic changes well reflected physiological changes measured in the animals (DOC_ApoE_Ext_Verif_ Mixed_Panel). The third panel reviewed the results and conclusions from the *in vitro* organotypic and ApoE−/− THS 2.2 studies. Majority of the conclusions were supportive or very supportive towards the study design and methods as well as data evaluation and translatability. Moreover, most experts deliberated that the interpretations and conclusions of the publications were sound and well supported by the data DOC_ApoE_Organo_Ext_Verif_ Mixed_Panel.

In conclusion, 22/26 key opinion leaders were confident that both switching to THS 2.2 and cessation leas to similar biological impact based on the evidence they reviewed.

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7.5.3 Conclusions

Overall, the results of the *in vitro* studies showed that exposure of organotypic tissues and endothelial cells to THS 2.2 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 reference cigarette smoke. The chronic exposure studies in both rats and mice were consistent in terms of directionality (THS aerosol has less biological impact/effect than cigarette smoke exposure), consistency across animal models and of a similar magnitude to sham exposure (air). Additionally, animals switched to THS and ambient air conditions after exposure to smoke show reversal of most biological network perturbations as well as histopathological endpoints.Therefore, taking these preclinical findings into account, it is most likely that a human adult using a THS 2.2 will not be exposed to additional hazards to those presented by smoking conventional cigarettes.

The biological effects of exposure to THS 2.2 aerosol in to exposure to reference cigarette smoke were also investigated with a focus on relevant mechanisms involved in smoking-related diseases. Analysis of the differential expression of genes that encode xenobiotic-metabolizing enzymes and transporters that are relevant to the respiratory tract showed that exposure to THS 2.2 aerosol had weaker effects than exposure to 3R4F reference cigarette smoke in all of the pre-clinical models that were tested. The perturbation of the biological network model that captures the xenobiotic metabolism response in the respiratory tract, was weaker after exposure to THS 2.2 aerosol than after exposure to cigarette smoke at the same nicotine concentration. The response in the organotypic tissue culture models exposure to THS 2.2 aerosol was transient and limited 4 h after exposure, whereas the response to exposure of 3R4F reference cigarette smoke decreased slowly and remained elevated for 72 h after exposure. Taken together, the nonclinical *in vitro* and *in vivo* model results showed that exposure to THS 2.2 or THS 2.2M aerosol affected xenobiotic metabolism less than did exposure to cigarette smoke. The responses in nonclinical exposure studies could be compared on a qualitative and quantitative basis with the results for human smokers using the network modeling approach, and a high degree of translational correlation was found between the systems (DOC_Xenobiotic_Metabolism).

In addition to triggering a xenobiotic metabolism response in target cells and tissues, exposure to cigarette smoke caused a broad spectrum of oxidative insults to biomolecules. These oxidative insults cause molecular signaling pathways to be perturbed, triggering oxidative stress responses in the exposed cells. Sustained oxidative stress responses, with or without overt injury, have been linked to a number of mechanisms involved in the pathogenesis of chronic obstructive pulmonary disease and cardiovascular disease. In the nonclinical assessment of THS 2.2 aerosol, oxidative stress was determined by measuring various apical endpoints *in vivo* (including oxidized lipids in lung and blood plasma and the metabolites of the lipids in urine) and *in vitro* (through high-content screening for multiple oxidative stress and damage parameters) (Gonzalez-Suarez et al, 2016). The systems toxicology approach included extensive molecular

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profiling (using lipidomics, proteomics, and transcriptomics) of the exposed tissues and cell cultures and computational evaluations, including dedicated causal network models related to oxidative stress signaling (Boue et al, 2015). Overall, the effects of oxidative stress were substantially weaker in tissues and cells exposed to THS 2.2 aerosol than in tissues and cells exposed to 3R4F smoke, and this was indicated by a range of exposure and effect biomarkers and molecular signaling perturbations (DOC_Oxidative_stress).

Abnormal and chronic inflammatory processes resulting from prolonged cellular stress are fundamental smoking-related mechanisms in the pathogenesis of chronic obstructive pulmonary disease and cardiovascular disease (Barnes, 2013; Barnes et al, 2003; Libby et al, 2002; Packard & Libby, 2008). Numerous inflammatory biomarkers and endpoints were investigated in the *in vivo* models that were used to assess THS 2.2 aerosol. Transcriptomics, proteomics, and lipidomics combined with computational modeling were the basis for the molecular analysis leveraging the dedicated causal network models of inflammatory processes. In all of the pre-clinical studies, exposure to 3R4F smoke induced inflammatory responses, resulting in changes in disease-related endpoints and perturbations in the network models underlying tissue-specific and common inflammatory responses. Exposure to THS 2.2 aerosol induced only marginal inflammatory responses and changes in disease-related endpoints, and markedly attenuated network perturbations compared with controls exposed to air (DOC_Inflammation).

Similar conclusions about the weaker effects of exposure to THS 2.2 aerosol than of exposure to cigarette smoke were drawn from the results of lipid metabolism studies. Many lipid species that were found at increased concentrations in the aortic walls of *ApoE*-deficient mice exposed to cigarette smoke have previously been found to be enriched in human aortic plaques. Most of the changes in lipid profiles and lipid metabolism were substantially weaker (or were absent) after exposure to THS 2.2 or THS 2.2M aerosol than after exposure to cigarette smoke with or without menthol at the same nicotine concentration. Switching to THS 2.2 aerosol for six months after being exposure to cigarette smoke for two months markedly decreased the cigarette-smoke-related lipid changes, and this effect was similar to the effect of ceasing smoking (DOC_Lipid_Biology).

Finally, independent verification of the results obtained from the preclinical studies provides reassurance that the conclusions that have been made on the effect of exposure to THS 2.2 aerosol are valid and interpreted correctly.

The overall scientific studies produced **coherent findings**, results that hold together in terms of biological relevance and plausibility. The data consistently showed that the biological impact of switching to THS aerosol was **directionally aligned with cessation** and of **similar magnitude to cessation**. It was also important that these findings were **consistent across multiple biological systems** (e.g. molecular, cellular, tissue, organ, whole organism), **consistent across potential pathways of disease causation** (e.g. inflammation, oxidative stress, cell proliferation, apoptosis) and **biologically plausible** in their relevance to the risk of harm and tobacco-related diseases.

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