NON-CLINICAL ASSESSMENT SUMMARY

1 Pre-clinical Toxicity Assessment

The pre-clinical toxicity assessment of EHTP consists of a comprehensive evaluation of the EHTP aerosol's biological activity in vitro and in vivo. This assessment is conducted for two purposes: 1) to assess the toxicity of the EHTP aerosol compared with CC smoke and 2) to ensure that the product is safe for use by smokers in clinical studies. The assessment includes a range of well-established and internationally recognized toxicological assays. The regular and mentholated variants of EHTP were assessed in separate studies.

1.1 Pre-clinical Assay Overview

Three *in vitro* assays - the Neutral Red Uptake cytotoxicity assay (measuring mammalian cell toxicity), the Ames bacterial mutagenicity assay (measuring bacteria cell mutations) and the Mouse Lymphoma mammalian mutagenicity assay (measuring mutations in mammalian cells) - were performed to assess the cytotoxicity and the genotoxicity of EHTP aerosol fractions, total particulate matter (TPM) and gas vapor phase (GVP) in comparison with the corresponding fractions of 3R4F smoke. In the Ames bacterial mutagenicity assay, only TPM was tested. The sub-chronic toxicity of the aerosol *in vivo* was evaluated in two 90-day inhalation studies. All aerosols (and reference CC smoke) were generated according to the Health Canada Intense smoking protocol. All pre-clinical assessment studies were conducted in accordance with the OECD GLP principles.

1.1.1 *In vitro* Studies

1.1.1.1 Cytotoxicity - Neutral Red Uptake Assay (NRU)

The *in vitro* cytotoxicity of the TPM and GVP of the EHTP (Regular and Menthol) aerosol and the smoke from 3R4F reference cigarette were assessed in the Neutral Red Uptake (NRU) assay in accordance with the INVITTOX protocol No. 3 (INVITTOX, 1990). The exposure was performed by incubating mouse embryo BALB/c 3T3 cells for 24 hours in culture medium to which solutions/suspensions of TPM and GVP from either THS or 3R4F were added at increasing concentrations. At the end of the exposure, the culture medium containing THS aerosol fractions or CC smoke fractions was replaced by medium containing the vital dye neutral red. After a 3-hour incubation period, the neutral red, which was taken up only by viable cells, was determined photometrically. Concentrations of TPM and GVP that have reduced the number of viable cells by

This appendix to the Philip Morris Products S.A. Technical & Scientific Dossier For the Electrically Heated Tobacco Product (EHTP) as part of the Tobacco Heating System (THS), Version 2.0, dated 27 May 2016, has been reformatted for publication on pmiscience.com.

50% (EC_{50}) were determined for EHTP aerosol and 3R4F smoke. The reciprocal EC_{50} values were calculated on a per mg nicotine basis and on a per Tobacco Stick basis.

1.1.1.2 Genotoxicity - Bacterial Cell Reverse Mutation Assay (Ames assay)

The Bacterial Cell Reverse Mutation assay (Ames assay) was performed with TPM from 3R4F cigarette smoke and EHTP aerosol from both the regular and menthol variants in accordance with OECD Test Guideline 471 in five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, and TA 1537) with and without metabolic activation using S9. In each test, concurrent positive controls and negative controls (solvent), with and without S9, were evaluated and used to confirm the test performance. The test designs used two TPM batches of each of EHTP and 3R4F. Each batch was tested separately, one batch per assay. The yields of TPM and nicotine were used to calculate the mutagenicity on a per mg nicotine and per stick basis.

1.1.1.3 Genotoxicity - Mammalian Cell Gene Mutation Assay (Mouse Lymphoma assay)

The Mouse Lymphoma Assay (MLA) was performed in accordance with OECD Test Guideline 476. The mouse lymphoma cells were exposed to TPM and GVP from EHTP aerosol and 3R4F cigarette smoke, both with and without metabolic activation using S9 (4 hours ± S9 and 24 hours - S9), and sub-cultured to determine cytotoxicity and allow phenotypic expression of induced mutations prior to mutant selection. The test design used two TPM and GVP batches of each of EHTP and 3R4F. Each batch was tested separately, one batch per assay.

1.1.2 *In vivo* Studies

1.1.2.1 Sub-chronic 90-day inhalation toxicity study

The objective of sub-chronic 90-day inhalation studies is to compare the *in vivo* toxicity of the EHTP aerosol with that of the smoke of a reference CC. Towards this end, two independent studies, one with a regular and one with a EHTP Menthol variant, were performed in accordance with OECD guideline 413 (OECD, 2009).

Sprague-Dawley rats were nose-only exposed to three target concentrations of either 3R4F reference cigarette smoke or EHTP aerosol for six hours/day, five days/week for thirteen weeks. A control (sham) group exposed to conditioned air under the same experimental conditions was included in both studies. Furthermore, in the study conducted with the EHTP Menthol variant, two mentholated combustible cigarettes (CC) were included as additional references. As the EHTP Menthol variant was design to yield 1.2 mg/stick menthol in smoke (MIS) when smoked according to ISO standard 3308 (ISO, 2012), the two mentholated reference CCs were produced to 3R4F

specifications and designed to yield 1.2 mg/stick (DDA3 1XMIS) and 2.4 mg/stick MIS (DDA3 2XMIS) when smoked according to ISO standard 3308 (ISO, 2012).

During the studies, the CC smoke and the EHTP aerosol were generated according to the Health Canada Intensive Smoking Protocol (Health Canada, 1999): This protocol is based on the ISO standard 3308 (ISO, 2012) with the exceptions of the puff volume (55ml), puff duration (2 seconds), puff frequency (one puff every 30 seconds) and closing off ventilation holes where applicable. In both studies, the target concentrations of 3R4F smoke and EHTP aerosol were adjusted to deliver test atmosphere nicotine concentrations of 8, 15, 23 µg/l and 15, 23 and 50 µg/l respectively. This ensures that the highest nicotine concentration of the EHTP aerosols was twice as high as the highest smoke nicotine concentrations from the reference cigarettes. Satellite groups exposed under the same conditions to the highest target concentrations were included to investigate the degree of reversibility of test substance-related effects after a 42-day recovery period. Based on OECD testing guideline 413 and previous experiments on the same endpoints (Terpstra et al., 2003; Vanscheeuwijck et al., 2002), the sample size was fixed to 10 male and 10 female rats per group for all OECD groups with the exception of the Sham and 3R4F recovery groups where only 6 male and 6 female rats per group were allocated.

To confirm the appropriate exposure of the animals, the smoke and aerosol exposures were monitored by on-line carbon monoxide measurements and daily determinations of both nicotine and total particulate matter (TPM) concentrations in samples taken from the breathing zone of the exposure chambers. Biomonitoring was performed through both blood carboxyhemoglobin measurements and the quantification of the main nicotine metabolites in urine and/or in blood samples.

The biological activity of the test items was assessed by evaluating the following endpoints according to OECD Test Guideline 413: in-life health status, mortality, body weight development, food consumption, respiratory physiology (indirect plethysmography), ophthalmoscopy, hematology, clinical chemistry, organ weight, gross pathology, histopathology of the respiratory tract, histopathology of the non-respiratory tract organs and in addition, bronchoaveolar lavage fluid (BALF) analysis.

1.2 Pre-clinical Study Summaries

The pre-clinical assessment of EHTP was conducted in comparison with 3R4F and included (Table 1):

- *in vitro*: the evaluation of cytotoxicity (NRU) and genotoxicity (Ames and MLA),

- *in vivo*: the sub-chronic toxicity (90-day inhalation study).

The study reports are listed in Table 1.

Table 1: Pre-clinical assessment of EHTP.

Test System	**Smoke/aerosol generation regimen**	**Tobacco Sticks tested**	**Study report number**
NRU	Health Canada Intense (HCI)	Platform 1 – ZRH / C3.1 / DORADO I / CL / Menthol / Flavor AC Mint Vinny	RLS-ZRH-2015-249 (Appendix NC-1)
		Platform 1 – ZRH / C3 / DORADO II / CL / Flavor Ron / DELI / 9A222D SI / C3 white	RLS-ZRH-2015-250 (Appendix NC-2)
Ames	Health Canada Intense (HCI)	Platform 1 – ZRH / C3.1 / DORADO I / CL / Menthol / Flavor AC Mint Vinny	RLS-ZRH-2015-253 (Appendix NC-3)
		Platform 1 – ZRH / C3 / DORADO II / CL / Flavor Ron / DELI / 9A222D SI / C3 white	RLS-ZRH-2015-254 (Appendix NC-4)
MLA	Health Canada Intense (HCI)	Platform 1 – ZRH / C3.1 / DORADO I / CL / Menthol / Flavor AC Mint Vinny	RLS-ZRH-2015-251 (Appendix NC-5)
		Platform 1 – ZRH / C3 / DORADO II / CL / Flavor Ron / DELI / 9A222D SI / C3 white	RLS-ZRH-2015-252 (Appendix NC-6)
90-day inhalation study	Health Canada Intense (HCI)	ZRH/C3/F Reform 1/Cast Leaf– CL/Flavor/Reynaldo	15006 (Appendix NC-7)
		ZRH/Deli/C3/F Reform 1/Cast Leaf-	15025 (Appendix NC-8)

Table 1: Pre-clinical assessment of EHTP.

Test System	Smoke/aerosol generation regimen	Tobacco Sticks tested	Study report number
		CL/Menthol/Flavor/Mint Veronica	

1.2.1 *In vitro* Studies

1.2.1.1 Cytotoxicity - Neutral Red Uptake Assay (NRU)

The *in vitro* cytotoxicity of the aerosol fractions TPM and GVP of both EHTP Regular and Menthol (test items) were compared with that of the corresponding smoke fractions of the research cigarette 3R4F (reference item) using the NRU assay conducted with mouse embryo BALB/c 3T3 cells.

Concurrent controls confirmed acceptable assay performance. A clear dose-dependent decrease in cell viability was observed both for the test item aerosol fractions and reference cigarette smoke fractions. The concentrations used in the tests covered a range spanning from the maximum to no, or little cytotoxicity, allowing an accurate determination of EC50 values. These values enable a direct comparison of the cytotoxicity of the test item aerosol fractions with the corresponding reference cigarette smoke fractions. Cytotoxicity was determined on a *per test item* basis (Table 2). Using the nicotine concentration in the TPM fraction, the cytotoxicity was also calculated per on a *per mg nicotine* basis (Table 2).

On a per mg nicotine basis, the *in vitro* cytotoxicity of the regular EHTP aerosol fractions was reduced by approximately 90% (91.7 % for TPM and 90.2 % for GVP) compared with the 3R4F reference item (Table 2) (Figure 1).

Similarly, on a per mg nicotine basis, the in vitro cytotoxicity of the mentholated EHTP aerosol TPM and GVP was reduced by approximately 90% (91.8 % for TPM and 90.6 % for GVP) compared to the 3R4F reference item (Table 2) (Figure 1).

Table 2: THS Regular and Menthol: Neutral Red Uptake Assay: Cytotoxicity of cigarette smoke and aerosol fractions and percent relative difference (Health Canada Intense smoking conditions)

Aerosol/Smoke fraction	$1/EC_{50}$ (mean ± SE) 3R4F	$1/EC_{50}$ (mean ± SE) EHTP	Relative difference EHTP to 3R4F (%)
PER STICK BASIS (mL/cig.)			
THS2.2 Regular TPM	360.47	16.87	95.3
THS2.2 Regular GVP	500.28	27.59	94.5
PER NICOTINE BASIS (mL/mg nic.)			
THS2.2 Regular TPM	208.55	17.34	91.7
THS2.2 Regular GVP	289.06	27.59	90.2
PER STICK BASIS (mL/cig.)			
THS2.2 Menthol TPM	415.55	18.87	95.5
THS2.2 Menthol GVP	479.56	24.97	94.8
PER NICOTINE BASIS (mL/mg nic.)			
THS2.2 Menthol TPM	239.51	19.73	91.8
THS2.2 Menthol GVP	276.21	26.07	90.6

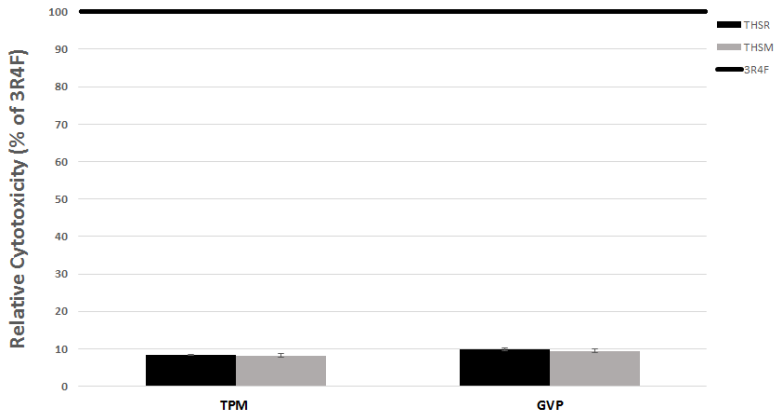


Figure 1: Cytotoxicity of both regular (black bars) and menthol (grey bars) EHTP aerosol fractions TPM (left) and GVP (right) relative to that of 3R4F smoke fractions on a per mg nicotine basis. Error bars express ± S.E.M. The cytotoxic activity (1/EC50) of the aerosol fractions from 3R4F was set to 100 %. Percent values indicate the remaining activities relative to 3R4F.

1.2.1.2 Genotoxicity - Bacterial Cell Reverse Mutation Assay (Ames assay)

The Bacterial Cell Reverse Mutation assay (Ames assay) was performed with TPM from 3R4F cigarette smoke and EHTP aerosol from both the Regular and Menthol variants. The yields of TPM and nicotine were used to calculate the mutagenicity on a per mg nicotine and per stick basis.

All bacterial strains used showed the expected response with the applied positive control substances. In the bacterial strains TA98, TA100 and TA1537 reproducible mutagenic responses were observed for the TPM from 3R4F in the presence of S9. In contrast, no biologically relevant mutagenicity was found with TA102 or TA1535 (data not shown).

Under the assay conditions used, TPM from THS 2.2 Regular and THS 2.2 Menthol did not show any mutagenic activity in the different strains tested irrespective of the presence or absence of S9.

1.2.1.3 Genotoxicity - Mammalian Cell Gene Mutation Assay (Mouse Lymphoma assay)

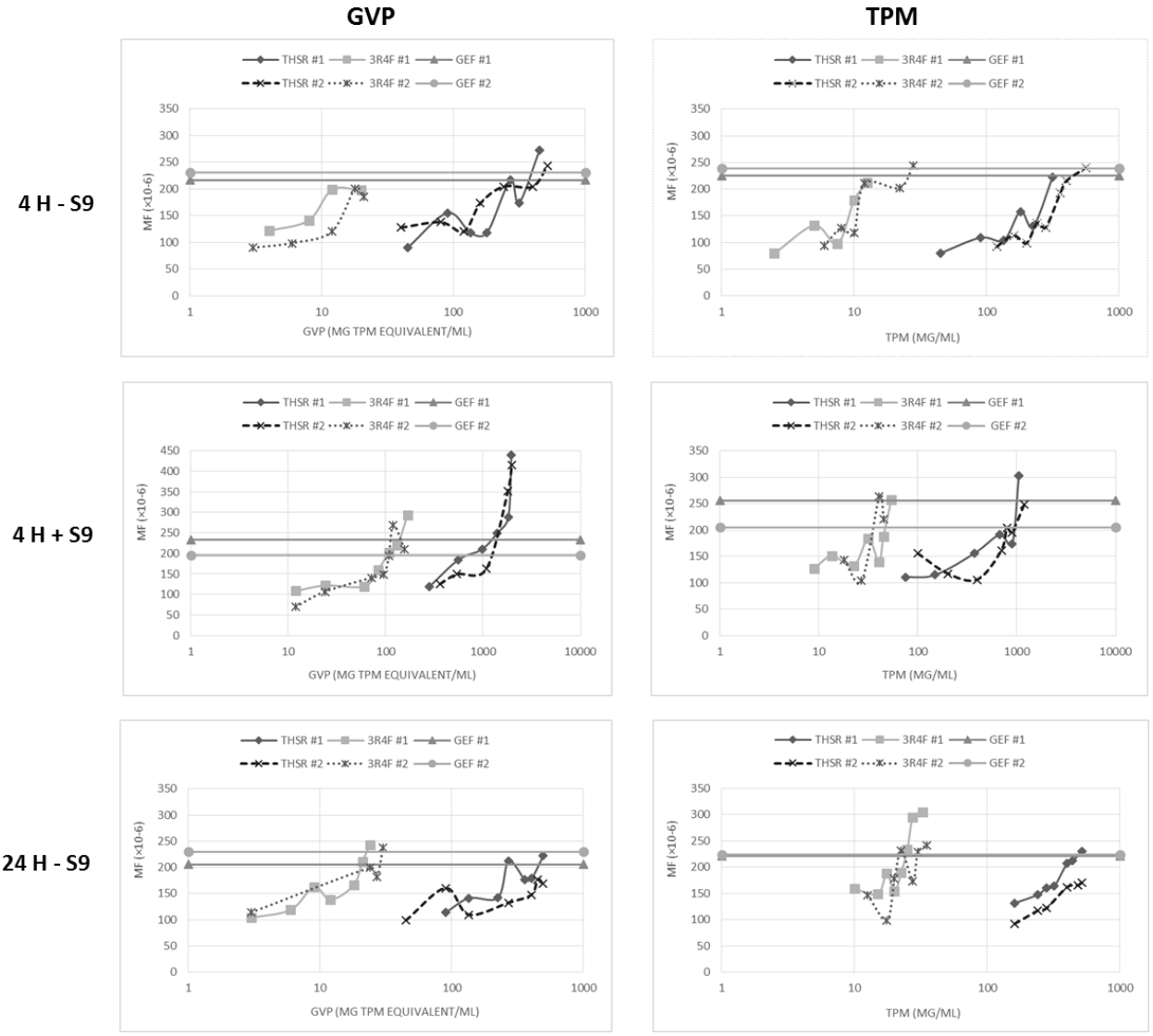
The mouse lymphoma assay (MLA) was performed with the TPM and GVP fractions of EHTP aerosol (both regular and menthol variants) and 3R4F cigarette smoke. All tests were performed in duplicate. The yields of TPM and nicotine were used to calculate the mutagenicity on a per mg TPM and nicotine basis.

Concurrent controls confirmed acceptable assay performance and the TPM and GVP aerosol fractions from EHTP aerosol and 3R4F cigarette smoke induced a concentration-dependent increase in mutagenicity. All aerosol fractions induced a biologically-relevant mutagenic response in at least one of the three treatment conditions as classified by the global evaluation factor (GEF) approach (Moore et al., 2006; Moore et al., 2007). Mutagenic potency, expressed as Lowest Observed Genotoxic Effect Levels (LOGELs) was markedly lower for EHTP aerosol fractions than for 3R4F somke fractions, i.e. LOGELs of the EHTP aerosols were depending on the fraction and test condition 8 to 29-fold higher compared with LOGELs from counterpart 3R4F smoke fractions. In particular, on a per mg TPM basis, LOGELs were found to be markedly different between EHTP regular-derived TPM (between 19.4 and 29.6-fold higher) and GVP (between 8.3 and 15-fold higher) compared with LOGELs from counterpart fractions of 3R4F (Figure 2A). Similar results were obtained considering the results on a per mg nicotine basis. Indeed, LOGEL values for EHTP regular-derived TPM were between 14 and 21.9-fold higher compared with LOGELs values for 3R4F-derived TPM.

Similarly, on a mg TPM basis, LOGELs were found to be markedly different between EHTP menthol-derived TPM (between 15.4 and 22.9-fold higher) and GVP (between 14.4 and 23.6 fold higher) compared with LOGELs from counterpart fractions of 3R4F (Figure 2B). Comparable results were obtained considering the results on a per mg nicotine basis. Indeed, LOGEL values for THS 2.2 menthol-derived TMP were between 9.1 and 13.1-fold higher compared with LOGELs values for 3R4F-derived TPM.

In conclusion, although the TPM and GVP aerosol fractions derived from both EHTP Regular and Menthol variants displayed a mutagenic response in the MLA, their mutagenic potency *in vitro* was markedly lower compared to the corresponding fractions derived from 3R4F.

A


GVP
TPM
4 H - S9
4 H + S9
24 H - S9
THSR #1
3R4F #1
GEF #1
THSR #2
3R4F #2
GEF #2
MF (×10-6)
GVP (MG TPM EQUIVALENT/ML)
TPM (MG/ML)

B

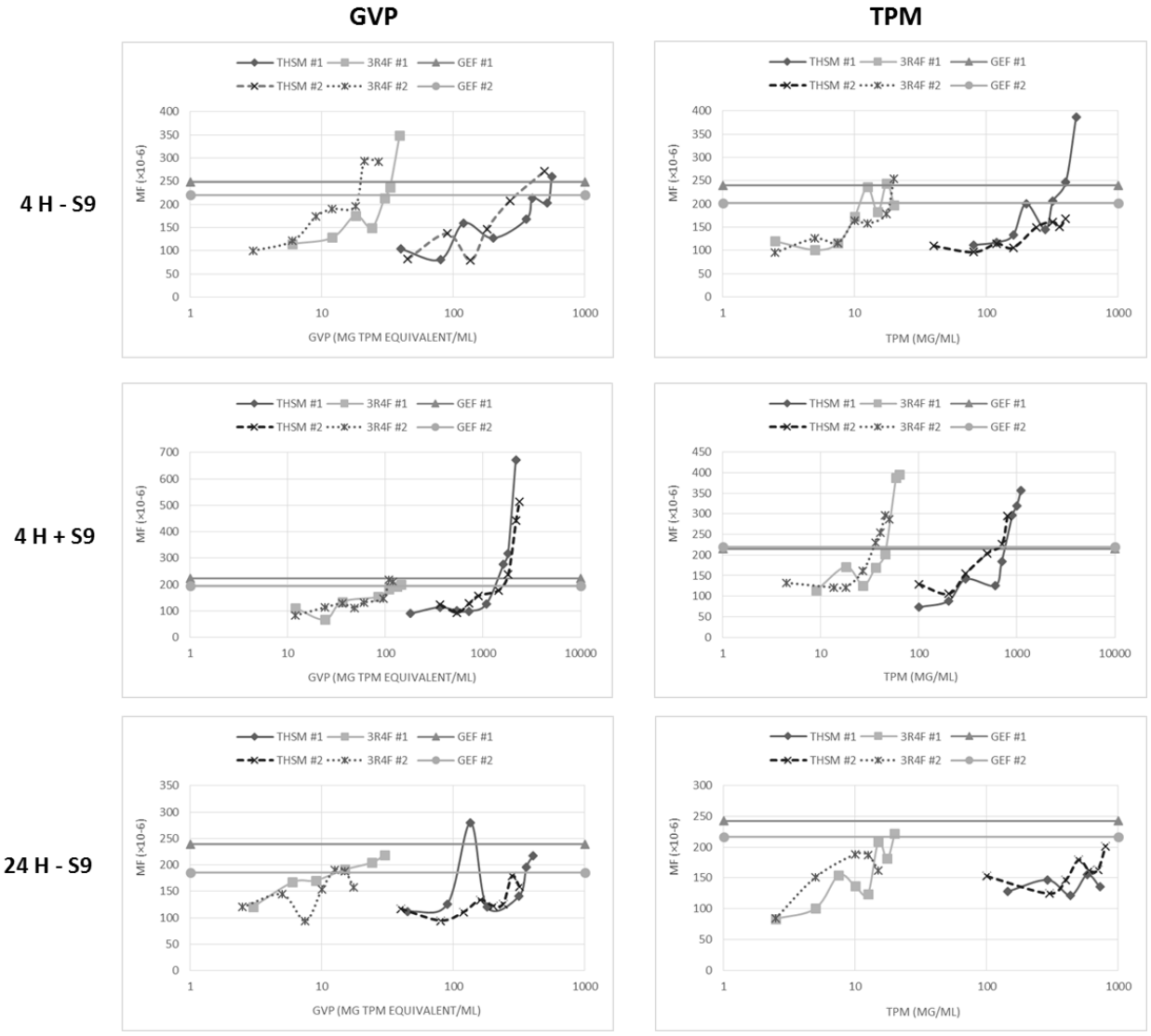


Figure 2: Mutagenicity in the MLA of the TPM and GVP fractions of EHTP aerosols: (A) Regular and (B) Menthol, compared with the corresponding fractions of 3R4F smoke (for GVP the TPM equivalent concentration is used). The results are graphed for incubations with (4 hours) and without (4 and 24 hours) S9 Metabolic Activation. #1= replicate1; #2 replicate2. Legend: GEF; Global Evaluation Factor level; THSR = EHTP Regular, THSM = EHTP Menthol. The GEF

threshold represents the background level plus the global evaluation factor value of 126 (mutation frequency).

1.2.2 *In vivo* Studies

1.2.2.1 Sub-chronic 90-day inhalation toxicity studies

Aerosol generation was reproducible throughout the exposure period and both EHTP Regular and Menthol aerosols were taken up by the animals as indicated by the biomonitoring parameters. Results of carboxyhemoglobin, nicotine and metabolites of other urinary aerosol constituent correlated well with the concentrations of these constituent in the test atmospheres.

At equal test atmosphere nicotine concentrations, nicotine uptake of a EHTP Regular and Menthol variant was higher than that of 3R4F because of the lowered respiratory frequency and minute volume in 3R4F-exposed rats due to irritation of the upper respiratory tract. No additional remarkable clinical (in-life) observations were observed in EHTP-exposed rats compared with 3R4F-exposed rats or mentholated cigarette smoke-exposed rats. The biological effects from the test and reference items were limited mainly to hematological changes (increased neutrophil counts), changes in blood chemistry (rise in liver enzyme activities, reduced total protein, albumin and triglycerides in females, reduced cholesterol), and changes in organ weights (notably in lungs, livers, adrenal glands, thymus, uterus) (Table 3). Some of these changes were more pronounced in the rats exposed to the highest concentration of EHTP aerosol. Inflammation and histopathological changes seen in the respiratory tract organs of CC-exposed animals were adaptive and degenerative changes that were within expectations upon exposure to conventional cigarettes. Histopathological changes observed in the cigarette smoke-exposed animals were either not observed or their severity was significantly lower in the regular or menthol EHTP-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. In addition, no remarkable effects were noted due to the added levels of menthol and similar results were observed in the 3R4F or mentholated CC-exposed rats in the study with the EHTP Menthol variant.

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
MORTALITY	No death related to exposure up to 23 µg/L nicotine in test atmosphere.	No death related to exposure up to 50 µg/L nicotine in test atmosphere.	No death related to exposure up to 50 µg/L nicotine in test atmosphere.
BODY WEIGHT	Reduced body weight gain.	Reduced body weight gain but less pronounced when compared to 3R4F.	Reduced body weight gain but less pronounced when compared to 3R4F.
RELATIVE/ABSOLUTEORGAN WEIGHTS	Concentration-dependent increase of lung, larynx and trachea weight.	Increase in lung, larynx and trachea weight less pronounced when compared to 3R4F.	Relative weight of lungs (with trachea and larynx) were lower in THS 2.2 Menthol groups when compared to reference groups for both genders.
	Increase of liver weight at 23 µg/L	Typical nicotine exposure-related changes in	Increase of liver weight compared to sham.

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	nicotine (nicotine side effect).	absolute and/ or relative weights of liver were observed in THS 2.2 Regular-exposed animals compared to Sham group.	Absolute liver weight was higher in THS 2.2 Menthol high group when compared to 3R4F
	Increase of adrenal gland weight.	Increase of adrenal gland weight compared to Sham group.	Increase of adrenal gland weight compared to Sham group.
	Concentration-dependent decrease of thymus weight.	Decrease in thymus weight less pronounced compared to 3R4F.	Decrease in thymus weight less pronounced compared to Sham group.
	Concentration-dependent	The aerosol induced reduction in relative spleen weights in females reversed in the post-	Decrease of spleen weight. When comparing THS 2.2 Menthol groups to reference groups,

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	decrease of spleen weight.	exposure recovery phase.	only relative spleen weight was observed to be higher in THS 2.2 Menthol High group when compared to 3R4F group.
	Concentration-dependent decrease of uterus weight.	Concentration-dependent decrease of uterus weight.	Decrease of uterus weight compared to Sham group. When comparing THS 2.2 Menthol groups to reference groups, only relative uterus weight was observed to be lower in THS 2.2 Menthol High group when compared to 3R4F group.

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
RESPIRATORY PHYSIOLOGY	Concentration-dependent reduction in respiratory minute volume compared to Sham group	No change in respiratory minute volume compared to Sham group	Respiratory frequency similar to that of Sham-exposed animals.
LUNG INFLAMMATION	Concentration-dependent increase in immune cell counts present in bronchoalveolar lavage compared to Sham group	Marginal increase in immune cell counts present in bronchoalveolar lavage compared to Sham group	No significant changes in immune cell counts present in bronchoalveolar lavage compared to Sham group
CLINICAL CHEMISTRY	Concentration-dependent increase in liver	Concentration-dependent increase in liver	Concentration-dependent increase in liver

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	enzymes (nicotine side effect). Concentration-dependent decrease in triglycerides compared to Sham group	enzymes (possibly linked to nicotine exposure). Decrease of triglycerides at the highest tested concentration compared to Sham group	enzymes (possibly linked to nicotine exposure). Decrease of triglycerides at the highest tested concentration compared to Sham group
HISTOPATHOLOGY OF THE RESPIRATORY TRACT ORGANS	Nose Reserve cell hyperplasia of the respiratory epithelium. Squamous epithelial metaplasia of the respiratory epithelium and olfactory epithelium. Cornification of the metaplastic	Nose Nasal effects observed in response to 3R4F exposure were significantly lower in THS 2.2 Regular-exposed animals. Changes observed in 3R4F-exposed animals were high in severity score in nose level 1 and moderate to high	Nose Effects observed in 3R4F-exposed animals were significantly lower in THS 2.2 Menthol-exposed animals. The findings observed in 3R4F/DDA3 1XMIS/DDA3 2XMIS-exposed animals were similar and with severity scores in

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	respiratory epithelium. Presence of neutrophilic granulocyte. Loss of goblet cells at the septum. Presence of amorphous eosinophilic material and necrotic cells in the lumen. Ulceration and atrophy of olfactory epithelium. Loss of nerve bundles at the lamina propria of olfactory epithelium. Edema and mixed inflammatory	score in nose level 4. Changes observed in THS 2.2 Regular-exposed animals were moderate in severity score in nose level 1 but absent in nose levels 2 to 4.	nose level 1 and moderate to high severity scores in nose level 4. Changes observed in THS 2.2 Menthol-exposed animals were moderate in nose level 1 when compared to reference 3R4F-exposed animals and absent in nose levels 3 to 4.

This appendix to the Philip Morris Products S.A. Technical & Scientific Dossier For the Electrically Heated Tobacco Product (EHTP) as part of the Tobacco Heating System (THS), Version 2.0, dated 27 May 2016, has been reformatted for publication on pmiscience.com.

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	cells at the lamina propria of the olfactory epithelium. Exudate and necrotic cells in the lumen of olfactory region. Larynx Squamous epithelial metaplasia at base and distal base of epiglottis, at ventral depression and floor of arytenoid projection and at upper medial region of vocal cords. Cornification at mid base and distal base of epiglottis, at ventral	Larynx In THS 2.2 Regular-exposed animals, reserve cell hyperplasia at mid and distal base of epiglottis and at pseudostratified epithelium of vocal folds were observed instead of squamous epithelial metaplasia that was seen in 3R4F-exposed animals.	Larynx Changes observed were significantly lower in THS 2.2 Menthol-exposed animals (low severity score) when compared to reference 3R4F-exposed animals.

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	depression and floor of arytenoid projection, at lower medial region of vocal cords, at squamous epithelium of vocal folds. Dilatation of sub-epithelial glands at mid base of epiglottis (female only). Hyperplasia at lower medial region of vocal cords. Reserve cell hyperplasia at pseudostratified epithelium of vocal folds. Hyperplasia of squamous epithelium of vocal folds.	Similarly, hyperplasia at floor of arytenoid projections were observed in THS 2.2 Regular-exposed animals instead of squamous epithelial metaplasia that was seen in 3R4F-exposed animals.	

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
	Epithelial thickness at the floor of the larynx and at the lower medial region of vocal cords.		
	Tracheal ring and bifurcation		
	Reserve cell hyperplasia.	Tracheal ring and bifurcation	Tracheal ring and bifurcation
	Goblet cell hyperplasia at tracheal epithelium.	Changes observed in response to 3R4F-exposure (low severity score) absent in THS 2.2 Regular-exposed animals.	Changes in the tracheal ring and bifurcation were mainly observed in response to 3R4F/DDA3 1XMIS/DDA3 2XMIS-exposure (low severity score) while changes observed in THS 2.2 Menthol-exposed animals were

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
			considered incidental.
	Left lung Presence of macrophages with and without yellow pigmentation in the alveolar lumen. Presence of neutrophilic granulocytes in the alveolar lumen. Goblet cell hyperplasia at the main bronchus.	Left lung In the THS 2.2 Regular-exposed animals, macrophages with and without yellow pigmentation in the alveolar lumen were statistically lower compared to 3R4F as well as the presence of neutrophilic granulocytes. Similarly, goblet cell hyperplasia at the main bronchus was lower in THS 2.2	Left lung In the THS 2.2 Menthol-exposed groups, all observed findings were of similar incidence and/or severity to the sham control group, and were considered incidental

Table 3. Systemic toxicity and histopathology of male and female rats exposed to mainstream smoke from 3R4F and mainstream aerosol from THS 2.2 Regular and THS 2.2 Menthol in a 90-day inhalation study.

Biological Endpoints	3R4F	THS 2.2 Regular	THS 2.2 Menthol
		Regular-exposed animals.	

In conclusion, the totality of the data form the 90-day inhalation studies shows that exposure to the aerosol of EHTP elicits (irrespective of the addition of menthol), in general, a much lower biological response than exposure to the smoke of 3R4F, even at much higher test atmosphere nicotine concentrations. For instance, systemic toxicity parameters responsive to 3R4F smoke are much less affected by EHTP aerosol, histomorphological changes of the respiratory tract are significantly less pronounced in EHTP aerosol- than 3R4F smoke-exposed rats and, the inflammatory response is significantly less extensive in the lungs of EHTP aerosol- than 3R4F smoke-exposed rats. Moreover, most findings in both respiratory and non-respiratory tract organs were very similar to those observed in previous published studies where animals were exposed to nicotine and nicotine salt solutions at equivalent nicotine test atmosphere concentration (Phillips B. et al, 2015) suggesting that most of the observed effects are likely due to nicotine.

2 Systems toxicology assessment

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 3).

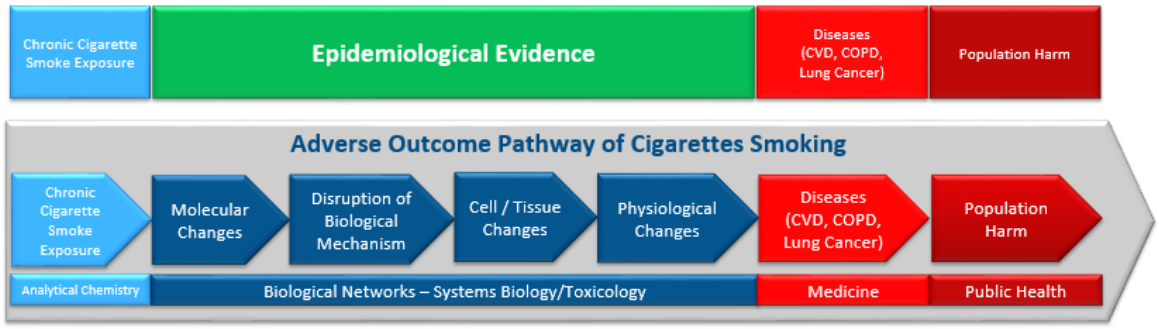


Figure 3: Chronic exposure to cigarette smoke affects a number of biological networks associated with smoking-related diseases in a causal chain of events know as an Adverse Outcome Pathway (Sturla et al., 2014 and references therein).

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 defines PMI's Systems Toxicology-informed risk assessment approach. Using computable biological network models (Boue et al., 2015) of the key mechanisms affected by toxicants, Systems Toxicology enables the quantification of the biological Network Perturbation Amplitudes (NPA) (Martin et al., 2014) caused by exposure to such toxicants and their numerical aggregation into an overall Biological Impact Factor (BIF) (Thomson et al., 2013). This approach permits a systematic and quantitative mechanism-based comparison of the biological impact of switching to a novel tobacco product compared to continued cigarette smoking as well as benchmarking the impact of switching to smoking cessation in animal models of disease, which ultimately allows for more rapid emulation of disease progression and reversibility. PMI has conducted several studies using a Systems Toxicology-based approach (Hoeng et al., 2012; Sturla et al., 2014).

2.1 Systems Toxicology Assessment *in vitro*

The first study conducted *in vitro* used primary normal human bronchial epithelial cells and demonstrated that exposure to EHTP aerosol is up to 15 times less effective in triggering key endpoints of cellular toxicity than 3R4F smoke (Gonzalez-Suarez et al., 2016). The second study conducted *in vitro* demonstrated that EHTP aerosol is 10 to 20 times less effective in triggering the adhesion of human monocyte to human endothelial cells (Poussin et al., 2016) than 3R4F smoke. The third study demonstrated that *in vitro*, EHTP aerosol has a much reduced effect (approx.18

times less) on monocyte chemotaxis and transendothelial migration (van der Toorn et al., 2015) than 3R4F smoke.

2.2 Systems Toxicology Assessment *in vivo*

PMI also conducted an 8-month study *in vivo* using Apoe-/- mice exposed to either 3R4F smoke, EHTP aerosol or fresh air. Furthermore, two groups of mice were first exposed for two months to 3R4F smoke and then for 6 months to fresh air (cessation group) or EHTP aerosol (switching group). The Apoe-/- mouse model of disease as it permits the concomitant evaluation of pulmonary and cardiovascular endpoints and associated mechansisms (Lo Sasso et al., 2016a). This study shows that unlike 3R4F smoke which causes emphysema and an acceleration of atherosclerotic plaque growth, EHTP aerosol has a minimal impact on these endpoints. Furthermore, switching to EHTP aerosol following 3R4F smoke exposure significantly reduces the progression of these disease endpoints, to a level approaching the effects of cessation (Phillips et al., 2016). This study also shows that the molecular mechanisms as well as the cellular and histological endpoints underpinning these disease endpoints are only marginally affected by EHTP aerosol as compared to 3R4F smoke (Phillips et al., 2016; Titz et al., 2016; Lo Sasso et al., 2016b).

3 Non-clinical Assessment Conclusions

The toxicity data reviewed above provide robust *in vitro* and *in vivo* evidence of the reduced toxicity of the EHTP aerosol compared to cigarette smoke. This is demonstrated by markers for genotoxicity and cytotoxicity, as well as reduced sub-chronic inhalation toxicity. Furthermore, innovative systems toxicology assessments show that EHTP aerosol has a significantly reduced impact on disease mechanisms and disease progression relative to cigarette smoke, further supporting reduced risk potential of the EHTP.

Results from the overall non-clinical assessment program a) confirm the potential of the EHTP to reduce risk compared to continued smoking of CC; b) support the reduced risk potential of EHTP, indicating that a risk profile approaching that of smoking cessation is possible; and c) indicate, but do not substantiate, that by switching from CC to EHTP, smokers have the potential to reduce their exposure to HPHCs.

4 References

Boue, S., M. Talikka, J. W. Westra, W. Hayes, A. Di Fabio, J. Park, W. K. Schlage, A. Sewer, B. Fields, S. Ansari, F. Martin, E. Veljkovic, R. Kenney, M. C. Peitsch and J. Hoeng (2015). Causal biological network database: a comprehensive platform of causal biological network

models focused on the pulmonary and vascular systems. Database 2015: bav030. (PMID: 25887162).

Gonzalez Suarez, I., F. Martin, D. Marescotti, E. Guedj, S. Acali, S. Johne, R. Dulize, K. Baumer, D. Peric, D. Goedertier, S. Frentzel, N. V. Ivanov, C. Mathis, J. Hoeng and M. C. Peitsch (2016). In vitro systems toxicology assessment of a candidate modified risk tobacco product shows reduced toxicity compared to a conventional cigarette. Chem Res Toxicol. 29:3-18. (PMID: 26651182).

Health Canada (1999) Health Canada Method T-115: Determination of "Tar", Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke. Available at (https://www.iso.org/obp/ui/#iso:std:iso:tr:19478:-2:ed-1:v1:en).

Hoeng J, Kenney RD, Pratt D, Martin F, Sewer A, Thomson TM, Drubin DA, Waters CA, de Graaf D and Peitsch MC (2012) A Network-Based Approach to Quantify the Impact of Biologically Active Substances. Drug Discovery Today, 17:413-418. (PMID: 22155224).

International Organization for Standardization (2012) Routine analytical cigarette-smoking machine — Definitions and standard conditions, Geneva, Switzerland, ISO 3308. Available at (https://www.iso.org/obp/ui/#!iso:std:60404:en).

INVITTOX (1990), Protocol Number 3a, The frame modified neutral red uptake cytotoxicity test.

Lo Sasso, G., Schlage, W.K., Boué, S., Veljkovic, E., Peitsch, M.C. and Hoeng, J. (2016a) The Apoe−/− mouse model: a suitable model to study cigarette smoke-induced cardiovascular and respiratory diseases. Journal of Translational Medicine, 14:146. (PMID: 27207171).

Lo Sasso, G., Titz, B., Nury, C., Boué, S., Phillips, B., Belcastro, V., Schneider, T., Dijon, S., Baumer, K., Peric, D., Dulize, R., Elamin, A., Guedj, E., Buettner, A., Leroy, P., Kleinhans, S., Vuillaume, G., Veljkovic, E., Ivanov, N.V., Martin, F., Vanscheeuwijck, P., Peitsch M. C. and Hoeng, J. (2016b). Effects of cigarette smoke, cessation and switching to a candidate modified risk tobacco product on the liver in Apoe$^{-/-}$ mice – a systems toxicology analysis. Inhal Toxicol. 28:226-240. (PMID: 27027324).

Martin, F., Sewer, A., Talikka, M., Xiang, Y., Hoeng, J. and Peitsch, M.C. (2014) Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models. BMC Bioinformatics. 15:238. (PMID: 25015298).

Moore, M.M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J., Delongchamp, R., Durward, R., Fellows, M., Gollapudi, B., Hou, S., Jenkinson, P., Lloyd, M., Majeska, J., Myhr, B., O'Donovan, M., Omori, T., Riach, C., San, R., Stankowski, L.F.

Jr., Thakur, A.K., Van Goethem, F., Wakuri, S. and Yoshimura, I. (2006) Mouse lymphoma thymidine kinase gene mutation assay: follow-up meeting of the International Workshop on Genotoxicity Testing--Aberdeen, Scotland, 2003--Assay acceptance criteria, positive controls, and data evaluation. Environ Mol Mutagen. 47:1-5. (PMID:15991242).

Moore, M.M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J., Clay, P., Doppalapudi, R., Fellows, M., Gollapudi, B., Hou, S., Jenkinson, P., Muster, W., Pant, K., Kidd, D.A., Lorge, E., Lloyd, M., Myhr, B., O'Donovan, M., Riach, C., Stankowski, L.F. Jr., Thakur, A.K., Van Goethem, F., and Mouse Lymphoma Assay Workgroup, IWGT. 2007. Mouse lymphoma thymidine kinase gene mutation assay: meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. Mutat Res. 627:36-40. (PMID: 17157054).

Organization for Economic Cooperation and Development, Environment Directorate, Chemicals Group and Management Committee (1998) OECD Series on Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

Organization for Economic Cooperation and Development (2009), Test No. 413: Subchronic Inhalation Toxicity: 90-day Study. Available from: http://www.oecd-ilibrary.org/environment/test-no-413-subchronic-inhalation-toxicity-90-day-study_9789264070806-en

Phillips, B., Esposito, M., Verbeeck, J., Boué, S., Iskandar, A., Vuillaume, G., Leroy, P., Krishnan, S., Kogel, U., Utan, A., Schlage, W.K., Bera, M., Veljkovic, E., Hoeng, J., Peitsch, M.C. and Vanscheeuwijck, P. (2015) Toxicity of aerosols of nicotine and pyruvic acid (separate and combined) in Sprague–Dawley rats in a 28-day OECD 412 inhalation study and assessment of systems toxicology. Inhal Toxicol. 27:405-431. (PMID: 26295358).

Phillips, B., E. Veljkovic, S. Boue, W. K. Schlage, G. Vuillaume, F. Martin, B. Titz, P. Leroy, A. Buettner, A. Elamin, A. Oviedo, M. Cabanski, E. Guedj, T. Schneider, M. Talikka, N. V. Ivanov, P. Vanscheeuwijck, M. C. Peitsch and J. Hoeng (2016). An 8-month systems toxicology inhalation/cessation study in Apoe-/- mice to investigate cardiovascular and respiratory exposure effects of a candidate modified risk tobacco product, THS 2.2, compared with conventional cigarettes. Toxicol Sci. 149:411-432. (PMID: 26609137).

Poussin, C., A. Laurent, M. C. Peitsch, J. Hoeng and H. De Leon (2016). Systems toxicology-based assessment of the candidate modified-risk tobacco product THS2.2 for the adhesion of monocytic cells to human coronary arterial endothelial cells. Toxicology 339:73-86. (PMID: 26655683).

Sturla, S.J., Boobis, A.R., FitzGerald, R.E., Hoeng, J., Kavlock, R.J., Schirmer, K., Whelan, M., Wilks, M.F. and Peitsch, M.C. (2014) Systems Toxicology: from basic research to risk assessment. Chem. Res. Toxicol. 27:314-329. (PMID: 24446777).

Terpstra, P.M., Teredesai, A., Vanscheeuwijck, P.M., Verbeeck, J., Schepers, G., Radtke, F., Kuhl, P., Gomm, W., Anskeit, E. and Patskan, G. (2003) Toxicological evaluation of an electrically heated cigarette. Part 4: Subchronic inhalation toxicology. J Appl Toxicol. 23:349-362. (PMID: 12975774).

Thomson, T. M., A. Sewer, F. Martin, V. Belcastro, B. P. Frushour, S. Gebel, J. Park, W. K. Schlage, M. Talikka, D. M. Vasilyev, J. W. Westra, J. Hoeng and M. C. Peitsch (2013). Quantitative assessment of biological impact using transcriptomic data and mechanistic network models. Toxicol Appl Pharmacol. 272:863-878. (PMID: 23933166).

Titz, B., S. Boue, B. Phillips, M. Talikka, T. Vihervaara, T. Schneider, C. Nury, A. Elamin, E. Guedj, M. J. Peck, W. K. Schlage, M. Cabanski, P. Leroy, G. Vuillaume, F. Martin, N. V. Ivanov, E. Veljkovic, K. Ekroos, R. Laaksonen, P. Vanscheeuwijck, M. C. Peitsch and J. Hoeng (2016). Effects of cigarette smoke, cessation and switching to two heat-not-burn tobacco products on lung lipid metabolism in C57BL/6 and Apoe-/- mice - an integrative systems toxicology analysis. Toxicol Sci. 149:441-457. (PMID: 26582801).

Van der Toorn, M., S. Frentzel, H. De Leon, D. Goedertier, M. C. Peitsch and J. Hoeng (2015). Aerosol from a candidate modified risk tobacco product has reduced effects on chemotaxis and transendothelial migration compared to combustion of conventional cigarettes. Food Chem Toxicol 86: 81-87. (PMID: 26432920).

Vanscheeuwijck, P.M., Teredesai, A., Terpstra, P.M., Verbeeck, J., Kuhl, P., Gerstenberg, B., Gebel, S. and Carmines, E.L. (2002) Evaluation of the potential effects of ingredients added to cigarettes. Part 4: subchronic inhalation toxicity. Food Chem Toxicol. 40:113-131. (PMID: 11731041).