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The Science behind the Tobacco Heating System

A Summary of Published Scientific Articles

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Philip Morris International

Preamble

We recognize that cigarettes are a dangerous product, and it is well known that the best way to avoid the harms of smoking is never to start, or to quit. Nevertheless, based on the World Health Organization's own predictions, there will be more than one billion smokers by the year 2025. Therefore, alternative products that significantly reduce the risk of disease compared with cigarette smoking are a fundamental complement to the regulatory efforts aimed at reducing smoking prevalence.

WE HAVE BROUGHT TOGETHER OVER 300 WORLD-CLASS SCIENTISTS FROM 30 FIELDS OF EXPERTISE – INCLUDING TOXICOLOGY, SYSTEMS BIOLOGY AND MEDICINE – TO DEVELOP AND ASSESS PRODUCTS THAT HAVE THE POTENTIAL TO REDUCE INDIVIDUAL RISK AND POPULATION HARM COMPARED WITH SMOKING.

For this reason, PMI is investing in the development and rigorous assessment of a portfolio of potentially reduced-risk alternatives to cigarette smoking. In fact, our objective is to replace cigarettes with RRPs[1] as soon as possible. The scientific work we're conducting is at the heart of this transformation. Our approach is based on the acknowledgment that innovative products will benefit public health if they meet two conditions: first, they must significantly reduce risk of disease compared with cigarettes; and, second, they must be acceptable enough to smokers to encourage them to switch to such reduced-risk alternatives.

In order to demonstrate that switching to our RRPs results in a significant reduction in the risk of disease compared with cigarette smoking, we are following a rigorous scientific assessment program that we outline in this document. Our program is in line with the draft guidance from the U.S. Food and Drug Administration (FDA) for Modified-Risk Tobacco Products (MRTPs). In December 2016, we submitted our MRTP Application to the FDA.

We recognize that our scientific work must also be assessed by independent experts. We welcome such review and are committed to share scientific data for independent verification by qualified third parties. Towards this end we are utilizing a very contemporary platform called sbvIMPROVER to foster the verification of both our methods and results by independent scientists. We have also recently launched an Investigator Initiated Study program, a first step towards encouraging third parties to conduct studies with our RRPs.

In this document, you will find a summary of our scientific publications describing the assessment of THS[2]. If you have any comments or questions about our science, be it about the methods we use or the results we have obtained, let us know. We look forward to hearing from you.

Prof. Manuel C. Peitsch
Chief Scientific Officer

Declaration of Interest

All studies presented and referenced in this document were fully funded by Philip Morris International. All authors are (or were) employees of, or contracted by, Philip Morris International.

Electronic Version

This document is available from PMIScience.com at:

https://www.pmiscience.com/library/pmi-science-ths-executive-summary

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[1] Reduced-Risk Products ("RRPs") is the term we use to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switch to these products versus continued smoking. We have a range of RRPs in various stages of development, scientific assessment and commercialization. Because our RRPs do not burn tobacco, they produce far lower quantities of harmful and potentially harmful compounds than found in cigarette smoke.

[2] The studies described in this documents were conducted with THS 2.2. THS is commercialized under the name IQOS.

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The Scientific Assessment of THS[1]

Executive Summary

Philip Morris International R&D

Tobacco harm reduction and the need for innovative approaches

Worldwide, there are about 1.1 billion smokers (*Ng 2014*) and nearly six million deaths annually are attributed to tobacco smoke (*World Health Organization 2009*). For many decades, the primary strategies for reducing the harm associated with cigarette smoking was focused on preventing smoking initiation and promoting smoking cessation. Although smoking prevalence has declined in many countries over the last forty years, in the last ten years those declines have flattened in many countries. Smoking causes a number of serious diseases including cardiovascular diseases, lung cancer and chronic obstructive pulmonary disease (COPD).

Smoking cessation is clearly the most effective strategy for smokers to reduce their risk of harm and disease. However, the number of former smokers who relapse is high since approximately 80% of smokers who attempt to quit return to smoking within one month, and only approximately 5% of smokers successfully quit each year (*Tashkin 2015*). Smoking is both an addiction to nicotine and a conditioned habit triggered by various environmental cues, and smokers enjoy the rituals associated with smoking (*Fagerström 2012*). Nicotine replacement therapies (NRTs) (e.g. patches, gums, inhalers) are designed to deliver nicotine and thereby eliminate withdrawal symptoms and the sensations of craving during a smoking cessation attempt (*Fagerström 2014*). However, one of the limitations of NRTs is that they do not replace the sensory cues and rituals associated with cigarette smoking. Behavioral interventions (including simple advice) modestly improve sustained smoking cessation rates and the combination of counseling and pharmacotherapy is more effective than either one on its own (*Tashkin 2015*). Smokers who receive specialist one-to-one behavioral support are twice as likely to remain abstinent than those who are seen by a general practitioner and people who receive group behavioral support are three times more likely to stop smoking. However, at a 1-year follow-up, despite these interventions, only 8% of smokers remain abstinent from smoking (*Dobbie 2015*).

"Harm reduction is a strategy used in medicine and social policy to minimize harm to individuals and/or wider society from hazardous behaviors or practices that cannot be completely avoided or prevented" (*Royal College of Physicians 2016*). In this context, *Tobacco Harm Reduction* is starting to gain support from a range of stakeholders – including public health organizations, healthcare professionals and regulators – to complement the existing strategies to reduce smoking prevalence. According to the Royal College of Physicians, embracing such an approach could offer a means to prevent millions of deaths (*Royal College of Physicians 2007*). For example, in May 2014, 53 prominent public health experts and leading scientists from over 18 countries wrote an open letter to the World Health Organization (WHO) urging the organization to support Tobacco Harm Reduction[2]. They wrote:

> *"There are now rapid developments in nicotine-based products that can effectively substitute for cigarettes but with very low risks. These include, for example, e-cigarettes and other vapour products, low-nitrosamine smokeless tobacco such as snus, and other low-risk non-combustible nicotine or tobacco products that may become viable alternatives to smoking in the future."*

The letter concluded:

> *"The potential for tobacco harm reduction products to reduce the burden of smoking related disease is very large, and these products could be among the most significant health innovations of the 21st Century – perhaps saving hundreds of millions of lives."*

[1] The studies described in this documents were conducted with THS 2.2. THS is commercialized under the name IQOS.

[2] https://nicotinepolicy.net/documents/letters/MargaretChan.pdf

Furthermore, it is widely recognized that the adverse health effects caused by smoking are not triggered by nicotine, but primarily by the toxic substances generated during tobacco combustion (burning). The most recent report from the UK Royal College of Physicians (*Royal College of Physicians 2016*) notes that "*The ideal harm-reduction device should therefore deliver nicotine in a manner as similar as possible to cigarettes, while at the same time maximizing palatability and nicotine delivery to approximate the experience of cigarette smoking more closely*". The contemporary *Tobacco Harm Reduction* approach endorsed by public health and medical experts in the US, UK and Sweden, embraces the use of noncombustible, less toxic, nicotine-containing products as valid alternatives to limit smoking-related death and disease. Since 2013, The National Institute for Health and Care Excellence (*NICE 2013*) recommends the use of licensed nicotine containing products (NRTs). In Sweden, the long-standing availability of moist snuff (snus) has effectively reduced the prevalence of smoking among males, resulting in male smoking-related mortality to be amongst the lowest in Europe (*Rodu 2009; Lee 2013; Ramström 2016*).

There are, however, legitimate concerns about endorsing a *Tobacco Harm Reduction* approach. The benefits of alternative products could be offset by an increased risk of smoking initiation (known as gateway) or by making the act of smoking socially accepted again (renormalization), also if they discouraged quitting among smokers who would otherwise have quit or encouraged relapse amongst those smokers who have already quit. If these legitimate concerns are addressed it is nevertheless conceivable that the *Tobacco Harm Reduction* approach can adequately complement the existing strategies to reduce smoking related harm. For instance, the US FDA states that "*modified risk tobacco products provisions of the Family Smoking Prevention and Tobacco Control Act (FSPTCA) may be valuable tools in the effort to promote public health by reducing the morbidity and mortality associated with tobacco use, particularly if companies take advantage of these provisions by making bold, innovative product changes that substantially reduce, or even eliminate altogether either the toxicity or addictiveness of tobacco products or both*" (*US Food and Drug Administration 2012a*).

Through technological innovation and rigorous scientific assessment, PMI is developing a number of non-combustible nicotine and tobacco products that have the potential to reduce significantly individual risk and population harm when compared with smoking cigarettes. We refer to these products as Reduced-Risk Products[3] (RRPs), and PMI's declared ambition is to lead a full-scale effort to ensure that non-combustible products ultimately replace cigarettes.[4]

This article describes a novel tobacco-containing product, the Tobacco Heating System (THS), which electrically heats tobacco to deliver nicotine in an aerosol containing substantially reduced levels of the toxicants formed when tobacco is burned. It includes an outline of the approach used to assess scientifically whether such a product has the potential to reduce the risk of harm for smokers who completely switch to such products instead of continuing to smoke cigarettes. This article will conclude that based on the totality of the available evidence, switching from cigarette smoking to THS use both reduces the exposure to harmful toxicants and leads to improvements in clinical risk markers in ways that approach those of smoking cessation.

Heating rather than burning tobacco

For over a century, the basic design and use of cigarettes has not changed. Shredded tobacco leaves are burned to produce smoke which is inhaled by the smoker. The smoke contains nicotine and a large number of toxic substances, most of which are generated during the combustion of tobacco.

Cigarette smoke is formed when tobacco is burned at temperatures ranging from 600°C to 900°C as a complex mixture of solid particles and liquid droplets (*Baker 1975*). Over 6000 chemicals have been identified in cigarette smoke (*Rodgman 2013*). Several public health agencies (such as Health Canada and the US Food and Drug Administration) have developed lists of the subsets of these chemicals that are considered to be the harmful and potentially harmful

[3] Reduced-Risk Products ("RRPs") is the term we use to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switch to these products versus continued smoking. We have a range of RRPs in various stages of development, scientific assessment and commercialization. Because our RRPs do not burn tobacco, they produce far lower quantities of harmful and potentially harmful compounds than found in cigarette smoke.

[4] https://www.unglobalcompact.org/system/attachments/cop_2016/292971/original/Philip_Morris_International_Communication_on_Progress_2015.pdf?1466005977

constituents (HPHCs)[5] of cigarette smoke (*Health Canada 2000*, *US Food and Drug Administration 2012b*). Many of these HPHCs are formed during combustion (burning) of the tobacco. In contrast, heating tobacco at temperatures well below combustion, results in the formation of an aerosol with reduced levels of HPHCs (*Baker 1981, 1987, 2006; White 2001; Torikai 2005; Czegeny 2009; Dyakonov 2008; McGrath 2007*).

THS: A novel product that heats tobacco

THS is very different from cigarettes and has three distinct components (Figure 1): (1) a *Tobacco Stick* - a novel tobacco-containing product with processed tobacco made from tobacco powder, (2) a *holder,* which heats the tobacco by means of an electronically controlled heating blade, and (3) a *charger* that is used to recharge the holder after each use.

Figure 1: The three components of the THS product.

To operate the THS product, the user inserts a *Tobacco Stick* into the *holder* and turns on the device by means of a switch. This initiates the heating of the tobacco via the heating blade inserted into the tobacco. The tobacco never ignites nor burns (*Appendix A, section I*). The electronically controlled heating, in combination with the uniquely processed tobacco, prevents combustion from occurring. Heat is supplied to the tobacco stick for a fixed period of approximately 6 min and allows up to 14 puffs to be taken during that time. The temperature of the heating blade is carefully controlled and the energy supply to the blade is cut if its operating temperature exceeds 350°C (*Appendix A, section I*).

The operating temperature of the THS product is substantially lower than that required to cause ignition and combustion of tobacco and the temperature measured in the tobacco does not exceed 300°C. Since combustion does not occur, the structural integrity of the *Tobacco Stick* is retained after use (*Appendix A, section I*). The tobacco is not consumed as in a cigarette and no ash is formed. This absence of combustion, because of controlled heating, is designed to reduce significantly the formation of HPHCs by the THS product compared with cigarettes.

Scientific assessment of the Tobacco Heating System

Decades of epidemiological data have demonstrated that smoking causes serious diseases such as lung cancer, chronic obstructive pulmonary disease and cardiovascular diseases. The development of smoking-related diseases is triggered by the chronic inhalation of the HPHCs in cigarette smoke. Epidemiology has also demonstrated that smoking cessation – effectively eliminating the exposure to HPHCs – is the optimal way for a smoker to reduce the risk of harm and smoking-related disease.

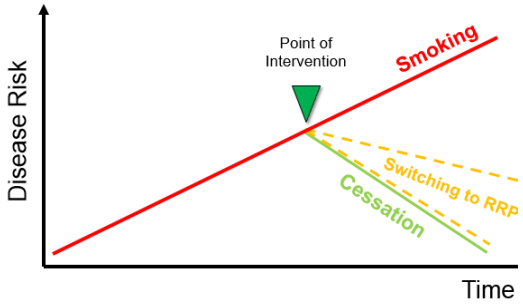

Figure 2: Risk framework for RRP assessment. Conceptual depiction of the cumulated risk of smoking (red line) and the effect of cessation (green line) over time. These represent the two boundaries for the assessment of a candidate RRP (yellow lines): 1) comparing switching to a novel product with continued smoking and 2) benchmarking switching against smoking cessation (gold standard). Note that the straight lines used in this figure are for illustration purposes only as the accumulation of disease risk and the reduction upon cessation and switching to a novel product follow different trajectories for specific diseases.

[5] Harmful and Potentially Harmful Constituent includes any chemical or chemical compound in a tobacco product or in tobacco smoke that (a) is, or potentially is, inhaled, ingested, or absorbed into the body, including as an aerosol (vapor) or any other emission; and b) causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products. Examples of constituents that have the "potential to cause direct harm" to users or non-users of tobacco products include constituents that are toxicants, carcinogens, and addictive chemicals and chemical compounds (*US Food and Drug Administration 2016*).

This provides the fundamental framework for the assessment of RRPs. Given that smoking cessation is always the best way for smokers to minimize their disease risk, an effective RRP (THS) must therefore have a risk reduction profile approaching that of smoking cessation. In the diagram shown in Figure 2, the red line represents a cigarette smoker's increased disease risk over time and the green line that smoker's decreasing disease risk following smoking cessation. The yellow lines in the diagram show the objective for the effect of switching to such an RRP – that is, to achieve changes in disease risk that approach those seen following smoking cessation, regarded by the U.S. Institute of Medicine (*Institute of Medicine 2012*) as the "*gold standard*" for such a risk assessment.

PMI takes a thorough, systematic and stepwise approach to assess candidate RRPs within this framework (*Smith 2016*). The assessment program integrates seven steps, designed to provide five levels of evidence to address two objectives (Figure 3):

1. The first objective is to demonstrate that a novel product significantly reduces harm and the risk of tobacco-related disease to individual smokers.
2. The second objective is to show that the novel product, which meets the first objective, benefits the health of the population as a whole, taking into account both smokers and nonsmokers.

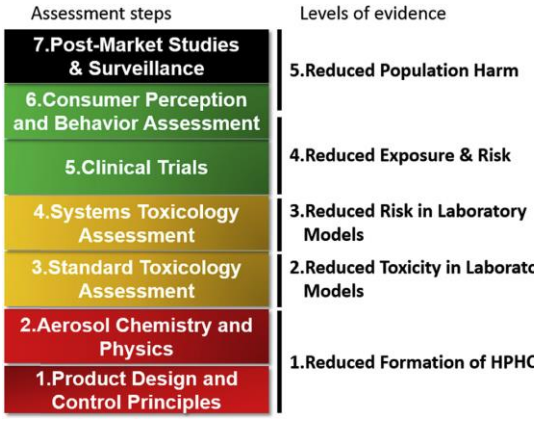

Figure 3: The RRP assessment program. Seven steps of assessment lead to five levels of evidence. Taken together, these levels of evidence provide the scientific evidence to demonstrate that a novel product significantly reduces harm and the risk of tobacco-related disease to individual smokers and benefits the health of the population as a whole, taking into account both smokers and nonsmokers.

The primary objective of this document is to summarize the research conducted in the context of the first of these two objectives (Steps 1-5). Furthermore, a high-level summary of the results of perception and behavior assessment studies (Step 6) conducted to assess the THS product in the context of the second objective is also provided.

THS development and assessment

Scientific standards

The Aerosol Chemistry studies were conducted under ISO 17025 (accredited by Swiss authority), while the standard toxicology studies were performed under OECD GLP (accredited by Swiss and Singaporean authorities). The systems toxicology studies were conducted under a Quality Management Systems based on GLP. Our *in vivo* laboratory in Singapore is accredited by AAALAC. (More details in the section on our *Quality Management System*).

The clinical studies are all reviewed and approved by Institutional Review Boards or Ethics Committees and conducted according to the International Conference on Harmonization (ICH) guidelines for Good Clinical Practice (GCP), Declaration of Helsinki, and local requirements. All studies were registered with ClinicalTrials.gov.

Product design and control (Step 1).

Following the principles outlined above, the THS was designed with two main criteria in mind (1) the THS should significantly reduce or eliminate the formation of HPHCs found in cigarette smoke; and (2), the THS should preserve as much as possible the sensory experience, nicotine delivery profile and ritual characteristics of cigarettes to facilitate the conversion of adult smokers. This first step of the THS assessment is designed to ensure that the product meets these criteria and that it is manufactured to appropriate quality standards.

Aerosol chemistry and physics (Step 2).

In the second step of the THS assessment, we compared the chemical composition of the THS aerosol with that of the smoke from a reference cigarette (known as 3R4F) supplied by the University of Kentucky. Standardized and validated analytical methods were used to quantify the most important HPHCs known to be carcinogens, respiratory and cardiovascular toxicants or involved in other toxic effects.

These HPHCs are reduced on average by over 90% in THS aerosol compared with the reference

cigarette smoke (*Schaller 2016a*) (Figure 4, Table 1) and commercial cigarettes (*Jaccard 2017*) (*Appendix A, section II*). Importantly, the carcinogens (IARC group 1) are reduced by over 95% in THS aerosol. Additionally, no new hazardous substances were detected.

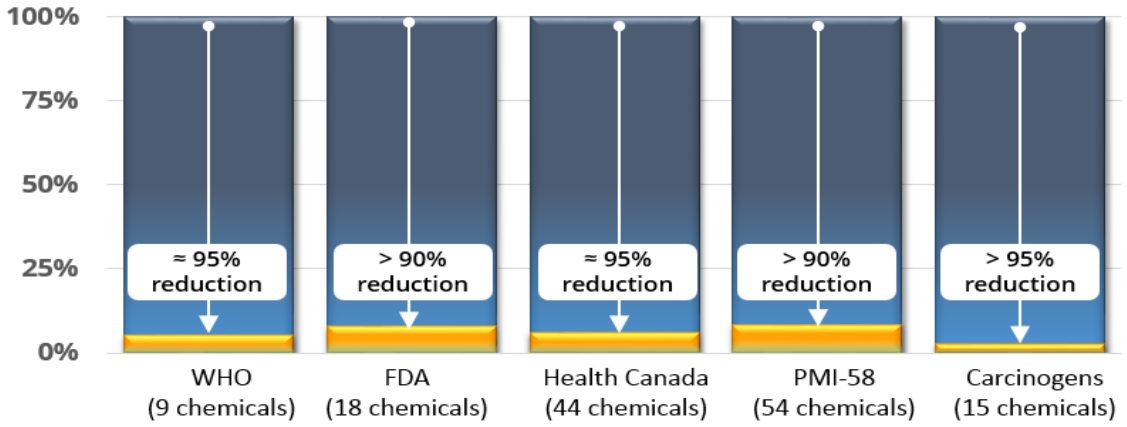

Figure 4: Reduced formation of HPHCs by the THS. The relative level of HPHCs in THS aerosol is shown by the yellow bars and is compared with the 100% in smoke from the 3R4F reference cigarette (dark blue bars). The level of reduction for each group of HPHCs is shown.

Aerosol collection with Health Canada Intense Smoking Regime: 55 mL puff volume, 2-second puff duration, 30-second interval puff. Comparison on a per-stick basis. Reduction calculations exclude nicotine and total particulate matter. The PMI 58 list includes the WHO 9, FDA 18, Health Canada 44 and 15 carcinogens of the IARC Group 1.

Table 1: Comparison in HPHC yields of 3R4F cigarettes and THS Tobacco Sticks.

HPHC[a]	Unit / Stick	3R4F Reference cigarette	THS Tobacco Stick	THS as percentage of 3R4F
Acetaldehyde	µg	1555	219	14.1%
Acrolein	µg	154	11.3	7.3%
Acrylonitrile	µg	31.9	0.258	0.8%
4-Aminobiphenyl	ng	3.26	< 0.051	< LOQ
1-Aminonaphthalene	ng	20.8	0.077	0.4%
2-Aminonaphthalene	ng	11.0	0.046	0.4%
Ammonia	µg	39.3	14.2	36.1%
Benzene	µg	97.6	0.649	0.7%
Benzo[a]pyrene	ng	14.2	< 1.00	< LOQ
1,3-Butadiene	µg	63.8	0.294	0.5%
Carbon monoxide	mg	32.8	0.531	1.6%
Crotonaldehyde	µg	68.8	4.14	6.0%
Formaldehyde	µg	56.5	5.53	9.8%
Isoprene	µg	798	2.35	0.3%
NNK	ng	266	6.7	2.5%
NNN	ng	309	17.2	5.6%
Toluene	µg	188	2.59	1.4%

[a]HPHCs from the FDA 18 list, excluding nicotine.
Abbr.: <LOQ = below level of quantification, NNN = N-nitrosonornicotine; NNK = 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone.

Standard toxicology assessment (Step 3).

The third step of the THS assessment was to determine whether the reduced formation of HPHCs leads to reduced toxicity in laboratory models. Toxicological studies were conducted both *in vitro* and *in vivo*. First, three commonly used *in vitro* assays (Neutral Red Uptake, Bacterial

mutagenicity [Ames] and Mouse Lymphoma) were used to assess the cytotoxicity and genotoxicity of the THS aerosol in comparison with the smoke from the 3R4F reference cigarette.

The Neutral Red Uptake (NRU) assay results showed that the *in vitro* cytotoxicity of the THS aerosol was reduced by approximately 90% compared with the smoke of the 3R4F reference cigarette. The absence of activity in the Ames assay for bacterial mutagenicity and the decreased activity in the Mouse Lymphoma Assay (MLA) for mammalian mutagenicity are consistent with reductions of over 95% for the mutagenicity of the THS aerosol compared to the smoke from 3R4F. Taken together, these results show that THS aerosol is significantly less cytotoxic and genotoxic than the smoke from cigarettes in these studies (*Schaller 2016a*) (Figure 5).

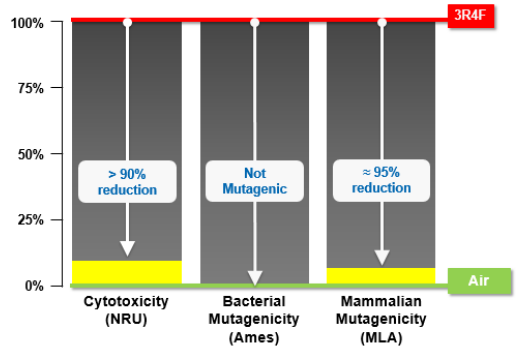

Figure 5: Reduced *in vitro* toxicity of THS aerosol. The relative *in vitro* toxicity of THS aerosol is shown by the yellow bars and is compared with the 100% of smoke from the 3R4F reference cigarette (red). The level of reduction for each test is shown in blue numbers.

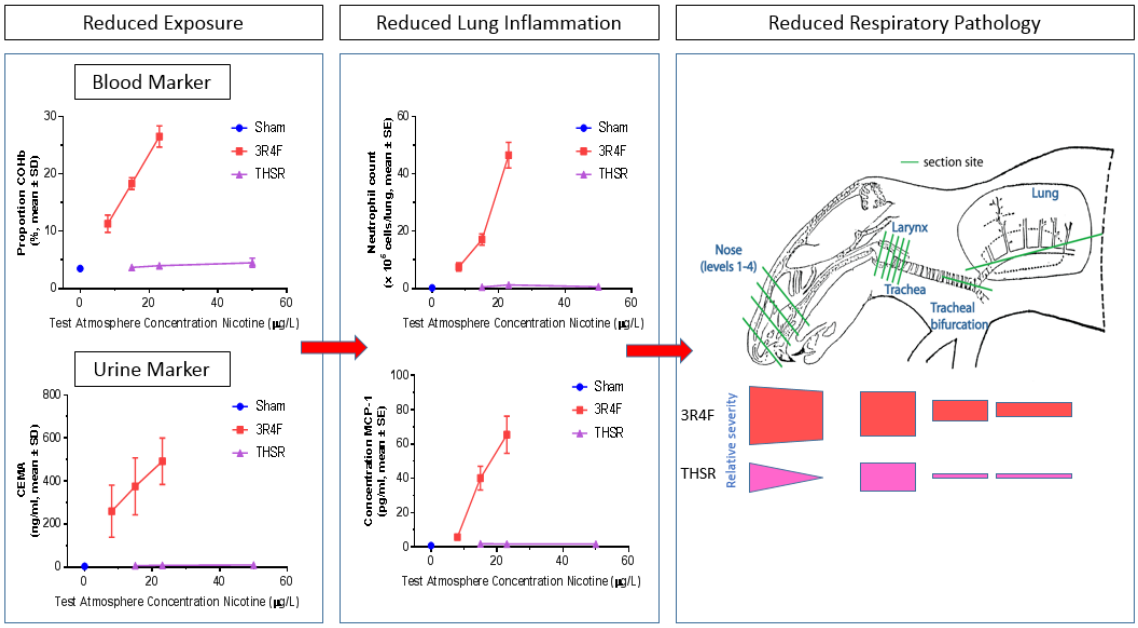

Figure 6: Summary of the 90-day inhalation study results. This *in vivo* study was conducted in rats to compare the toxicity of the THS aerosol with that of 3R4F smoke and a sham group (held in same conditions and exposed to filtered air). The reduced exposure to HPHCs leads to reduced lung inflammation, which in turn leads to reduced respiratory pathology findings. Abbr.: 3R4F = Reference Cigarette 3R4F, THSR = Tobacco Heating System, COHb = carboxyhemoglobin, CEMA = 2-cyanoethylmercaturic acid, MCP-1 = monocyte chemoattractant protein 1.

Second, the inhalation toxicity of THS aerosol was analyzed *in vivo* according to the testing guidelines from the Organization for Economic Co-operation and Development (*OECD 2009*). The study was conducted in rats and the effects of increasing doses of THS aerosol and 3R4F smoke were compared after 90 days of nose-only inhalation exposure. The study results show that the reduced exposure to HPHCs achieved with THS aerosol exposure leads to a significantly reduced lung inflammation and respiratory toxicity of the THS aerosol compared with cigarette smoke (*Wong 2016; Sewer 2016; Oviedo 2016; Kogel 2016*) (Figure 6).

Systems toxicology assessment (Step 4).

Our systems toxicology approach allows us to determine whether reduced toxicity leads to reduced risk in laboratory models (*Appendix B*). Systems toxicology enables a detailed assessment

of the disease-relevant biological mechanisms affected by exposure to toxicants (*Sturla 2014*). Systems toxicology heavily relies on state-of-the-art high-throughput experimental technologies and advanced computational sciences.

First, systems toxicology is applied to identify the biological mechanisms that are altered by cigarette smoke, capturing this knowledge in biological network models. These models are then used to analyze the datasets we generate for product assessment, allowing us to compare the network alterations caused by the aerosols of the THS with those caused by cigarette smoke. Furthermore, the approach allows us to quantitatively compare the overall biological impact of these exposures in the context of toxicological and disease endpoints (*Hoeng 2012*).

We conducted several systems toxicology studies using human-derived *in vitro* cell cultures and organotypic tissue cultures. These studies showed that, compared with 3R4F smoke, THS aerosol has a significantly reduced impact on key mechanisms involved in respiratory diseases (*Gonzalez Suarez 2016; Iskandar 2017a; Zanetti 2016; Iskandar 2017b; Zanetti 2017*) and cardiovascular diseases (*van der Toorn 2015; Poussin 2016*).

For instance, a meta-analysis of the studies performed with the THS in nasal (*Iskandar 2017a*), buccal (*Zanetti 2016*) and bronchial (*Iskandar 2017b*) organotypic epithelial tissue cultures was performed to compare the effects of THS aerosol with those of 3R4F smoke (*Iskandar 2017c*). The results show that THS aerosol causes significantly less perturbation of all biological networks perturbed by 3R4F smoke at equivalent nicotine doses (Figure 7). These results correlate well with physiological measurements such as cytotoxicity in all three tissue cultures and cilia beating in both nasal and bronchial epithelial cultures.

Two *in vitro* mechanistic assays were performed to compare the impact of THS aerosol with that of 3R4F smoke on biological mechanisms related to the initial steps leading to atherosclerosis.

The first study evaluated the adhesion of monocytes to endothelial cells in response to THS aerosol or 3R4F smoke. In this assay (*Poussin 2016*), Primary Human Coronary Artery Endothelial Cells (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 either THS aerosol or 3R4F smoke for 2 h (indirect treatment).
- Unconditioned media (direct treatment).
- Fresh aqueous extracts of THS aerosol and 3R4F smoke extracts (fresh direct treatment).

The cigarette smoke extract promoted the adhesion of monocytes to HCAECs via distinct direct and indirect concentration-dependent mechanisms. Ten- and 20-fold higher concentrations of THS aerosol extract were necessary to elicit effects (adhesion and molecular changes) similar to those measured with 3R4F smoke extract in both fresh direct and indirect treatments, respectively.

The second assay measured monocyte (human THP-1 cell line) chemotaxis and their trans-endothelial migration in response to treatment with aqueous THS aerosol and cigarette smoke extracts. Both 3R4F smoke and THS aerosol induced concentration-dependent decreases in the integrity of the HCAEC monolayer. However, the changes induced by THS aerosol were more than one order of magnitude lower than those induced by 3R4F smoke. In addition, 3R4F significantly inhibited the efflux of monocytic cells across the HCAEC monolayer, whereas the inhibitory effect of THS aerosol extracts on monocyte efflux was approximately 18 times lower (*van der Toorn 2015*). Overall, these results support findings from other studies that THS aerosol could pose much less risk of cardiovascular disease compared with tobacco smoke.

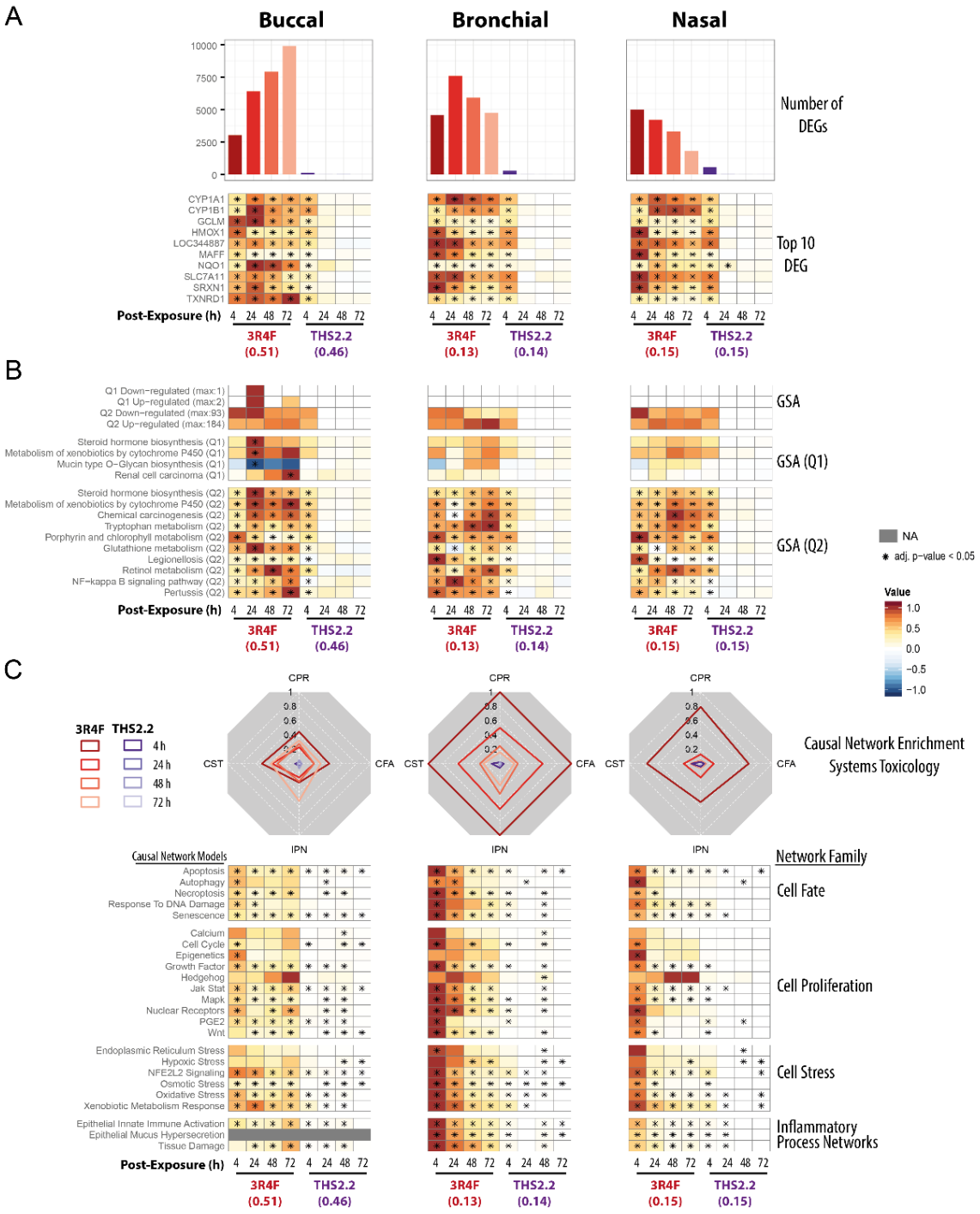

Figure 7. Mechanistic investigation of the exposure impact based on transcriptomics data.

(A) Barplots showing the number of significantly differentially expressed genes (DEGs) across the exposure conditions (FDR-adjusted p-value < 0.05). The heatmaps indicate the expression profiles of the top ten genes. The log2(fold-changes) compared with the respective air control groups are color-coded and the statistical significance level is indicated (FDR-adjusted p-value). (B) Gene set analysis (GSA) was performed with the KEGG gene-set collection using absolute log2(fold-changes) as the gene-level and the mean as the gene-set level statistics. Significance with respect to the treatment effect (Q2, compared with the air control) and dominant effects of individual gene sets (Q1) was assessed with Benjamini-Hochberg based FDR adjustment (FDR adj. p-value < 0.05). The numbers of significantly up- and down regulated gene sets for Q1 and Q2 are shown in the top panel, and the top gene sets are shown in the bottom panels. (C) The causal network enrichment approach for the analysis of the transcriptomics datasets. For each network category, the relative biological impact factor is shown in radar plots (CFA, Cell Fate; CPR, Cell Proliferation; CST, Cell Stress; IPN, Inflammatory Process Networks). The heatmaps show the network perturbation amplitudes for each network in the collection, across all conditions. Full details of the comparative analyses for all doses of the exposure are given in *Iskandar 2017c*.

We also conducted a systems toxicology study in an animal model of disease (Apoe$^{-/-}$ mouse) that develops atherosclerotic plaque and emphysema when exposed to cigarette smoke (*Lo Sasso 2016a*). In this study, mice were exposed to either 3R4F smoke or THS aerosol for 8 months. Furthermore, a group of mice was first exposed for two months to 3R4F smoke and then randomized to either THS aerosol (switching) or fresh air (cessation) to mimic the framework illustrated in Figure 2.

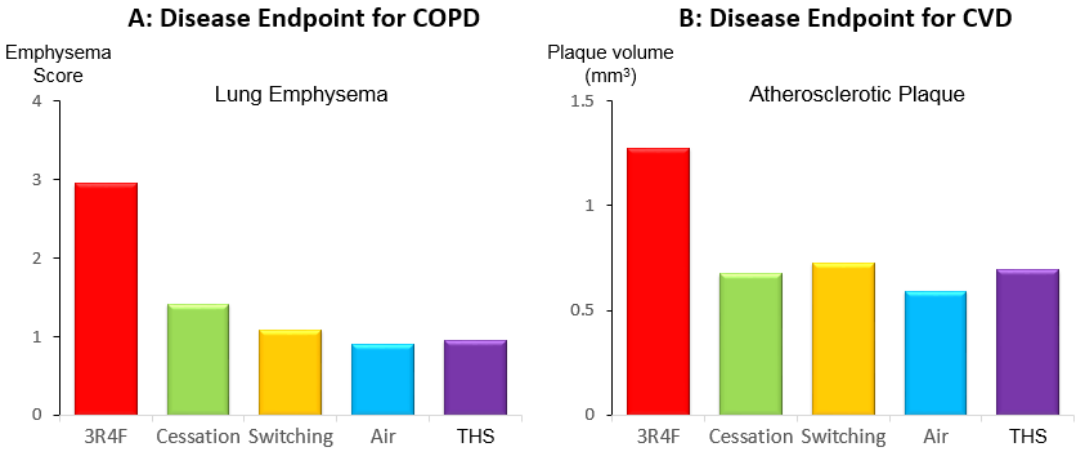

Figure 8: Disease endpoints in a mouse switching study. Lung emphysema (A) and atherosclerotic plaque volume (B) in Apoe$^{-/-}$ mice that were exposed for 8 months to either 3R4F smoke (red bars) or THS aerosol (magenta bars). A group of mice was first exposed for two months to 3R4F smoke and then switched to either THS aerosol (orange bars) or fresh air (green bars). The fresh air control is depicted by blue bars. Lung emphysema scores were assessed by histopathology after 8 months of exposure, atherosclerotic plaque volumes were measured by micro-CT after 7 months of exposure.

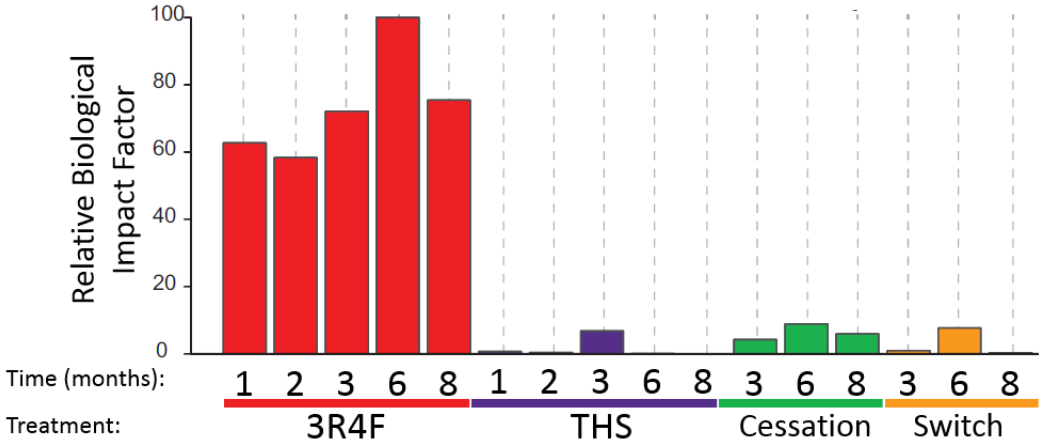

Figure 9: Network-based relative biological impact factor (RBIF) analysis of the lung. RBIF for each treatment group compared to air. The percentages show the relative biological impact, which is derived from the cumulated network perturbations caused by the treatment relative to the air control. RBIF is computationally derived from gene expression data in the context of causal biological networks (*Thomson 2013; Martin 2014; Boué 2015*)

We observed that switching to THS aerosol following two months of cigarette smoke exposure reduces the development of both atherosclerosis and emphysema in a manner similar to smoking cessation (*Phillips 2016; Lo Sasso 2016b*) (Figure 8). A detailed analysis of the molecular mechanisms affected by smoke exposure in the lung showed that switching to THS aerosol reduces the overall biological impact in a way that approaches cessation and that long-term exposure to THS aerosol has little effect on these mechanisms compared with 3R4F smoke exposure (Figure 9).

Clinical assessment (Step 5).

The fifth step of the assessment program utilizes clinical studies to assess the pharmacokinetic (PK) profile of nicotine and emerging pharmacodynamics (PD) effects of the THS and to evaluate whether THS use leads to reduced exposure to HPHCs of adult smokers compared with cigarette smoking. As stated above, these studies are all reviewed and approved by Institutional Review Boards or Ethics Committees and conducted according to the International Conference on Harmonization (ICH) guidelines for Good Clinical Practice (GCP), Declaration of Helsinki, and local requirements. All studies were registered with ClinicalTrials.gov.

Pharmacokinetic profile of THS

Four randomized cross-over, PK/PD studies including 62 study participants in each, were conducted to determine the plasma nicotine absorption profile over 24 hours following single use of the THS as compared to cigarette and NRT use. These studies have been conducted in 2013 in Europe (NCT01967732)[6], Japan (NCT01959607 and NCT01967706) (*Brossard 2017*), and the US (NCT01967719). The studies showed a similar profile of nicotine uptake (e.g. time to maximum nicotine concentration (T_{max}) and maximum nicotine concentration (C_{max}) between THS and cigarettes following single use, except in the US study where C_{max} was lower relative to cigarettes (*Brossard 2017; Marchand 2017*). Suppression of urge-to-smoke between the two products showed similar patterns, except in the US study in line with the lower nicotine intake. Furthermore, the two studies performed in Japan (NCT01959607 and NCT01967706), showed that the urge-to-smoke time profiles were different for Gum (maximum suppression 45–60 min after start of product use) compared to both THS and CC (maximum suppression 15–30 min after first puff). Also, maximum urge-to-smoke suppression was lower for Gum than for THS and CC (*Brossard 2017*).

Reduced exposure to harmful and potentially harmful smoke constituents

In line with the assessment framework illustrated in Figure 2, four clinical studies, each with 160 study participants, were conducted in adult cigarette smokers randomized into three groups: (1) continue smoking their own brand of cigarettes, (2) smoking abstinence, or (3) switch to THS. The exposure period lasting five days to three months. Fifteen biomarkers of exposure to selected HPHCs (Table 2) and nicotine exposure were assessed in these studies with the aim to demonstrate reduced exposure to HPHCs when switching to THS use relative to cigarette smoking.

Table 2: List of measured Biomarker of Exposure to selected HPHCs.

HPHC		HPHC list	Biomarker [Matrix]	Phase	Health Risk
1	1,3-butadiene	FDA, WHO	Monohydroxybutenyl-mercapturic acid (MHBMA) [Urine[1]]	Gas	CA, RT, RDT
2	1-aminonaphthalene	FDA	1-Aminonaphthalene (1-NA) [Urine[1]]	Particulate	CA
3	2-aminonaphthalene	FDA, WHO	2-Aminonaphthalene (2-NA) [Urine[1]]	Particulate	CA
4	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	FDA, WHO	Total 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanol (total NNAL) [Urine[1]]	Particulate	CA
5	4-aminobiphenyl	FDA, WHO	4-Aminobiphenyl (4-ABP) [Urine[1]]	Particulate	CA
6	Acrolein	FDA, WHO	3-Hydroxypropyl- mercapturic acid (3-HPMA) [Urine[1]]	Gas	RT, CT
7	Acrylonitrile	FDA, WHO	2-Cyanoethylmercaturic acid (CEMA) [Urine[1]]	Gas	CA, RT
8	Benzene	FDA, WHO	S-Phenyl-mercapturic acid (S-PMA) [Urine[1]]	Gas	CA, CT, RDT
9	Benzo[a]pyrene	FDA, WHO	Total 3-Hydroxybenzopyrene (3-OH-B[a]P)[Urine[1]]	Particulate	CA
10	Carbon monoxide	FDA, WHO	Carboxyhemoglobin (COHb) [Blood[2]]	Gas	RDT, CT
11	Crotonaldehyde	FDA, WHO	3-Hydroxy-1-methylpropyl-mercapturic acid (3-HMPMA) [Urine[1]]	Gas	CA

[6] NCT codes refer to entries in http://clinicaltrials.gov.

HPHC		HPHC list	Biomarker [Matrix]	Phase	Health Risk
12	Ethylene oxide	FDA	2-Hydroxyethyl-mercapturic acid (HEMA) [Urine[(1)]]	Gas	CA, RT, RDT
13	Nicotine	FDA	Nicotine (NIC-P) [Plasma[1]] Cotinine (COT-P) 3-OH-Cotinine (3OHCOTP) [Plasma[1]] Nicotine equivalents (NEq) [Urine[1]]	Particulate	RDT, AD
14	N-nitrosonornicotine (NNN)	FDA, WHO	Total N-nitrosonornicotine (total NNN) [Urine[1]]	Particulate	CA
15	*o*-toluidine	FDA	o-Toluidine (o-tol) [Urine[1]]	Gas	CA
16	Pyrene	PMI-58	Total 1-hydroxypyrene (1-OHP) [Urine[1]]	Particulate	Nontoxic

[1]Analytical methods: liquid chromatography-tandem mass spectrometry (LC-MS/MS); [2]Analytical method: spectrophotometry.
Abbr.: AD = addictive, CA = carcinogen, CT = cardiovascular toxicant, FDA = Food and Drug Administration, PMI = Philip Morris International, RT = respiratory toxicant, RDT = reproductive & developmental toxicant, WHO =World Health Organization

Two clinical studies with a five day exposure duration, in confinement were conducted with THS in Europe (NCT01959932) (*Haziza 2016a; Haziza 2017*) and Japan (NCT01970982) (*Haziza 2016b*). Use of a confinement study design ensures that the compliance to arm allocation is fully controlled, and therefore provides useful information on reduction of exposure under optimal conditions.

Subsequently, two 3-month studies (Figure 10), which included a five-day exposure period under confined conditions followed by an ambulatory period of 85 days, were conducted with the mentholated version of THS in Japan (NCT01970995) (*Haziza 2016c; Lüdicke 2017a*) and the US (NCT01989156) (*Haziza 2016d*). The ambulatory study period was intended to assess if reductions in exposure observed in a confined setting were sustained under more "real world" conditions, where confounding factors such as environment, diet, passive smoking and use of CC in combination with THS (dual-use) could influence the levels of exposure to HPHCs. Furthermore, these studies provided further insights in product use and acceptance, as well as early indications of changes in smoking-related disease risk endpoints over a prolonged period. In the Japanese study, compliance of study participants with THS use was of at least 82% on a monthly basis while a lower compliance in the US study was observed (at least 42.5% reaching 55% during the last month).

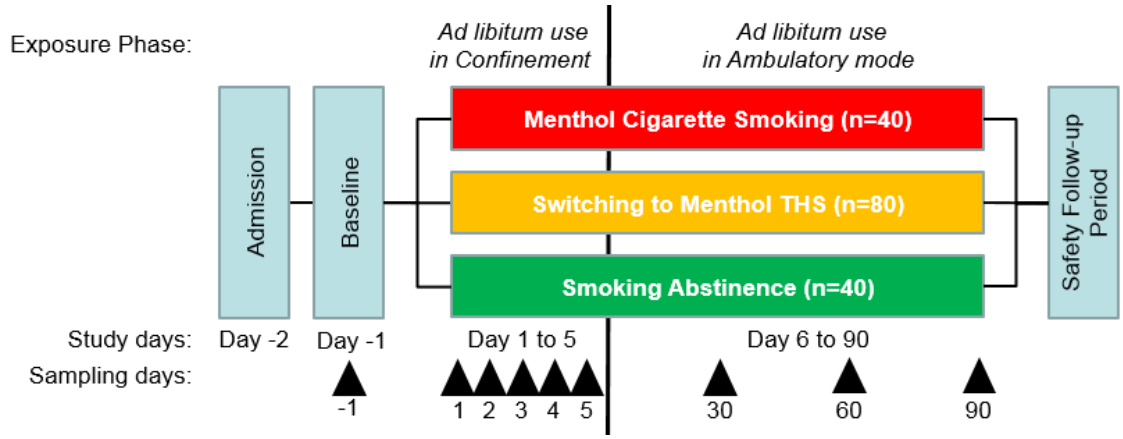

Figure 10: Study design of the two 3-month reduced exposure studies.

All four studies showed a significant reduction (ranging from 47% to 96% relative to continuing smoking cigarettes) in the fifteen biomarkers of exposure in adult smokers who switched to THS

use after 5 days of use in confinement, which approached that of those who abstained from smoking for the duration of the study. The ambulatory period of the two three-month studies, showed sustained reduction of biomarkers of exposure levels after 3 months (ranging from 34% to 94% relative to continuing smoking cigarettes). Figure 11 and Table 3 show the results of the two three-month studies at the end of the study. The time course of the exposure reduction is illustrated by two biomarkers of exposure, COHb and Total NNAL for both 3-month studies (Figures 12 and 13).

The profile of product consumption differed slightly between the two 3-month studies (Figure 14). During the last month of the study, the daily consumption of THS *Tobacco Sticks* (12.7) was lower than cigarettes (15.2) in the Japanese study while it was comparable between the two products (14.2 THS *Tobacco Sticks* and 15.5 cigarettes) in the US study. Despite these differences, exposure to nicotine, as measured by nicotine equivalent in urine, was not different during the last month of the study between study participants using the THS and those smoking cigarettes (Figure 15). This suggests that study participants adapt their product consumption according to their nicotine need over the 3-month period of use.

To complement the biomarker of exposure measurements described above, we have used the previously reported whole blood-derived gene expression signature that can distinguish current smokers from either nonsmokers or former smokers with high specificity and sensitivity (*Martin 2015*). We tested the small signature consisting of only 11 genes on the blood transcriptome of subjects enrolled in the 5-day clinical study conducted in Europe (*Haziza 2016a*) and showed a reduced exposure response in subjects that either stopped smoking or switched to THS, compared with subjects who continued smoking their regular tobacco product (*Martin 2016*). The validity of this small gene expression signature and the results obtained with the data from the 5-day reduced exposure clinical studies were later verified by blinded crowdsourcing through a computational challenge conducted under sbvIMPROVER[7] (*Belcastro 2017, Poussin 2017*) (*Appendix B*).

[7] sbvIMPROVER stands for Systems Biology Verification combined with Industrial Methodology for Process Verification in Research. This approach aims to provide a measure of quality control of industrial research and development by verifying methods and results. The sbv IMPROVER project is a collaborative effort led and funded by PMI Research and Development. For more information please see the published descriptions of sbvIMPROVER in Nature Biotechnology (*Meyer 2011*) or Bioinformatics (*Meyer 2012*).

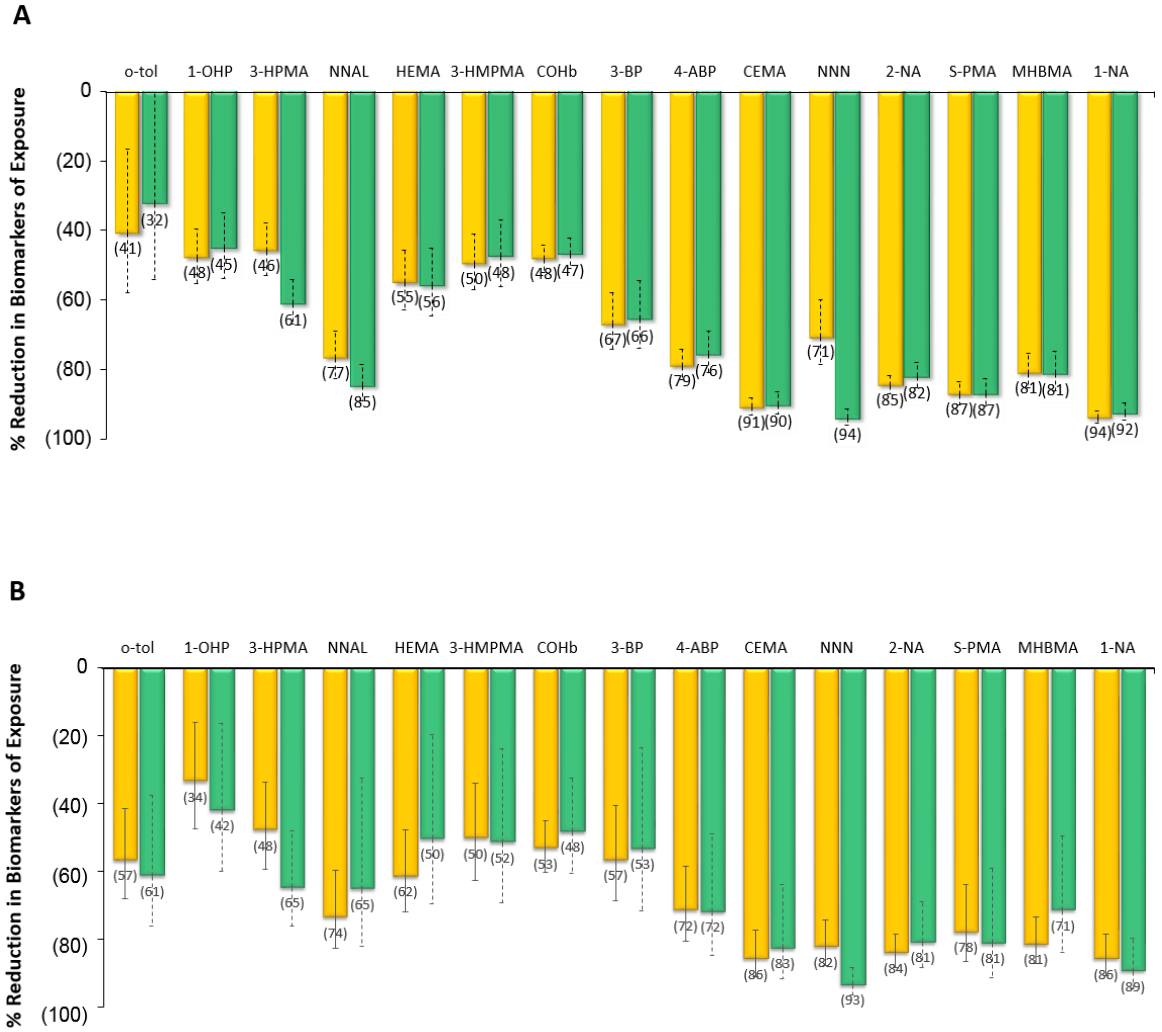

Figure 11: Percent reduction in biomarkers of exposure relative to continuing smoking cigarettes after switching to THS (yellow bars) and smoking abstinence (SA) (green bars) after three months. A: Study conducted in Japan, B: Study conducted in the US. For numerical values see Table 3.

Abbr.:
o-tol = o-toluidine;
1-OHP = total 1-hydroxypyrene;
3-HPMA = 3-hydroxypropylmercapturic acid;
NNAL = total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
HEMA = 2-hydroxyethyl-mercapturic acid;
3-HMPMA = 3-hydroxy-1-methylpropyl-mercapturic acid;
COHb = carboxyhemoglobin;
3-BP = 3-hydroxybenzopyrene;
4-ABP = 4-aminobiphenyl;
CEMA = 2-cyanoethylmercaturic acid;
NNN = total N-nitrosonornicotine;
2-NA = 2-aminonaphthalene;
S-PMA = S-phenylmercapturic acid;
MHBMA = monohydroxybutenyl mercapturic acid;
1-NA = 1-aminonaphthalene.

Table 3: Reduction in Biomarker of Exposure relative to continuing smoking cigarettes after switching to THS and smoking abstinence after three months in the studies conducted in Japan and the US.

HPHC	Biomarker of Exposure	Japan Study		US Study	
		% Reduction THS vs CC (CI)[a]	% Reduction SA vs CC (CI)	% Reduction THS vs CC (CI)	% Reduction SA vs CC (CI)
1,3-butadiene	Monohydroxybutenyl-mercapturic acid (MHBMA)	–81% (–85; –75)	–81% (–86; –75)	–81% (–87; –73)	–71% (–84; –49)
1-aminonaphthalene	1-Aminonaphthalene (1-NA)	–94% (–95; –92)	–92% (–94; –90)	–86% (–91; –78)	–89% (–95; –80)
2-aminonaphthalene	2-Aminonaphthalene (2-NA)	–85% (–87; –82)	–82% (–85.2; –77.9)	–84% (–88; –78)	–81% (–88; –69)
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Total 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanol (Total NNAL)	–77% (–83; –67)	–85% (–89; –79)	–74% (–83; –60)	–65% (–82; –33)
4-aminobiphenyl	4-Aminobiphenyl (4-ABP)	–79% (–83; –74)	–76% (–81; –69)	–72% (–81; –58)	–72% (–85; –49)
Acrolein	3-Hydroxypropyl- mercapturic acid (3-HPMA)	–46% (–53; –38)	–61% (–66.8; –54.0)	–49% (–59; –34)	–65% (–76; –48)
Acrylonitrile	2-Cyanoethylmercaturic acid (CEMA)	–91% (–93; –88)	–90% (–93; –86)	–86% (–91; –77)	–83% (–92; –64)
Benzene	S-Phenyl-mercapturic acid (S-PMA)	–87% (–90; –83)	–87% (–90; –83)	–78% (–87; –64)	–81% (–91; –59)
Benzo[a]pyrene	Total 3-Hydroxybenzopyrene (3-OH-B[a]P)	–67% (–74; –58)	–66% (–74; –55)	–57% (–69; –40)	–53% (–72; –24)
Carbon monoxide	Carboxyhemoglobin (COHb)	–48% (–52; –44)	–47% (–51; –42)	–53% (–60; –45)	–48% (–60; –33)
Crotonaldehyde	3-Hydroxy-1-methylpropyl-mercapturic acid (3-HMPMA)	–50% (–57; –41)	–48% (-56; –37)	–52% (–69; –24)	–52% (–69; –24)
Ethylene oxide	2-Hydroxyethyl-mercapturic acid (HEMA)	–55% (–63; –46)	–56% (–65; –45)	–62% (–72; –48)	–51% (–70; –20)
N-nitrosonornicotine (NNN)	Total N-nitrosonornicotine (total NNN)	–71% (–79; –60)	–94.0% (–96; –91)	–82% (–88; –74)	–93% (–96; –88)
o-toluidine	o-Toluidine (o-tol)	–41% (–58; –17)	–32% (–54; 0)	–57% (–68; –47)	-61% (–76; –38)
Pyrene	Total 1-hydroxypyrene (1-OHP)	–48% (–55; –40)	–45% (–54; –35)	–34% (–47; –16)	–42% (–60; –17)

[a]CI = confidence interval

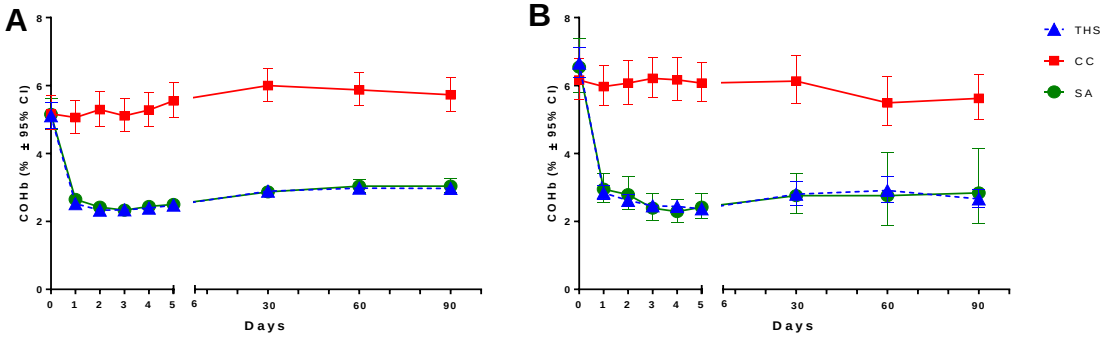

Figure 12: Time course of reduction in COHb in the THS group (THS), cigarette smoking group (CC), and smoking abstinence group (SA) over three months. A: Study conducted in Japan, B: Study conducted in the US.

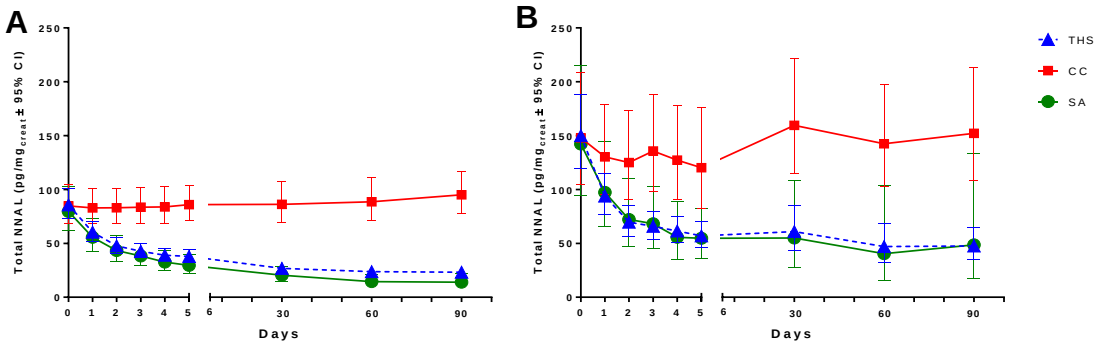

Figure 13: Time course of reduction in Total NNAL in the THS group (THS), cigarette smoking group (CC), and smoking abstinence group (SA) over three months. A: Study conducted in Japan, B: Study conducted in the US.

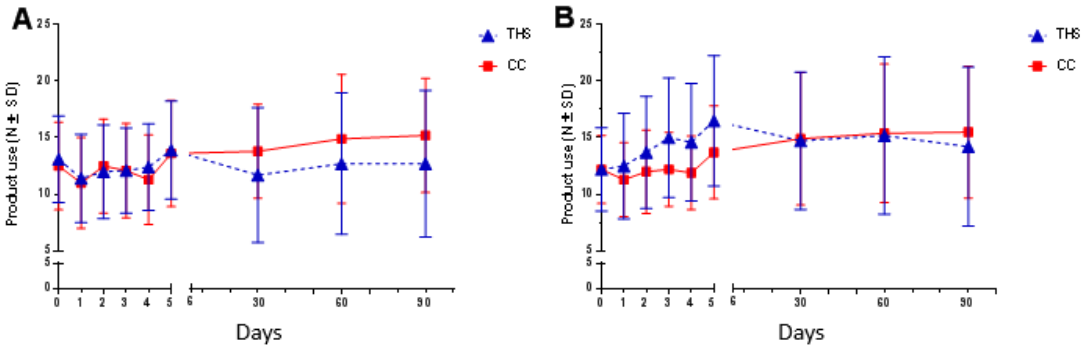

Figure 14: Product use (daily number of cigarette (CC) or THS tobacco sticks (THS)) throughout the two three-month studies. A: Study conducted in Japan, B: Study conducted in the US.

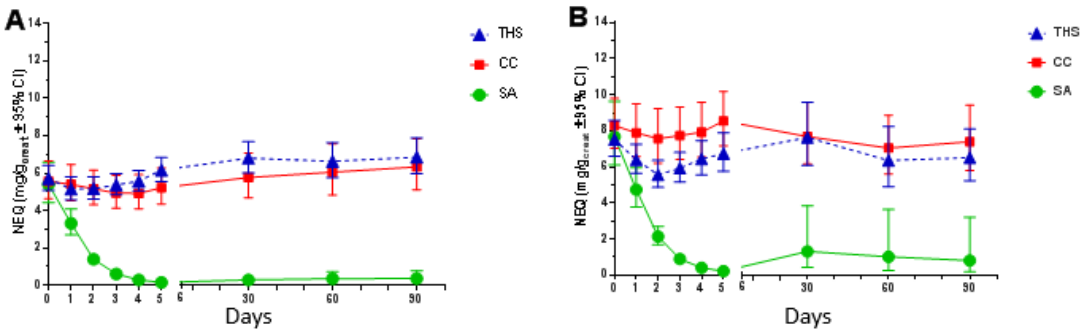

Figure 15: Urinary nicotine equivalent levels (NEQ, is calculated based on the sum of all nicotine metabolites that are measured) in the cigarette smokers (CC), THS users (THS) or smoking abstinent (SA) subjects, throughout the two three-month studies. A: Study conducted in Japan, B: Study conducted in the US.

In both of these three-month studies, six clinical risk markers were also measured (*Lüdicke 2017b*). They are reflective of disease mechanisms known to be affected by smoking and to reverse upon cessation. While these clinical studies were primarily designed to focus on biomarkers of exposure, the results are generally consistent with the expected direction of change and indicate that switching completely to the THS led to an overall improvement of clinical risk markers affected by smoking after only three months (Table 4).

Table 4: Clinical risk markers in two three-month studies conducted in Japan and the US

Disease Mechanisms	Marker	Expected Direction of Change	Japan THS versus CC	US THS versus CC
Lipid Metabolism	HDL-C	↑	4.53 mg/dL ↑	1.40 mg/dL↑
Inflammation	WBC	↓	-0.57 GI/L ↓	0.17 GI/L →
Airway Impairment	FEV_1	↑	1.91 % pred* ↑	0.53 % pred ↑
Endothelial Dysfunction	sICAM-1	↓	8.72% ↓	10.59% ↓
Oxidative Stress	8-epi-$PGF_{2\alpha}$	↓	12.71% ↓	13.46% ↓
Clotting	11-DTX-B_2	↓	9% ↓	3. 56% ↓

*pred = predicted

PMI is conducting additional clinical studies with THS. For instance, to establish a one-year "gold standard" of cessation for assessing novel products, a Smoking Cessation Response study across several countries is currently ongoing (NCT02432729), in order to measure the improvement of clinical risk markers when adult smokers quit smoking for one year. Furthermore, an Exposure Response study (NCT02396381, NCT02649556) primarily designed to measure clinical risk markers when adult smokers switch to the THS for a 12-month period is also ongoing. The results of this study will then be benchmarked against those of the Smoking Cessation Response study. All results will be published.

Perception and Behavior Studies (Step 6).

The second objective of the assessment program is to demonstrate that the THS, as it is actually used by consumers will benefit the health of the population as a whole taking into account both smokers and nonsmokers.

Population harm reduction depends on both the availability of significantly lower risk products and whether a significant proportion of adult daily smokers are willing to accept and switch to these products (*Smith 2016*). New tobacco products will realize their maximum population harm reduction potential when they are used in lieu of more hazardous tobacco products such as cigarettes. The maximum reduction in risk will be achieved by those smokers who completely switch to THS. At the same time, to benefit the health of the population as a whole, a new tobacco product should not be attractive to non-users of tobacco products, both never users and former users. The opportunity for a new tobacco product like the THS to lower the morbidity and mortality associated with use of tobacco products can be blunted or diminished by attracting significant numbers of never or former smokers. This may happen if nonusers believe that the product is risk free and transition to tobacco use, even with a significantly lower risk tobacco product.

In this context, PMI developed a program of Perception and Behavior Assessment (PBA) studies aimed at (i) developing understandable and scientifically accurate consumer messages, (ii) assessing the comprehension of these messages and the risk perception of the THS among various adult consumer groups, (iii) assessing the suitability of the THS as a substitute for cigarettes among adult smokers.

The results of these studies showed that all adult consumer groups that were tested (adult smokers with no intention to quit, adult smokers with intention to quit, adult former smokers, adult never smokers and young adult never smokers) were able to understand properly the following key points regarding THS:

1. THS presents less risk to health than cigarettes.
2. THS is for adult smokers with no intention to quit; it is not intended for former smokers or never smokers.
3. THS is not risk free.
4. Quitting the use of all tobacco is the best way to reduce the risk of tobacco-related disease.
5. THS contains nicotine, which is addictive.
6. THS is perceived to have (i) less risk than cigarettes, (ii) a similar risk to e-cigarettes and (iii) more risk than quitting the use of tobacco.

The studies also showed that approximately one third of adult smokers without intention to quit would use the THS, while less than 5% former smokers and less than 1% of never smokers were interested by the THS. Finally, the THS did not change the intention of adult smokers with intention to quit.

The combined results of the PBA program indicate that the presented communication materials provide scientifically accurate information that is clear and easily understandable. They allow consumers from different tobacco use experiences to make informed decisions about the use of the THS in a manner that is consistent with an overall reduction in population harm and the risk of harm and tobacco-related disease.

Post-Market Studies and Surveillance (Step 7).

Once a novel product is on the market, it is necessary to conduct post-market studies to understand how the product is used and by whom. In essence, these studies are necessary to confirm the results of the pre-market PBA program, to ensure that, once on the market, the THS does not attract significant numbers of never and former smokers, while a significant portion of current adult smokers switch completely to the THS. Towards this end, cross-sectional surveys are used to measure the prevalence of tobacco and nicotine product usage among the population following market introduction of the THS and cohort studies are used to determine the behavior of smokers who switched to THS. Additional clinical studies will be conducted to determine the health outcome of switching to the THS compared with ongoing smoking and to cessation. Furthermore, passive surveillance measures are used to gather spontaneous reports of adverse events related to novel product usage.

Safety

Safety was monitored in all the clinical studies and additional safety information were collected through post-market studies and passive safety surveillance. So far observed adverse events associated with or related to the THS seems to be very similar with the safety profile of smoking cessation pharmacotherapies such as NRT, as reported in NRT clinical trials.

Conclusions

Offering current adult smokers a portfolio of products with the potential to reduce disease risk when compared to smoking cigarettes should be pursued in addition to preventing initiation and promoting smoking cessation. To date, the scientific assessment of the THS has demonstrated that the THS yields significantly reduced levels of HPHCs compared with

cigarettes. This reduced HPHC formation led to the reduced toxicity of the THS aerosol as well as a reduced risk of disease in non-clinical laboratory models. In clinical studies, the reduced HPHC formation by the THS led to the reduced exposure of adult smokers who switched from cigarette smoking to THS use. The clinical evidence also showed that switching to THS use reduced the exposure to HPHCs in a way that approached smoking abstinence, which in turn led to positive changes in clinical risk markers in adult smokers who switched to it completely in comparison to smokers who continued smoking.

The results of the perception and behavior assessment studies furthermore show that adult smokers groups have a good understanding that the THS presents less risk than cigarettes but that it is not risk-free, that the THS is not intended for non-smokers (never smokers and former smokers) and that quitting the use of all tobacco is the best way to reduce the risk of tobacco-related disease.

The totality of the scientific evidence available to date indicates that the THS has the potential to present less risk of harm compared to continued smoking for adult smokers who switch to it completely.

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For more information, visit PMIScience.com

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Appendix A: Absence of Combustion in THS Leads to Reduced HPHC Formation and Emission

Summary of Scientific Evidence

Philip Morris International R&D

I. Evidence for absence of combustion.

Combustion in a conventional cigarette

Combustion is a defining characteristic of cigarettes. When a cigarette is lit, the combination of tobacco and paper (fuels) with oxygen (oxidant) and heat (energy) generates a self-sustaining combustion (exothermic) process that consumes the tobacco. This process results in the formation of ash and smoke that contains solid particles and high levels of harmful and potentially harmful constituents (HPHCs).

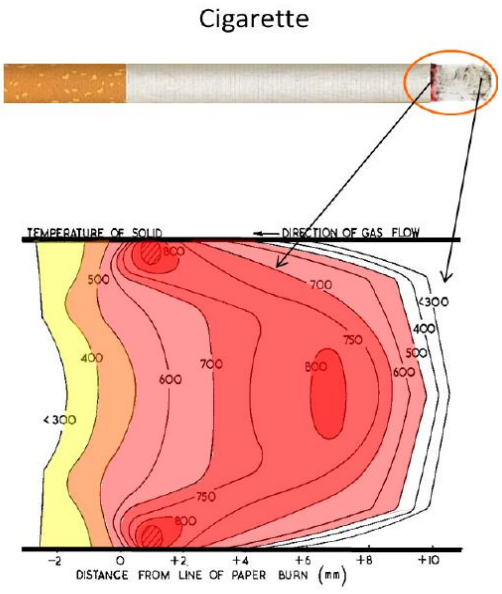


Figure 1: Tobacco temperature in a lit conventional cigarette

During the natural smoldering of a cigarette (between puffs), temperatures between 600°C and 800oC occur in the center of the burning cone (Figure 1) (*Baker 1975*). During a puff, fresh air (and therefore oxygen) is drawn through the lit-end of the cigarettes, and hence the temperature increases to more than 900°C at the periphery of the burning zone.

Two indicators are required to detect unambiguously biomass and/or tobacco combustion:

1. Presence of relevant amounts of nitrogen oxides in the gaseous products, which are not formed from the decomposition of nitrates already present in the original biomass/tobacco substrate.
2. Clear evidence of an exothermic process.

Evidence that the THS does not cause combustion

In contrast to lit-end cigarettes, which generate smoke, the THS is designed to reduce the formation of HPHCs by electronically heating the tobacco, which generates an aerosol (*Smith 2016*). PMI has generated several lines of evidence demonstrating that no combustion takes place in THS:

1. Ash: Since combustion does not occur, the structural integrity of the Tobacco Stick is retained after use. The tobacco is not consumed, as it is in a cigarette, and no ash is formed (Figure 2).
2. Temperature: The highest observed temperature of the tobacco in the Tobacco Stick is approximately 300°C (measured 0.2 mm from the heater blade) and cannot exceed 350°C (the programmed maximum temperature of the heater) (Figure 3). This is well below the temperature required for tobacco combustion (known to be in excess of 400°C; *Barontini 2013*); in fact, the temperature of most of the tobacco is significantly below 250°C. Indeed the

temperature of the tobacco reaches <230°C at 0.5 mm from the heater blade, <190°C at 1.7 mm from the heater blade and < 120°C at 3.4 mm from the heater blade.

Figure 2: Comparison of unused and used THS Tobacco Stick (left) and conventional cigarettes (right).
The structural integrity of the Tobacco Stick is retained after use and no ash is formed (left pictures), in contrast to a cigarette (right pictures).

Abbr.: THS = Tobacco Heating System

3. Exothermic process: Contrary to the increase in temperature that occurs when a puff is taken with a lit-end cigarette there is significant drop in the temperature of the tobacco in the Tobacco Stick when a puff is taken (Figure 3). Furthermore, when the energy source is switched off, the temperature of the tobacco begins to decrease (Figure 3B). Because combustion is a self-sustaining process, the decrease in temperature indicates an absence of combustion. These facts demonstrate the absence of an exothermic process.

A

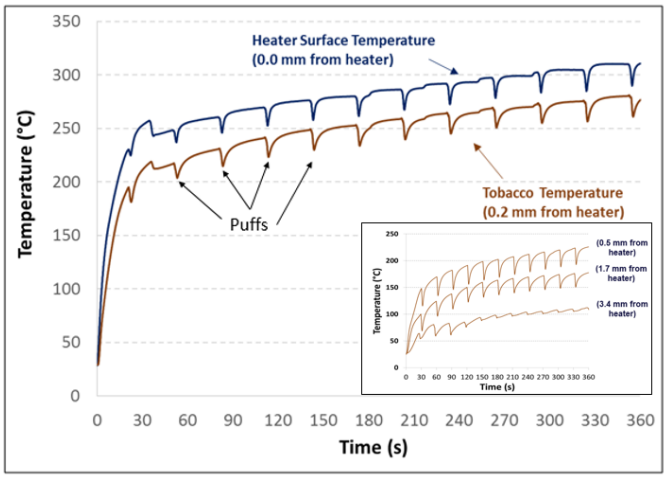
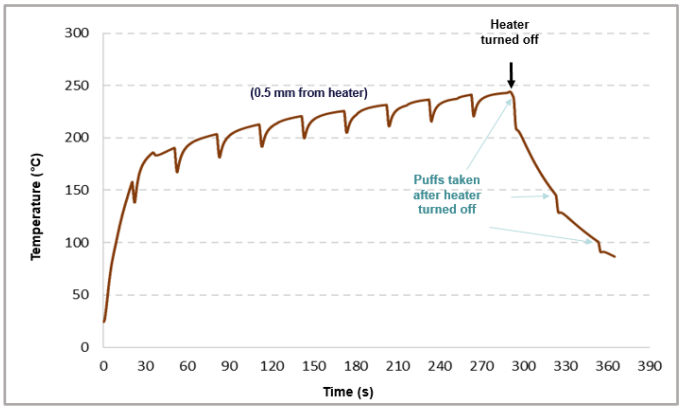


Figure 3: THS temperature profiles

A. Temperature profiles were measured at the interface between the heater and the tobacco substrate (blue line) and in the tobacco plug (at 0.2 mm from the heater) (brown line) during product use. The reported temperatures are the average of five replicates. The inset shows further temperature profiles that were measured at 0.5 mm, 1.7 mm and 3.4 mm from the heater.

B



B. Temperature profile measured 0.5 mm from the heater. The heater was turned off after 300 seconds of operation.

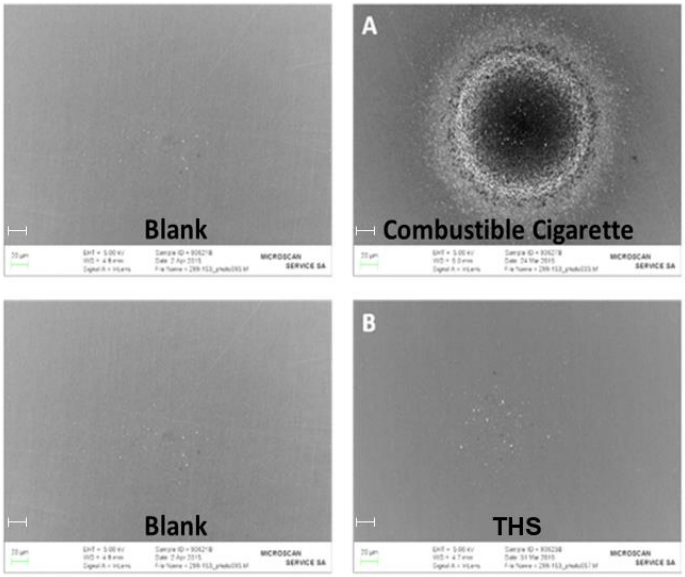


Figure 4: Comparison of scanning electron microscopy images determined for combustible cigarette smoke (3R4F reference cigarette) (A) and THS aerosol (B)

The scanning electron microscopy image of the respective blanks run prior to cigarette smoke and THS aerosol generation are shown on the left. The white scale bar (bottom left of each panel) corresponds to 20µm.

Abbr.: THS = Tobacco Heating System

4. Oxygen: Oxygen is the necessary oxidant of tobacco combustion. PMI tested the THS in a chamber with air and in a chamber filled only with nitrogen, where one of the essential elements of combustion (oxygen) was absent. The aerosol generated by the THS in an atmosphere of pure nitrogen (where combustion cannot occur) contained equivalent levels of HPHCs than the aerosol generated in air (21% oxygen). The aerosol was equivalent under both atmospheres supporting the view that combustion does not occur during THS use.
5. Solid particles: Combustion of tobacco in cigarettes generates solid ultra-fine particles with a median diameter below 100 nm (*Pratte 2016*), which have been shown to be cytotoxic (*Fariss 2013*). PMI analyzed both THS aerosol and cigarette smoke for the presence of solid particles by stripping them of their volatile constituents. This was achieved by passing the aerosol and smoke through a commercial Dekati thermo-denuder operating at 300°C. The analysis of the materials collected during this process by scanning electron microscopy revealed that the THS aerosol does not contain solid particles, and confirmed that cigarette smoke does (each 3R4F cigarette contains approx. 1012 ultra-fine particles) (Figure 4) (*Pratte 2016*).
6. Carbon monoxide and nitrogen oxides: The THS aerosol contains substantially lower levels of Harmful and Potentially Harmful Constituents compared with cigarette smoke (*Schaller 2016a*). Importantly, nitrogen oxides (NO_x) and carbon monoxide (CO), two important combustion markers, were reduced by over >96% (Table 1) (*Schaller 2016a*). Furthermore, their levels were shown to be equivalent when the THS was operated under nitrogen, demonstrating that their origin is not linked to combustion.

Table 1: Analyte yields from THS and 3R4F obtained under Health Canada Intensive machine-smoking conditions and expressed on a per-cigarette/tobacco stick basis.

HPHCs	Unit / Stick	THS[a]	3R4F[a]	% Reduction
Carbon monoxide (CO)	mg	0.531 ± 0.068	32.8 ± 2.4	-98.4%
Nitrogen oxides (NO_x)	µg	17.3 ± 2.6	537 ± 43	-96.8

[a] Mean ± CI 95%

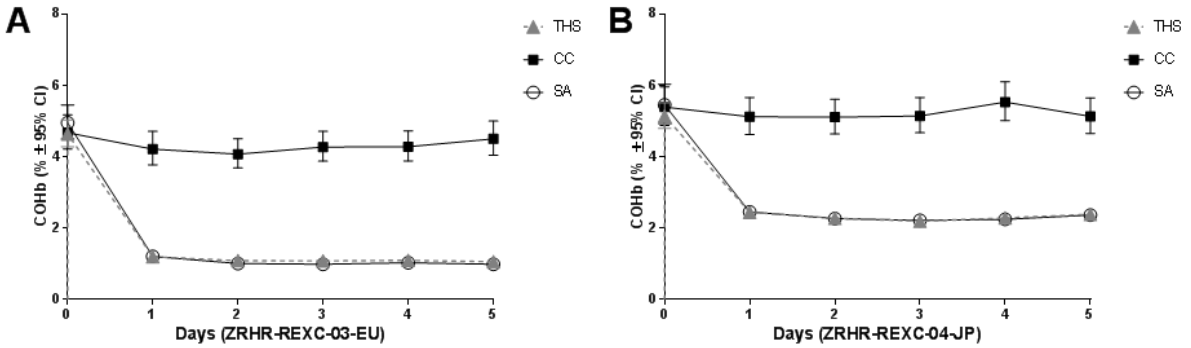


Figure 5: Reduced exposure to carbon monoxide in clinical studies
Two clinical studies with a five day exposure duration, in confinement were conducted in (A) Europe (Clinicaltrials.gov ID: NCT01959932) (*Haziza 2016a*) and (B) Japan (Clinicaltrials.gov ID: NCT01970982) (*Haziza 2016b*).
Abbr.: CC = Combustible Cigarettes; SA = Smoking abstinence; THS = Tobacco Heating System

7. Reduced exposure to CO: The reduction in CO formation by the THS led to the reduction in exposure measured in clinical studies. The levels of blood COHb in smokers who switched from cigarette smoking to THS use was indistinguishable from the level of blood COHb in smokers who abstained from smoking for the duration of these studies (Figure 5). This demonstrates that the drastically reduced levels of CO measured in laboratory-based aerosol chemistry analyses are confirmed in clinical studies

In conclusion, there is convincing evidence that no combustion occurs in the THS. This conclusion is furthermore supported by several expert opinions (*Cozzani 2014, Gierycz 2015, Fujita 2015, Wojtowicz 2015, Rein 2017*).

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II. Evidence that the THS yields reduced levels of HPHCs.

The absence of combustion in the THS leads to a reduction in HPHC formation compared with cigarettes. The difference in aerosol compositions is supported by several lines of evidence.

Aerosol color.

The color of cigarette smoke particulate matter is dark while the particulate matter of THS aerosol is not (Figure 6). This difference in color is indicative of the absence of combustion and of black carbon particles in the THS aerosol. This is supported by the absence of carbon-based solid particles in the aerosol of the THS (Evidence for absence of combustion, Point 5, Figure 4).

Aerosol Composition.

While cigarette smoke contains <50% water, the aerosol of THS is mainly composed of water (80%). Conversely, THS aerosol contains <10% other constituents in contrast to 3R4F smoke (>45%) (Figure 6).

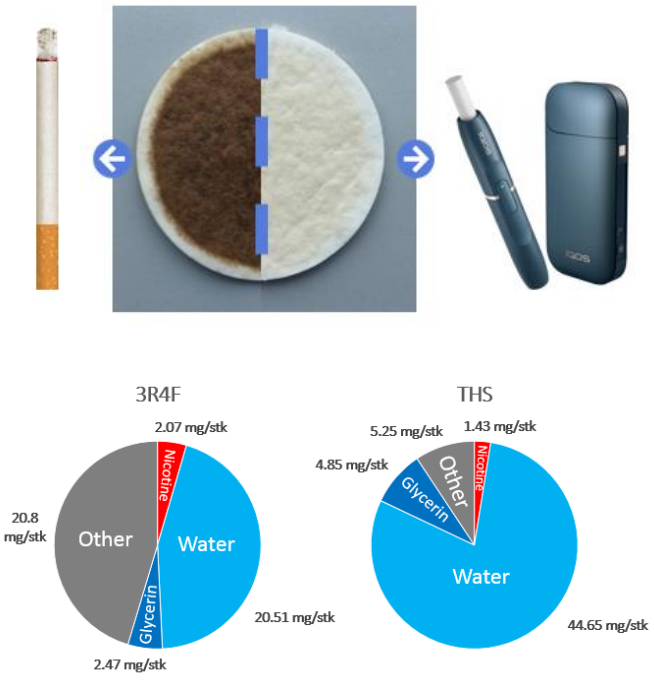

Figure 6: Top: comparison between the dark color of the particulate matter of cigarette smoke (left) and the very light color of the THS aerosol (right) after collection on Cambridge glass-fiber filter pads. Bottom: relative quantities of water (light blue), nicotine (red), glycerin (dark blue) and other constituents (grey) in 3R4F smoke (left) and THS aerosol (right) expressed in mg/stick.

Table 2: PMI-58 list of analytes (excluding product-specific analytes)

Analyte/Constituent	Health risk	Analyte/Constituent	Health risk
Acetaldehyde**,#	CA, RT, AD	Hydroquinone	(CA?)
Acetamide	CA	Isoprene**	CA
Acetone	RT	Lead	CA, CT, RDT
Acrolein**,#	RT, CT	Mercury	CA, RDT
Acrylamide	CA	Methyl ethyl ketone	RT
Acrylonitrile**	CA, RT	4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)**,#	CA*
3-aminobiphenyl	NA	Nickel	CA*, RT
4-aminobiphenyl**	CA*	Nicotine**	RDT, AD
1-aminonaphthalene**	CA	Nicotine Free Dry Particulate Matter (NFDPM)	
2-aminonaphthalene**	CA*	Nitric oxide (NO)	NA
Ammonia**	RT	Nitrobenzene	CA, RT, RDT
Arsenic	CA*, CT, RDT	Nitrogen oxides (NOx)	RT, CT, RDT
Benz(a)anthracene	CA, CT	N-nitrosoanabasine (NAB)	CA
Benzene**,#	CA*, CT, RDT	N-nitrosoanatabine (NAT)	NA
Benzo(a)pyrene**,#	CA*	N-nitrosonornicotine (NNN)**,#	CA*
1,3-butadiene**,#	CA*, RT, RDT	Phenol	RT, CT
Butyraldehyde	RT, CT	Propionaldehyde	RT, CT
Cadmium	CA*, RT, RDT	Propylene oxide	CA, RT
Carbon monoxide**,#	RDT	Pyrene	(CA?)
Catechol	CA	Pyridine	RT
Chromium	CA*, RT, RDT	Quinoline	CA
m-Cresol	CA, RT	Resorcinol	RT
o-Cresol	CA, RT	Selenium	RT
p-Cresol	CA, RT	Styrene	CA
Crotonaldehyde**	CA	Toluene**	RT, RDT
Dibenz(a,h)anthracene	CA	*o*-Toluidine	CA*
Ethylene oxide	CA*, RT, RDT	Total particulate matter (TPM)^	
Formaldehyde**,#	CA*, RT	Vinyl chloride	CA*
Hydrogen cyanide	RT, CT	Water^	

Abbr.: AD = Addictive, CA = Carcinogen, CT =Cardiovascular Toxicant, NA = Not Attributed, RDT = Reproductive or Developmental Toxicant, RT = Respiratory Toxicant, * denotes IARC group 1 carcinogens, ** denotes the 18 HPHCs mandated for reporting by FDA. # denotes the 9 HPHCs mandated for reporting by WHO (*WHO 2008*).
^ TPM consists of the total mass of aerosol captured on a filter pad (known as Cambridge filter). NFDPM is equal to the TPM minus the quantity of water and nicotine. TPM and NFDPM may contain HPHCs, but are not standalone HPHCs. Water is not an HPHC.

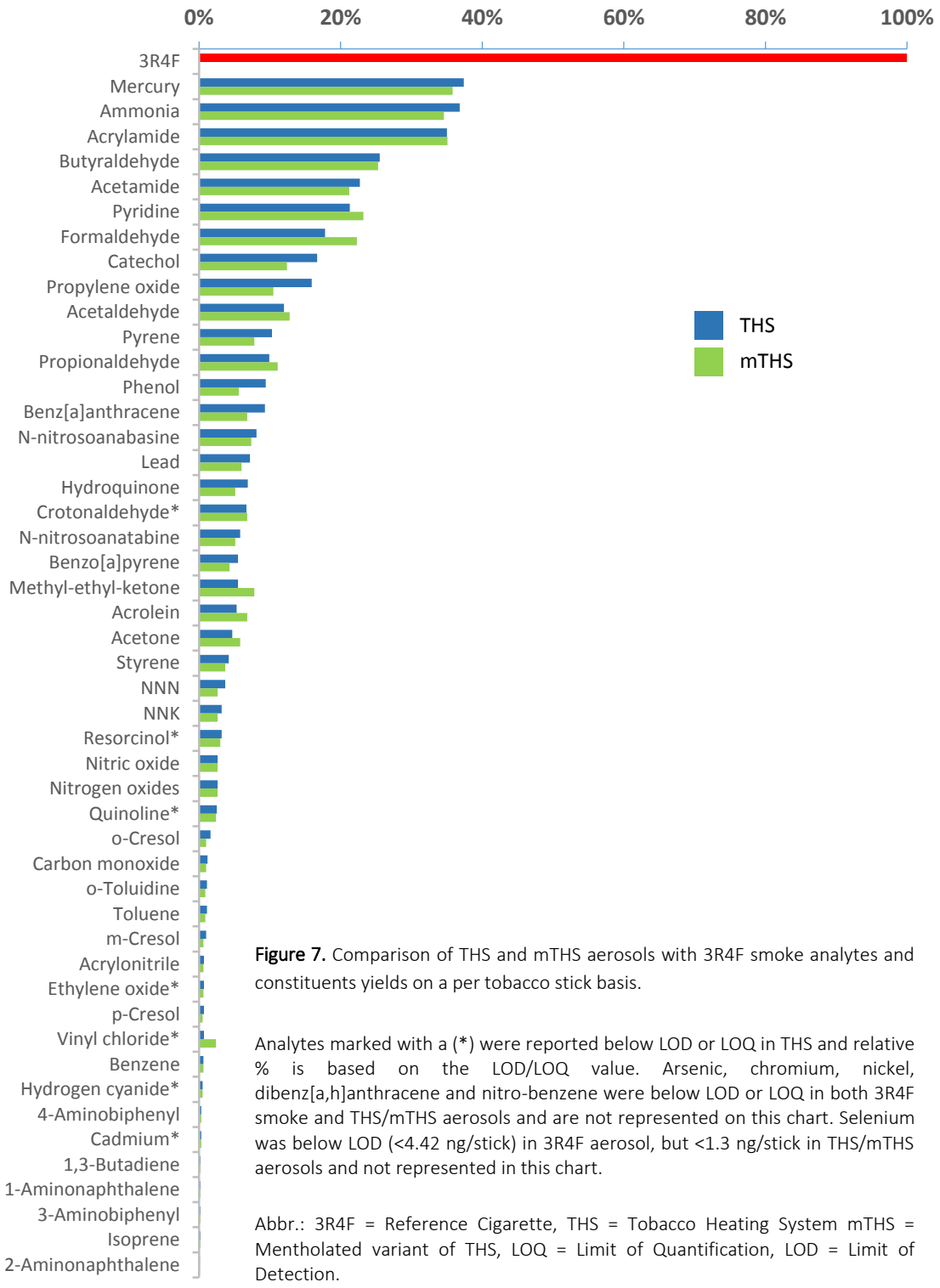

Figure 7. Comparison of THS and mTHS aerosols with 3R4F smoke analytes and constituents yields on a per tobacco stick basis.

Analytes marked with a (*) were reported below LOD or LOQ in THS and relative % is based on the LOD/LOQ value. Arsenic, chromium, nickel, dibenz[a,h]anthracene and nitro-benzene were below LOD or LOQ in both 3R4F smoke and THS/mTHS aerosols and are not represented on this chart. Selenium was below LOD (<4.42 ng/stick) in 3R4F aerosol, but <1.3 ng/stick in THS/mTHS aerosols and not represented in this chart.

Abbr.: 3R4F = Reference Cigarette, THS = Tobacco Heating System mTHS = Mentholated variant of THS, LOQ = Limit of Quantification, LOD = Limit of Detection.

Non-targeted semi-quantitative analysis of these other constituents of the THS aerosol revealed that 80% of their mass (4.07 mg) is explained by just 23 constituents – 5% of the total number of constituents (>400), most of which are naturally present in tobacco and not formed by heating.

THS aerosol was further analyzed to identify and characterize its HPHC profile in comparison with that of cigarette smoke (*Schaller 2016a, Jaccard 2017*). PMI chose to create a list of HPHCs that fulfilled the following criteria:

1. Priority toxicants in tobacco smoke as listed by regulatory bodies
2. Smoke constituent with established biomarkers of exposure (smoke/aerosol constituents or metabolites) and are not already included in criterion 1
3. HPHCs which are predominantly formed below 400°C and are not already included in criterion 1
4. HPHCs which are predominantly formed above 400°C, and are not already included in criterion 1
5. Product-specific analytes (such as glycerol and menthol)
6. Availability of well-established testing and analytical methods

In total, PMI assessed 58 analytes, which are listed in Table 2. These 58 analytes include 54 HPHCs, water, nicotine, Total Particulate Matter (TPM) and Nicotine Free Dry Particulate Matter (NFDPM). The quantification of the 58 analytes was performed in compliance with published international standards and practices. The levels of HPHCs found in the THS Regular (THS) and THS Menthol (mTHS) aerosols were compared with the levels in the 3R4F reference cigarette smoke. All aerosols and smoke samples were generated according to international standards, using the Health Canada Intense machine-smoking regimen (*Health Canada 2002*).

First, PMI measured the nicotine content in 3R4F smoke and in both THS and mTHS aerosols. While the nicotine content of 3R4F smoke is on average 1.8 mg/stick, the THS and mTHS aerosols contain respectively 1.3 mg nicotine/stick and 1.2 mg nicotine/stick.

Second, PMI compared the constituent yields of 3R4F with those of THS and mTHS to demonstrate that the THS aerosols contain significantly reduced levels of HPHCs. On a per-stick basis, the majority of measured constituents was reduced by 90% to 99% (Figure 7). The average reduction over all HPHCs (excluding nicotine) was greater than 90%. On an equivalent nicotine basis, the average reduction over all HPHCs (excluding nicotine) was greater than 89%. Some HPHCs were below the limit of quantification or detection. As shown in Table 2, each HPHC is associated with one or more health risks, the majority being known or probable human *carcinogens*. On a per-stick basis, these carcinogenic HPHCs were reduced on average by more than 90% in both THS and mTHS aerosols compared with 3R4F smoke. Among them, tobacco-specific nitrosamines (TSNAs) are of special interest as they are not generated by combustion but directly transferred from tobacco to smoke in cigarettes or to aerosol in the THS. For instance, NNN and NNK are reduced by >95% in both THS and mTHS aerosols compared with 3R4F smoke. While their yields are influenced by parameters such as tobacco blend composition and manufacturing processes, their markedly reduced yields in THS aerosol are most likely due to a reduced level of evaporation in the THS compared with 3R4F. Indeed, a recent study (*Forster 2015*) showed that the percentage TSNAs released from the tobacco into the aerosol at temperatures between 100°C to 200°C was very low (<10%) compared to the amounts available in the tobacco rod.

The HPHCs classified as *cardiovascular* toxicants or *reproductive or developmental* toxicants (except nicotine) were reduced by more than 90% in both THS and mTHS aerosols compared with 3R4F smoke. *Respiratory* toxicants were reduced by more than 87% in both THS and mTHS aerosols compared with 3R4F smoke.

PMI has demonstrated that both the THS and the mTHS generate aerosols with significantly reduced levels of HPHCs compared with cigarettes smoke (*Schaller 2016a, Jaccard 2017*); the differences between the THS and mTHS aerosols are ≤ 6% on a per-stick basis. This was a significant first step towards demonstrating that switching from cigarettes to either the THS or the mTHS reduces harm and the risk of smoking related diseases, particularly as many of these 58 analytes and constituents are linked to the most serious health effects of tobacco use.

In conclusion, there is convincing evidence that the THS yields on average >90% lower levels of HPHCs than cigarettes.

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III. Evidence that THS does not negatively impact indoor air chemistry.

PMI conducted studies to assess the effects of THS use on indoor air chemistry quality (IAQ) in comparison with cigarette smoking. Unlike the lit-end of a cigarette, which produces side stream smoke, which is the main source of environmental tobacco smoke, the THS does not produce side stream aerosol and only minimal amounts of emissions. Therefore, the THS has, by design, a substantially lower impact on IAQ than cigarettes.

The objective of the indoor air chemistry and quality studies was to assess the impact of the THS compared with cigarette emissions. For that purpose, four environmental conditions were simulated in a furnished controlled room (Figure 8), according to EN 15251:2007: "Hospitality", "Office", and two "Residential" settings briefly described in the legend of Figure 8 (*Goujon-Ginglinger 2016, Mitova 2016*).

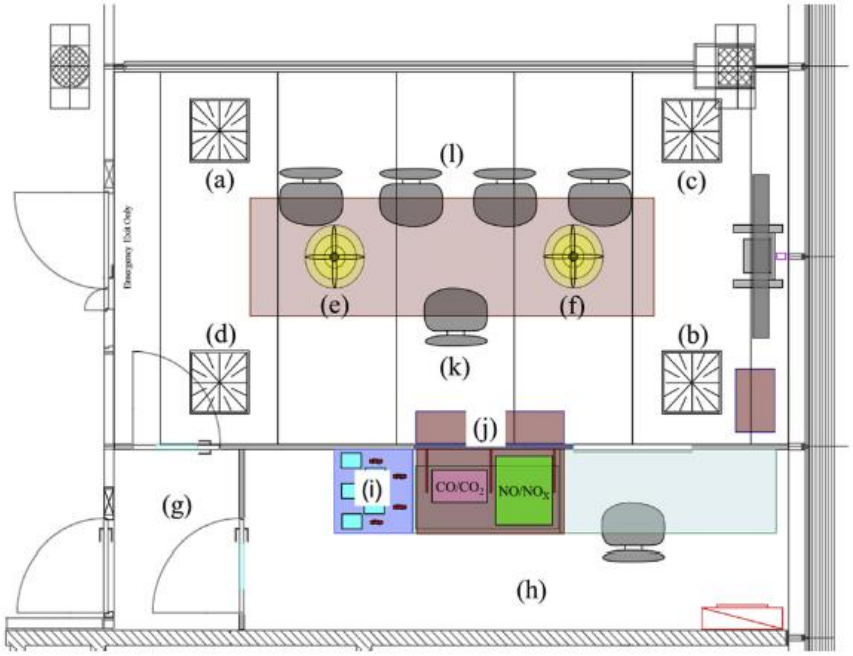

Figure 8: Layout of the environmentally controlled room and adjacent technical room. Air inlet ducts: a and b; air outlet ducts: c and d; electrical fans: e and f; air lock: g; technical room: h; membrane sampling pumps: i; sampling traps: j; PMI staff representative chair: k; volunteer panelist chairs: l.

Environmental conditions: "Hospitality": 7.68 air changes/h, 4.8 m^2/person; "Office": 2.16 air changes/h, 8 m^2/person; "Residential I": 1.68 air changes/h, 8 m^2/person; "Residential II": 1.20 air changes/h, 8 m^2/person. The size of the room was 24.1 m^2 and 72.3 m^3.

The impact of the THS on IAQ was evaluated, using a panel of THS users. It was compared with that of smoking a lit-end cigarette (Marlboro Gold) (by a panel of smokers) under identical experimental conditions. The concentrations of eighteen indoor air constituents (respirable suspended particles (RSP) <2.5 µm in diameter), ultraviolet particulate matter (UVPM), fluorescent particulate matter (FPM), solanesol, 3-ethenylpyridine, nicotine, 1,3-butadiene, acrylonitrile, benzene, isoprene, toluene, acetaldehyde, acrolein, crotonaldehyde, formaldehyde, carbon monoxide, nitrogen oxide, and combined oxides of nitrogen) were measured.

All testing methods were ISO 17025 accredited. The background concentrations of all constituents were determined when the panelists were present in the environmentally controlled room under equivalent conditions, but did not smoke or use THS. The experimental details are described in *Mitova 2016*.

The results of these measurements are summarized in Figure 9 (*Goujon-Ginglinger 2016, Mitova 2016*). When the THS is used under any of the four simulated conditions, the concentrations of most studied analytes did not exceed their respective background concentrations. Only acetaldehyde and nicotine concentrations were increased above background concentrations under all simulated environmental conditions, but reaching a maximum of 5.09µg/m^3 and 1.81µg/m^3 respectively under "Residential II" conditions. This is most likely due to the aerosol exhaled by the panelists. In contrast, cigarette smoking resulted in far greater increases in acetaldehyde and nicotine concentrations reaching a maximum of

83.80µg/m³ and 29.10µg/m³ respectively under identical conditions. Furthermore, cigarette smoking resulted in a marked increase of all other measured indoor air constituents under all simulated environmental conditions. The maximum acetaldehyde concentration during use of THS under "Residential II" conditions was well below the minimal risk level for chronic exposure (140 µg/m³) of the California Office of Environmental Health Hazard Assessment (*OEHHA 2008*) and the proposed exposure limit of 200 µg/m³ in the European Union (*Kotzias 2005*). Similarly, the maximum nicotine concentration during use of THS under "Residential II" conditions was well below the indicative occupational exposure limit of 500 µg/m³ in the European Union (*EU and Council 2006*) and the permissible exposure limit of 500 µg/m³ defined by the U.S. Occupational Safety and Health Administration (*Occupational Safety and Health Administration 1978*).

In conclusion, there is convincing evidence that THS use does not negatively impact indoor air quality.

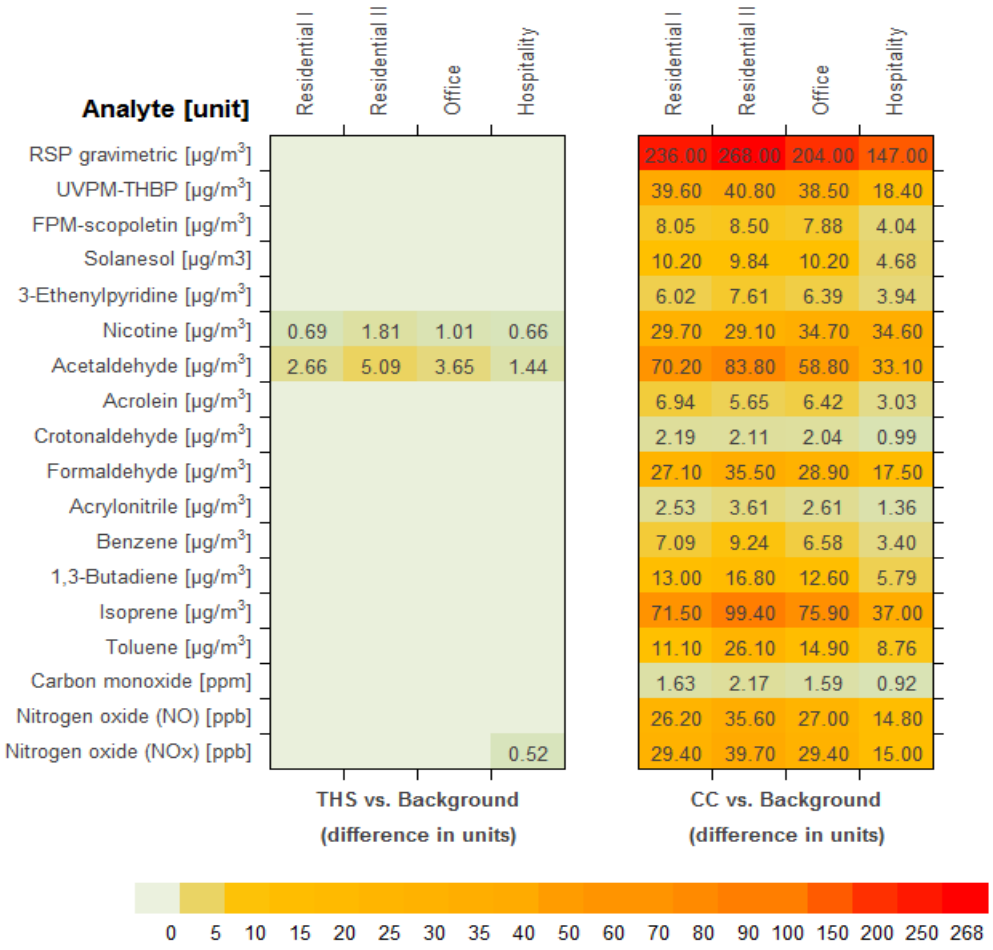

Figure 9: THS and cigarette indoor air chemistry assessment
Background levels are subtracted. Environmental conditions: "Hospitality": 7.68 air changes/h, 4 smokers consuming 2 items/h; "Office": 2.16 air changes/h, 2 smokers consuming 2 items/h; "Residential I": 1.68 air changes/h, 2 smokers consuming 1.5 items/h; "Residential II": 1.20 air changes/h, 2 smokers consuming 1.5 items/h. In each case, a non-smoking staff member was present in the room.
Abbr.: CC = Conventional Cigarette, FPM = Fluorescent Particulate Matter (FPM-scopoletin equivalent), RSP = Respirable Suspended Particles (RSP-gravimetric), THS = Tobacco Heating System, UVPM-THBP = Ultra-Violet Particulate Matter, VOCs = Volatile Organic Compounds. Items refer to Tobacco Sticks and Marlboro Gold cigarettes.

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Appendix B: Systems Toxicology & Verification

A short introduction

Philip Morris International R&D

I. A short introduction to systems toxicology

The pathway from smoke/HPHC exposure to disease manifestation can be depicted as a chain of causally linked key (biological) events known as an Adverse Outcome Pathway (AOP) (*Ankley 2010, Sturla 2014*) (Figure 1). This AOP begins with cigarette smoke/HPHC exposure that leads to molecular changes that cause the disruption of biological mechanisms, which in turn, cause cell/tissue changes. These changes then lead to physiological changes (e.g. organ/tissue damage), disease manifestations and population harm (e.g. mortality) (*Smith 2016*). Smoking cessation effectively removes the first causative event in the AOP, and hence leads to a reduction in molecular changes and its causally linked subsequent consequences at the cellular, tissue and organism level.

The impact of cigarette smoke on this chain of causally linked events can be quantified using both classical and advanced measurement methods such as analytical chemistry, –omics technologies (e.g. transcriptomics, proteomics, and metabolomics), cytology, histopathology, physiological measurement and eventually epidemiology. These data can be analyzed using statistical methods and advanced computational biology approaches (*Hoeng 2014a*). The outcome of these studies provides a detailed understanding of the effects caused by smoke exposure at each step along the AOP, and hence the mechanistic foundation for the assessment of RRPs. Conversely, the mechanistic effects of smoking cessation can also be evaluated in a biological system that is previously perturbed by chronic smoke exposure. Therefore, understanding the impact of chronic smoke exposure and how this impact changes upon smoking cessation ("gold standard for RRP assessment") provides the two mechanistic boundaries for the Risk Framework for RRP assessment (*Smith 2016*). Consequently, the effect of smoking cessation, and by extension that of a reduction in HPHC exposure, can be assessed on the causative chain of events that link exposure to disease risk and harm.

Based on these principles, the primary objective of PMI's systems toxicology assessment was to evaluate whether THS use reduces the risk of disease in laboratory models. The secondary objective was to evaluate whether switching from smoke to THS aerosol exposure leads to mechanistic changes that approach those caused by smoking cessation in laboratory models.

Towards this end, PMI conducted systems toxicology studies across several *in vitro* and *in vivo* systems, including animal models of disease. The *in vitro* and most *in vivo* studies provided comparative data about the effects of THS aerosol and cigarette smoke on disease-associated biological mechanisms. The *in vivo* study conducted in an animal model of disease enabled a comparative evaluation of the effects of THS aerosol, cigarette smoke, switching and cessation across the High Level Adverse Outcome Pathway (AOP) of cigarette smoking depicted in Figure 1.

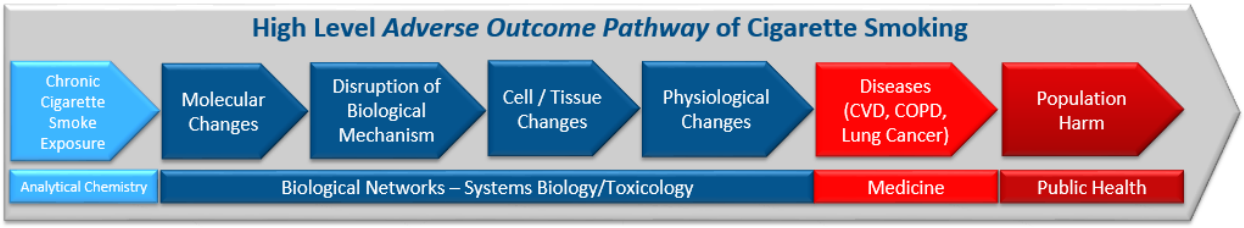

Figure 1. High Level Adverse Outcome Pathway (AOP) of Cigarette Smoking
Chronic exposure to cigarette smoke disrupts the biological homeostasis leading to adverse tissue changes and ultimately tobacco-related disease.

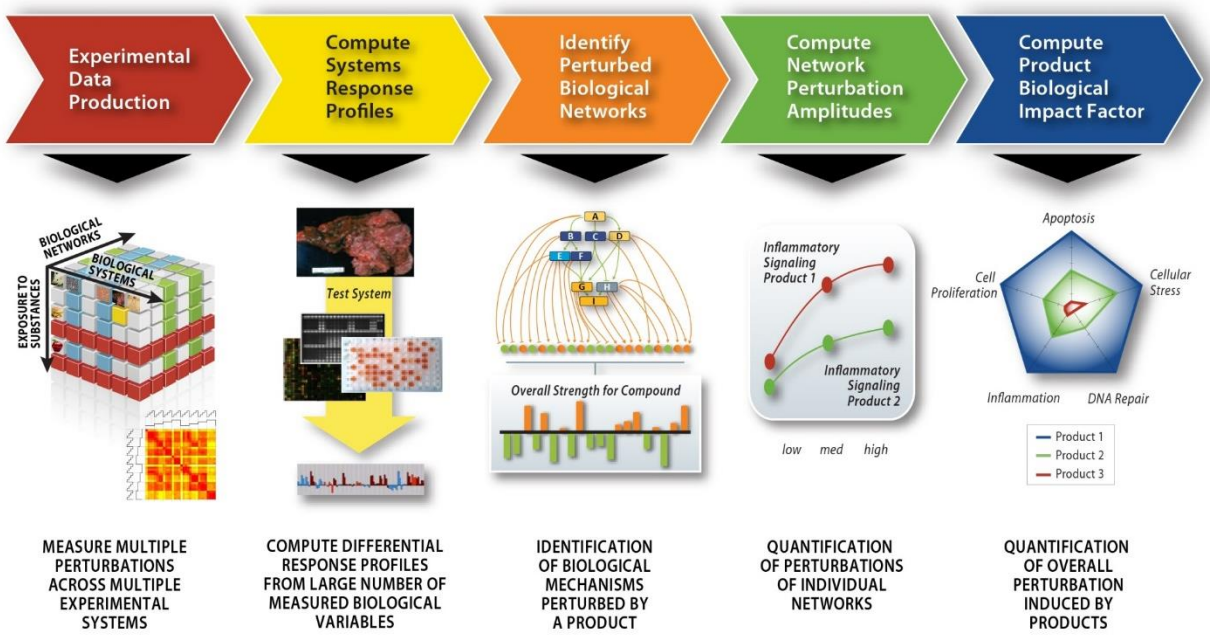

Figure 2. PMI Systems Toxicology Approach

Source: Hoeng 2012

Systems Toxicology is the integration of classical toxicology with quantitative analysis of large sets of molecular and functional measures of changes occurring across multiple levels of biological organization (*Sturla 2014*). Systems toxicology further informs on the observations/findings of traditional toxicological approaches by examining the changes in molecular pathways underlying adverse outcomes and disease causation (*Sturla 2014, Hoeng 2014a*). Systems toxicology allows for the identification of biological networks and molecular pathways that are affected by exposure to active substances. This provides a more comprehensive understanding of the exposure-induced molecular, cellular and tissue-level events and their causal relationships with adverse outcomes. This knowledge can then be applied to a detailed, mechanism-by-mechanism, assessment of product risk.

This approach is particularly useful when comparing the biological effects of RRPs with those of cigarettes since it can detect whether reductions in exposure translate into reduced perturbations of critical biological processes including Inflammation, Cell Stress, Cell Proliferation, Tissue Repair and Angiogenesis, and Cell Fate (*Boue 2015*). Many of these biological processes are strongly perturbed by cigarette smoke and have been causally linked to tobacco-related diseases such as cardiovascular, pulmonary and lung cancer. Sophisticated computational biology approaches allow for a quantification of the perturbation of each biological network (Network Perturbation Amplitudes) (*Martin 2012, Martin 2014*) and for

their aggregation into an overall relative biological impact factor (RBIF) (*Thomson 2013*). These approaches demonstrate how biological network perturbations are related to disease causation. A substantial reduction in perturbation of all relevant networks provides a solid mechanistic foundation for stating that an RRP is associated with lower risks of tobacco-related diseases.

PMI has applied this approach (Figure 2) (*Hoeng 2012, Hoeng 2014b, Sturla 2014*) across a variety of *in vivo* and *in vitro* studies to compare the impact of THS aerosol with that of cigarette smoke exposure and, when appropriate, to that of cessation (*in vivo* study in mouse model of disease). The results of several studies have been published in the peer-reviewed literature and are summarized in the executive summary.

II. A short introduction to verification

The assessment of an RRP is the responsibility of its manufacturer and aims at demonstrating that the RRP reduces the risk of harm compared to cigarettes. It is however essential to independently verify (at least some of) the key study results produced by the manufacturer to build confidence that the RRP is indeed a reduced risk alternative to cigarettes. From the manufacturer's perspective, there are both passive and proactive approaches to this verification.

Passive approaches include i) the conduct of independently funded studies without interaction with the manufacturer and ii) the in-depth review and inspection of the manufacturer's R&D and manufacturing processes, study documentations, data and premises by regulatory agencies upon regulatory submissions. For instance, the US FDA will conduct such an inspection following the submission of PMI's Modified Risk Tobacco Product Application in December 2016.

To complement these passive approaches, PMI has developed a strategy to build confidence in its scientific methods and results by taking several proactive steps:

1. Periodical inspection and renewal of ISO 17025 and GLP accreditations by national authorities.
2. Publish both methods and results in the peer-reviewed scientific literature. Scientific peer-review is a crucial component of quality control in science and is managed by the editorial office of scientific journals. Our publications describing both methods and study results have undergone critical review before publication. The list of articles describing the results of PMI's THS assessment studies is provided in Appendix C, section I. For a selection of published methods see Appendix C, section II.
3. Conduct crowdsourced verification of methods and study results through the sbvIMPROVER platform. This approach aims to provide a measure of quality control of industrial research and development by verifying methods and results (*Meyer 2011*). While this approach was first developed to verify methods and tools in systems biology, it was later extended to conduct anonymous in-depth reviews of studies through a double-blind process established by SciPinion. PMI has verified its non-clinical systems toxicology and PK/PD clinical studies through this process, while PMI's reduced exposure clinical studies will be verified in the coming months.
4. Proactively share the data from assessment studies to enable data analysis by independent third-parties through the INTERVALS platform (*Boué 2017*). This platform prototype was built to share results from *in vivo* inhalation studies and *in vitro* studies conducted by PMI. The platform will be later enhanced to share data from PMI's clinical studies. The Web-based portal allows users to browse the data by study or mechanism (e.g., inflammation, oxidative stress) and obtain information relevant to study design, methods, and the most important results.
5. Promote and support Investigator Initiated Studies (IIS) that independently advance scientific/medical knowledge or verify PMI science for our RRPs. This global program is open to researchers who have the relevant expertise and scientific credentials to conduct the proposed study, complying with local regulations and who are interested in receiving support for conducting their own research.

PMI is confident that this five-step strategy, combined with the two passive approaches, will verify that THS is an RRP and demonstrate the credibility of PMI's scientific approach to RRP assessment.

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Appendix C: Scientific References and Abstracts about THS 2.2 and Key Methods

Classified by assessment step

Philip Morris International R&D

Description of the assessment program

Figure 1: The RRP assessment program. Seven steps of assessment lead to five levels of evidence. Taken together, these levels of evidence provide the scientific evidence to demonstrate that a novel product significantly reduces harm and the risk of tobacco-related disease to individual smokers and benefits the health of the population as a whole, taking into account both smokers and nonsmokers. This program and the assessment framework are described in *Smith 2016*.

The scientific assessment of candidate RRPs follows a systematic and stepwise approach described in (*Smith 2016*). The program integrates seven steps, designed to provide five levels of evidence to address two objectives (Figure 1):

- The first objective is to demonstrate that a novel product significantly reduces harm and the risk of tobacco-related disease to individual smokers (Steps 1-5).
- The second objective is to show that the novel product, which meets the first objective, benefits the health of the population as a whole, taking into account both smokers and nonsmokers (Steps 6 & 7).

What follows is a list of references for the articles describing PMI's studies conducted with THS 2.2. Furthermore, the section is a list of references for the articles describing key methods developed and applied by PMI during the assessment of RRPs.

Smith M, Clark B, Lüdicke F, Schaller J-P, Vanscheeuwijck P, Hoeng J and Peitsch MC (2016) Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. Regul. Toxicol. Pharmacol. 81 Suppl 2:S17-S26. (PMID: 27450400).

Abstract: This publication introduces a series of eight other publications describing the non-clinical assessment and initial clinical study of a candidate modified risk tobacco product (MRTP) - the Tobacco Heating System 2.2 (THS 2.2). This paper presents background information on tobacco harm reduction, to complement the approaches aimed at increasing smoking cessation and reducing smoking initiation to reduce the morbidity and mortality caused by cigarette smoking. THS 2.2 heats tobacco without combustion, and the resulting formation of harmful and potentially harmful constituents (HPHC) is greatly reduced compared with cigarette smoke. Assessment of the THS 2.2 aerosol in vitro and in vivo reveals reduced toxicity and no new hazards. Additional mechanistic endpoints, measured as part of in vivo studies, confirmed reduced impact on smoking-related disease networks. The clinical study confirmed the reduced exposure to HPHCs in smokers switching to THS 2.2, and the associated transcriptomic study confirmed the utility of a gene expression signature, consisting of only 11 genes tested in the blood transcriptome of subjects enrolled in the clinical study, as a complementary measure of exposure response. The potential of THS 2.2 as an MRTP is demonstrated by the assessment and additional publications cited in this series.

I. THS 2.2 assessment studies

Aerosol Chemistry (Step 2)

Jaccard G, Tafin Djoko D, Moennikes O, Jeannet C, Kondylis A, Belushkin M (2017) Comparative assessment of HPHC yields in the Tobacco Heating System THS2.2 and commercial cigarettes. Regulatory Toxicology and Pharmacology, 90:1-8. (PMID: 28818540).

Abstract: There has been a sustained effort in recent years to develop products with the potential to present less risk compared with continued smoking as an alternative for adult smokers who would otherwise continue to smoke cigarettes. During the non-clinical assessment phase of such products, the chemical composition and toxicity of their aerosols are frequently compared to the chemical composition and toxicity of the smoke from a standard research cigarette – the 3R4F reference cigarette. In the present study, it is demonstrated that results of these analytical comparisons are similar when considering commercially available cigarette products worldwide. A market mean reduction of about 90% is observed on

average across a broad range of harmful and potentially harmful constituents (HPHC) measured in the aerosol of a candidate modified risk tobacco product, the Tobacco Heating System 2.2 (THS 2.2), compared against the levels of HPHC of cigarettes representative of selected markets; this mean reduction is well in line with the reduction observed against 3R4F smoke constituents in previous studies.

Mitova MI, Campelos PB, Goujon-Ginglinger CG, Maeder S, Mottier N, Rouget EG, Tharin M and Tricker AR (2016) Comparison of the impact of the Tobacco Heating System 2.2 and a cigarette on indoor air quality. Regul. Toxicol. Pharmacol. 80:91-101. (PMID: 27311683).

Abstract: The impact of the Tobacco Heating System 2.2 (THS 2.2) on indoor air quality was evaluated in an environmentally controlled room using ventilation conditions recommended for simulating "Office", "Residential" and "Hospitality" environments and was compared with smoking a lit-end cigarette (Marlboro Gold) under identical experimental conditions. The concentrations of eighteen indoor air constituents (respirable suspended particles (RSP) < 2.5 µm in diameter), ultraviolet particulate matter (UVPM), fluorescent particulate matter (FPM), solanesol, 3-ethenylpyridine, nicotine, 1,3-butadiene, acrylonitrile, benzene, isoprene, toluene, acetaldehyde, acrolein, crotonaldehyde, formaldehyde, carbon monoxide, nitrogen oxide, and combined oxides of nitrogen) were measured. In simulations evaluating THS 2.2, the concentrations of most studied analytes did not exceed the background concentrations determined when non-smoking panelists were present in the environmentally controlled room under equivalent conditions. Only acetaldehyde and nicotine concentrations were increased above background concentrations in the "Office" (3.65 and 1.10 µg/m(3)), "Residential" (5.09 and 1.81 µg/m(3)) and "Hospitality" (1.40 and 0.66 µg/m(3)) simulations, respectively. Smoking Marlboro Gold resulted in greater increases in the concentrations of acetaldehyde (58.8, 83.8 and 33.1 µg/m(3)) and nicotine (34.7, 29.1 and 34.6 µg/m(3)) as well as all other measured indoor air constituents in the "Office", "Residential" and "Hospitality" simulations, respectively.

Poget L, Campelos P, Jeannet C and Maeder S (2017) Development of models for the estimation of mouth level exposure to aerosol constituents from a heat-not-burn tobacco product using mouthpiece analysis. Beitr. Tabakforsch. Int. 27(5): 42-64. (Link).

Abstract: Philip Morris International has developed a heat-not-burn tobacco heating system (THS 2.2) that produces an aerosol without combustion. Adult smokers are anticipated to use the product with differing behaviors, such as puffing volume or puffing frequency, therefore it was important to find an easy way to study how users are exposed to the aerosol constituents. Thus, the intended outcome of this study was to propose and assess a simple approach for the estimation of THS users' exposure to harmful and potentially harmful constituents (HPHCs).

THS operates using tobacco sticks (HeatSticks) that include a mouthpiece and a tobacco plug which, when heated, generates an aerosol. The analysis of nicotine retained in the mouthpiece of the HeatSticks during use was identified as a potential approach to estimate users' mouth level exposure (MLE) to HPHCs. Consequently, the following study was conducted with the objectives 1.) to assess the correlation between the quantity of retained nicotine in the mouthpiece (Nicotine MP) of the HeatSticks and the nicotine delivered in the aerosol of machine-smoked products, 2.) to verify the practical range for Nicotine MP based on the analysis of used HeatSticks left by THS users, and 3.) to develop models describing the relationship between Nicotine MP and specific aerosol constituents measured in the aerosol of machine-smoked products. The regular non-mentholated HeatSticks variant was machine-smoked under various smoking regimens to cover the range of anticipated human puffing behaviors. The suitability of this practical range of machine-smoking conditions was verified by collecting used HeatSticks from two different trials conducted with THS users. The determined Nicotine MP distribution indicated that the machine smoked regimens encompassed the range observed for users.

Multiple Linear Regression (MLR) combined with a stepwise approach was used for selecting models describing the relationship between Nicotine MP and specific aerosol constituents. The stepwise approach interactively explores which amongst various tested predictors provides a good fit. The developed models showed good adjusted coefficients of determination (i.e., R2 adj. $ 0.75) for 28 out of the 43 investigated HPHCs.

Previously published studies showed that actual MLE can be estimated from cigarette filter analysis. This study demonstrated that the analysis of nicotine in THS mouthpiece (filter section) corresponded to an estimation of the upper limits of MLE, in line with maximum possible usage conditions.

Pratte P, Cosandey S and Goujon-Ginglinger C (2016) Investigation of solid particles in the mainstream aerosol of the Tobacco Heating System THS 2.2 and mainstream smoke of a 3R4F reference cigarette. Hum. Exp. Toxicol. E-pub ahead of print. (PMID: 27932538).

Abstract: Combustion of biomass produces solid carbon particles, whereas their generation is highly unlikely when a biomass is heated instead of being burnt. For instance, in the Tobacco Heating System (THS 2.2), the tobacco is heated below 350°C and no combustion takes place. Consequently, at this relatively low temperature, released compounds should form an aerosol consisting of suspended liquid droplets via a homogeneous nucleation process. To verify this assumption, mainstream aerosol generated by the heat-not-burn product, THS 2.2, was assessed in comparison with mainstream smoke produced from the 3R4F reference cigarette for which solid particles are likely present. For this purpose, a methodology was developed based on the use of a commercial Dekati thermodenuder operating at 300°C coupled with a two-stage impactor to trap solid particles. If any particles were collected, they were subsequently analyzed by a scanning electron microscope and an electron dispersive X-ray. The setup was first assessed using glycerine-based aerosol as a model system. The removal efficiency of glycerin was determined to be 86 ± 2% using a Trust Science Innovation (TSI) scanning mobility particle sizer, meaning that quantification of solid particles can be achieved as long as their fraction is larger than 14% in number. From experiments conducted using the 3R4F reference cigarette, the methodology showed that approximately 80% in number of the total particulate matter was neither evaporated nor removed by the thermodenuder. This 80% in number was attributed to the presence of solid particles and/or low volatile liquid droplets. The particles collected on the impactor were mainly carbon based. Oxygen, potassium, and chloride traces were also noted. In comparison, solid particles were not detected in the aerosol of THS 2.2 after passing through the

thermodenuder operated at 300°C. This result is consistent with the fact that no combustion process takes place in THS 2.2 and no formation and subsequent transfer of solid carbon particles is expected to occur in the mainstream aerosol.

Schaller J-P, Keller D, Poget L, Pratte P, Kaelin E, McHugh D, Cudazzo G, Smart D, Tricker AR, Gautier L, Yerly M, Pires RR, Le Bouhellec S, Ghosh D, Hofer I, Garcia E, Vanscheeuwijck P and Maeder S (2016a) Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47. (PMID: 27720919).

Abstract: The chemical composition, in vitro genotoxicity, and cytotoxicity of the mainstream aerosol from the Tobacco Heating System 2.2 (THS 2.2) were compared with those of the mainstream smoke from the 3R4F reference cigarette. In contrast to the 3R4F, the tobacco plug in the THS 2.2 is not burnt. The low operating temperature of THS 2.2 caused distinct shifts in the aerosol composition compared with 3R4F. This resulted in a reduction of more than 90% for the majority of the analyzed harmful and potentially harmful constituents (HPHCs), while the mass median aerodynamic diameter of the aerosol remained similar. A reduction of about 90% was also observed when comparing the cytotoxicity determined by the neutral red uptake assay and the mutagenic potency in the mouse lymphoma assay. The THS 2.2 aerosol was not mutagenic in the Ames assay. The chemical composition of the THS 2.2 aerosol was also evaluated under extreme climatic and puffing conditions. When generating the THS 2.2 aerosol under "desert" or "tropical" conditions, the generation of HPHCs was not significantly modified. When using puffing regimens that were more intense than the standard Health Canada Intense (HCI) machine-smoking conditions, the HPHC yields remained lower than when smoking the 3R4F reference cigarette with the HCI regimen.

Schaller J-P, Pijnenburg JPM, Ajithkumar A and Tricker AR (2016b) Evaluation of the Tobacco Heating System 2.2. Part 3: Influence of the tobacco blend on the formation of harmful and potentially harmful constituents of the Tobacco Heating Systems 2.2 aerosol. Regul. Toxicol. Pharmacol. 81 Suppl 2:S48-S58. (PMID: 27793747).

Abstract: The Tobacco Heating System (THS 2.2), which uses "heat-not-burn" technology, generates an aerosol from tobacco heated to a lower temperature than occurs when smoking a combustible cigarette. The concentrations of harmful and potentially harmful constituents (HPHCs) are significantly lower in THS 2.2 mainstream aerosol than in smoke produced by combustible cigarettes. Different tobacco types and 43 tobacco blends were investigated to determine how the blend impacted the overall reductions of HPHCs in the THS 2.2 mainstream aerosol. The blend composition had minimal effects on the yields of most HPHCs in the aerosol. Blends containing high proportions of nitrogen-rich tobacco, e.g., air-cured, and some Oriental tobaccos, produced higher acetamide, acrylamide, ammonia, and nitrogen oxide yields than did other blends. Most HPHCs were found to be released mainly through the distillation of HPHCs present in the tobacco plug or after being produced in simple thermal reactions. HPHC concentrations in the THS 2.2 aerosol may therefore be further minimized by limiting the use of flue- and fire-cured tobaccos which may be contaminated by HPHCs during the curing process and carefully selecting nitrogen rich tobaccos with low concentrations of endogenous HPHCs for use in the tobacco plug blend.

Pre-clinical Toxicology (Step 3)

Oviedo A, Lebrun S, Kogel U, Ho J, Tan WT, Titz B, Leroy P, Vuillaume G, Bera M, Martin FT, Rodrigo G, Esposito M, Dempsey R, Ivanov NV, Hoeng J, Peitsch MC and Vanscheeuwijck P (2016) Evaluation of the Tobacco Heating System 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with cigarette smoke. Regul. Toxicol. Pharmacol. 81 Suppl 2:S93-S122. (PMID: 27818348).

Abstract: The toxicity of a mentholated version of the Tobacco Heating System (THS 2.2M), a candidate modified risk tobacco product (MRTP), was characterized in a 90-day OECD inhalation study. Differential gene and protein expression analysis of nasal epithelium and lung tissue was also performed to record exposure effects at the molecular level. Rats were exposed to filtered air (sham), to THS 2.2M (at 15, 23 and 50 µg nicotine/l), to two mentholated reference cigarettes (MRC) (at 23 µg nicotine/l), or to the 3R4F reference cigarette (at 23 µg nicotine/l). MRCs were designed to meet 3R4F specifications. Test atmosphere analyses demonstrated that aldehydes were reduced by 75%-90% and carbon monoxide by 98% in THS 2.2M aerosol compared with MRC smoke; aerosol uptake was confirmed by carboxyhemoglobin and menthol concentrations in blood, and by the quantities of urinary nicotine metabolites. Systemic toxicity and alterations in the respiratory tract were significantly lower in THS 2.2M-exposed rats compared with MRC and 3R4F. Pulmonary inflammation and the magnitude of the changes in gene and protein expression were also dramatically lower after THS 2.2M exposure compared with MRCs and 3R4F. No menthol-related effects were observed after MRC mainstream smoke-exposure compared with 3R4F.

Schaller J-P, Keller D, Poget L, Pratte P, Kaelin E, McHugh D, Cudazzo G, Smart D, Tricker AR, Gautier L, Yerly M, Pires RR, Le Bouhellec S, Ghosh D, Hofer I, Garcia E, Vanscheeuwijck P and Maeder S (2016) Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47. (PMID: 27720919).

Abstract: See under *Step 2 of the assessment program.*

Wong E, Kogel U, Veljkovic E, Martin F, Xiang Y, Boue S, Vuillaume G, Leroy P, Guedj E, Rodrigo G, Ivanov NV, Hoeng J, Peitsch MC and Vanscheeuwijck P (2016) Evaluation of the Tobacco Heating System 2.2. Part 4: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects compared with cigarettes smoke. Regul. Toxicol. Pharmacol. 81 Suppl 2:S59-S81. (PMID: 27793746).

Abstract: The objective of the study was to characterize the toxicity from sub-chronic inhalation of test atmospheres from the candidate modified risk tobacco product (MRTP), Tobacco Heating System version 2.2 (THS 2.2), and to compare it with that of the 3R4F reference cigarette. A 90-day nose-only inhalation study on Sprague-Dawley rats was performed, combining classical and

systems toxicology approaches. Reduction in respiratory minute volume, degree of lung inflammation, and histopathological findings in the respiratory tract organs were significantly less pronounced in THS 2.2-exposed groups compared with 3R4F-exposed groups. Transcriptomics data obtained from nasal epithelium and lung parenchyma showed concentration-dependent differential gene expression following 3R4F exposure that was less pronounced in the THS 2.2-exposed groups. Molecular network analysis showed that inflammatory processes were the most affected by 3R4F, while the extent of THS 2.2 impact was much lower. Most other toxicological endpoints evaluated did not show exposure-related effects. Where findings were observed, the effects were similar in 3R4F- and THS 2.2-exposed animals. In summary, toxicological changes observed in the respiratory tract organs of THS 2.2 aerosol-exposed rats were much less pronounced than in 3R4F-exposed rats while other toxicological endpoints either showed no exposure-related effects or were comparable to what was observed in the 3R4F-exposed rats.

Systems Toxicology (Step 4)

In vitro studies relevant to the vascular system.

Poussin C, Laurent A, Peitsch MC, Hoeng J and De Leon H (2016) Systems toxicology-based assessment of the candidate modified-risk tobacco product THS 2.2 for the adhesion of monocytic cells to human coronary arterial endothelial cells. Toxicology 339:73-86. (PMID: 26655683).

Abstract: Alterations of endothelial adhesive properties by cigarette smoke (CS) can progressively favor the development of atherosclerosis which may cause cardiovascular disorders. Modified risk tobacco products (MRTPs) are tobacco products developed to reduce smoking-related risks. A systems biology/toxicology approach combined with a functional in vitro adhesion assay was used to assess the impact of a candidate heat-not-burn technology-based MRTP, Tobacco Heating System (THS) 2.2, on the adhesion of monocytic cells to human coronary arterial endothelial cells (HCAECs) compared with a reference cigarette (3R4F). HCAECs were treated for 4h with conditioned media of human monocytic Mono Mac 6 (MM6) cells preincubated with low or high concentrations of aqueous extracts from THS 2.2 aerosol or 3R4F smoke for 2h (indirect treatment), unconditioned media (direct treatment), or fresh aqueous aerosol/smoke extracts (fresh direct treatment). Functional and molecular investigations revealed that aqueous 3R4F smoke extract promoted the adhesion of MM6 cells to HCAECs via distinct direct and indirect concentration-dependent mechanisms. Using the same approach, we identified significantly reduced effects of aqueous THS 2.2 aerosol extract on MM6 cell-HCAEC adhesion, and reduced molecular changes in endothelial and monocytic cells. Ten- and 20-fold increased concentrations of aqueous THS 2.2 aerosol extract were necessary to elicit similar effects to those measured with 3R4F in both fresh direct and indirect exposure modalities, respectively. Our systems toxicology study demonstrated reduced effects of an aqueous aerosol extract from the candidate MRTP, THS 2.2, using the adhesion of monocytic cells to human coronary endothelial cells as a surrogate pathophysiologically relevant event in atherogenesis.

van der Toorn M, Frentzel S, De Leon H, Goedertier D, Peitsch MC and Hoeng J (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).

Abstract: Reduction of harmful constituents by heating rather than combusting tobacco is a promising new approach to reduce harmful effects associated with cigarette smoking. We investigated the effect from a new candidate modified risk tobacco product, the tobacco heating system (THS) 2.2, on the migratory behavior of monocytes in comparison with combustible 3R4F reference cigarettes. The monocytic cell line (THP-1) and human coronary arterial endothelial cells (HCAECs) were used to analyze chemotaxis and transendothelial migration (TEM). To assess the influence of aerosol extract from THS 2.2 and smoke extract from 3R4F on toxicity and inflammation, flow cytometry and ELISA assays were performed. The results show that treatment of THP-1 cells with extract from 3R4F or THS 2.2 induced concentration-dependent increases in cytotoxicity and inflammation. The inhibitory effects of THS 2.2 extract for chemotaxis and TEM were ~18 times less effective compared to 3R4F extract. Furthermore, extract from 3R4F or THS 2.2 induced concentration-dependent decreases in the integrity of HCAEC monolayer. For all examined endpoints, the extract from 3R4F showed more than one order of magnitude stronger effects than that from THS 2.2 extract. These data indicate the potential of a heat not burn tobacco product to reduce the risk for cardiovascular disease compared to combustible cigarettes.

In vitro studies relevant to the respiratory tract.

Gonzalez Suarez I, Martin F, Marescotti D, Guedj E, Acali S, Johne S, Dulize R, Baumer K, Peric D, Goedertier D, Frentzel S, Ivanov N, Mathis C, Hoeng J and Peitsch MC (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).

Abstract: Cigarette smoke increases the risk for respiratory and other diseases. Although smoking prevalence has declined over the years, millions of adults choose to continue to smoke. Modified risk tobacco products (MRTPs) are potentially valuable tools for adult smokers that are unwilling to quit their habit. Here, we investigated the biological impact of a candidate MRTP, the tobacco-heating system (THS) 2.2, compared to that of the 3R4F reference cigarette in normal primary human bronchial epithelial cells. Chemical characterization of the THS 2.2 aerosol showed reduced levels of harmful constituents compared to those of a combustible cigarette. Multiparametric indicators of cellular toxicity were measured via real-time cellular analysis and high-content screening. The study was complemented by a whole transcriptome analysis, followed by computational approaches to identify and quantify perturbed molecular pathways. Exposure of cells to 3R4F cigarette smoke resulted in a dose-dependent response in most toxicity end points. Moreover, we found a significant level of perturbation in multiple biological pathways, particularly in those related to cellular stress. By contrast, exposure to THS 2.2 resulted in an overall lower biological impact. At 3R4F doses, no toxic effects were observed. A toxic response was observed for THS 2.2 in some functional end points, but the responses occurred at doses between 3 and 15 times higher than those of 3R4F. The level of biological network perturbation was also significantly reduced following THS 2.2 aerosol exposure compared to

that of 3R4F cigarette smoke. Taken together, the data suggest that THS 2.2 aerosol is less toxic than combustible cigarette smoke and thus may have the potential to reduce the risk for smoke-related diseases.

Iskandar AR, Mathis C, Martin F, Leroy P, Sewer A, Majeed S, Kühn D, Trivedi K, Grandolfo D, Cabanski M, Guedj E, Merg C, Frentzel S, Ivanov NV, Peitsch MC and Hoeng J (2017a) 3-D nasal cultures: Systems toxicological assessment of a candidate Modified-Risk Tobacco Product. Altex 34:23-48. (PMID: 27388676).

Abstract: In vitro toxicology approaches have evolved from a focus on molecular changes within a cell to understanding of toxicity-related mechanisms in systems that can mimic the in vivo environment. The recent development of three dimensional (3-D) organotypic nasal epithelial culture models offers a physiologically robust system for studying the effects of exposure through inhalation. Exposure to cigarette smoke (CS) is associated with nasal inflammation; thus, the nasal epithelium is relevant for evaluating the pathophysiological impact of CS exposure. The present study investigated further the application of in vitro human 3-D nasal epithelial culture models for toxicological assessment of inhalation exposure. Aligned with 3Rs strategy, this study aimed to explore the relevance of a human 3-D nasal culture model to assess the toxicological impact of aerosols generated from a candidate modified risk tobacco product (cMRTP), the Tobacco Heating System (THS) 2.2, as compared with smoke generated from reference cigarette 3R4F. A series of experimental repetitions, where multiple concentrations of THS 2.2 aerosol and 3R4F smoke were applied, were conducted to obtain reproducible measurements to understand the cellular/molecular changes that occur following exposure. In agreement with "Toxicity Testing in the 21st Century - a Vision and a Strategy", this study implemented a systems toxicology approach and found that for all tested concentrations the impact of 3R4F smoke was substantially greater than that of THS 2.2 aerosol in terms of cytotoxicity levels, alterations in tissue morphology, secretion of pro-inflammatory mediators, impaired ciliary function, and increased perturbed transcriptomes and miRNA expression profiles.

Iskandar A, Mathis C, Schlage WK, Frentzel S, Leroy P, Xiang Y, Sewer A, Majeed S, Ortega Torres L, Johne S, Guedj E, Trivedi T, Kratzer G, Merg C, Elamin A, Martin F, Ivanov NV, Peitsch MC and Hoeng J (2017b) A systems toxicology approach for comparative assessment: Biological impact of an aerosol from a candidate modified-risk tobacco product and cigarette smoke on human organotypic bronchial epithelial cultures. Toxicol. In Vitro 39:29-51. (PMID: 27865774).

Abstract: This study reports a comparative assessment of the biological impact of a heated tobacco aerosol from the tobacco heating system (THS) 2.2 and smoke from a combustible 3R4F cigarette. Human organotypic bronchial epithelial cultures were exposed to an aerosol from THS 2.2 (a candidate modified-risk tobacco product) or 3R4F smoke at similar nicotine concentrations. A systems toxicology approach was applied to enable a comprehensive exposure impact assessment. Culture histology, cytotoxicity, secreted pro-inflammatory mediators, ciliary beating, and genome-wide mRNA/miRNA profiles were assessed at various time points post-exposure. Series of experimental repetitions were conducted to increase the robustness of the assessment. At similar nicotine concentrations, THS 2.2 aerosol elicited lower cytotoxicity compared with 3R4F smoke. No morphological change was observed following exposure to THS 2.2 aerosol, even at nicotine concentration three times that of 3R4F smoke. Lower levels of secreted mediators and fewer miRNA alterations were observed following exposure to THS 2.2 aerosol than following 3R4F smoke. Based on the computational analysis of the gene expression changes, 3R4F (0.13 mg nicotine/L) elicited the highest biological impact (100%) in the context of Cell Fate, Cell Proliferation, Cell Stress, and Inflammatory Network Models at 4 h post-exposure. Whereas, the corresponding impact of THS 2.2 (0.14 mg nicotine/L) was 7.6%.

Iskandar AR, Titz B, Sewer A, Leroy P, Schneider T, Zanetti F, Mathis C, Elamin A, Frentzel S, Schlage WK, Martin F, Peitsch MC and Hoeng J (2017c) Systems toxicology meta-analysis of in vitro assessment studies: Biological impact of a Modified-Risk Tobacco Product aerosol compared with cigarette smoke on human organotypic cultures of the respiratory tract. Toxicol. Res. (Camb.) *Accepted for publication*. doi: http://dx.doi.org/10.1039/C7TX00047B.

Abstract: Systems biology combines comprehensive molecular analyses with mathematical modeling to understand the characteristics of a biological system as a whole. Leveraging a similar approach, Systems Toxicology aims to decipher complex biological responses following exposures. This work reports a Systems Toxicology meta-analysis in the context of in vitro assessment of a modified-risk tobacco product (MRTP) using three human organotypic cultures of the respiratory tract (buccal, bronchial, and nasal). Complementing a series of functional measures, a causal network enrichment analysis of transcriptomics data was used to compare quantitatively the biological impact of aerosol from the Tobacco Heating System (THS) 2.2, a candidate MRTP, with 3R4F cigarette smoke (CS) at similar nicotine concentrations. Greater toxicity was observed in all cultures following exposure to 3R4F CS compared with THS 2.2 aerosol. Because of their morphological differences, a lesser exposure impact was observed in the buccal (stratified epithelium) compared with the bronchial and nasal (pseudostratified epithelium). However, the causal network enrichment approach supported a similar mechanistic impact of 3R4F CS across the three cultures, including the impact on xenobiotic, oxidative stress, and inflammatory responses. At comparable nicotine concentrations, THS 2.2 aerosol elicited reduced and more transient effects on these processes. To demonstrate the benefit of additional data modalities, we employed a newly established targeted mass-spectrometry marker panel to further confirm the reduced cellular stress responses elicited upon THS 2.2 aerosol compared with 3R4F CS in the nasal culture. Overall, this work demonstrates the applicability and robustness of the Systems Toxicology approach for an in vitro inhalation toxicity assessment.

Zanetti F, Sewer A, Mathis C, Iskandar A, Kostadinova R, Schlage WK, Leroy P, Majeed S, Guedj E, Trivedi K, Elamin A, Merg C, Ivanov NV, Frentzel S, Peitsch MC and Hoeng J (2016) Systems toxicology assessment of the biological impact of a candidate Modified Risk Tobacco Product on human organotypic oral epithelial cultures. Chem. Res. Toxicol. 29:1252-1269. (PMID: 27404394). *Based on recommendations by the journals' Editors, this*

article has also been selected to be featured in ACS Editors' Choice in addition to being published in Chemical Research in Toxicology.

Abstract: Cigarette smoke (CS) has been reported to increase predisposition to oral cancer and is also recognized as a risk factor for many conditions including periodontal diseases, gingivitis, and other benign mucosal disorders. Smoking cessation remains the most effective approach for minimizing the risk of smoking-related diseases. However, reduction of harmful constituents by heating rather than combusting tobacco, without modifying the amount of nicotine, is a promising new paradigm in harm reduction. In this study, we compared effects of exposure to aerosol derived from a candidate modified risk tobacco product, the tobacco heating system (THS) 2.2, with those of CS generated from the 3R4F reference cigarette. Human organotypic oral epithelial tissue cultures (EpiOral, MatTek Corporation) were exposed for 28 min to 3R4F CS or THS 2.2 aerosol, both diluted with air to comparable nicotine concentrations (0.32 or 0.51 mg nicotine/L aerosol/CS for 3R4F and 0.31 or 0.46 mg/L for THS 2.2). We also tested one higher concentration (1.09 mg/L) of THS 2.2. A systems toxicology approach was employed combining cellular assays (i.e., cytotoxicity and cytochrome P450 activity assays), comprehensive molecular investigations of the buccal epithelial transcriptome (mRNA and miRNA) by means of computational network biology, measurements of secreted proinflammatory markers, and histopathological analysis. We observed that the impact of 3R4F CS was greater than THS 2.2 aerosol in terms of cytotoxicity, morphological tissue alterations, and secretion of inflammatory mediators. Analysis of the transcriptomic changes in the exposed oral cultures revealed significant perturbations in various network models such as apoptosis, necroptosis, senescence, xenobiotic metabolism, oxidative stress, and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) signaling. The stress responses following THS 2.2 aerosol exposure were markedly decreased, and the exposed cultures recovered more completely compared with those exposed to 3R4F CS.

Zanetti F, Titz B, Sewer A, Lo Sasso G, Scotti E, Schlage WK, Mathis C, Leroy P, Majeed S, Ortega-Torres L, Keppler BR, Elamin A, Trivedi K, Guedj E, Martin F, Frentzel S, Ivanov NV, Peitsch MC and Hoeng J (2017) Comparative systems toxicology analysis of cigarette smoke and aerosol from a candidate modified risk tobacco product in organotypic human gingival epithelial cultures: a 3-day repeated exposure study. Food Chem. Toxicol. 101:15-35. (PMID: 28025120).

Abstract: Smoking is one of the major lifestyle-related risk factors for periodontal diseases. Modified risk tobacco products (MRTP) offer a promising alternative in the harm reduction strategy for adult smokers unable to quit. Using a systems toxicology approach, we investigated and compared the exposure effects of a reference cigarette (3R4F) and a heat-not-burn technology-based candidate MRTP, the Tobacco Heating System (THS) 2.2. Human gingival epithelial organotypic cultures were repeatedly exposed (3 days) for 28 min at two matching concentrations of cigarette smoke (CS) or THS 2.2 aerosol. Results showed only minor histopathological alterations and minimal cytotoxicity upon THS 2.2 aerosol exposure compared to CS (1% for THS 2.2 aerosol vs. 30% for CS, at the high concentration). Among the 14 proinflammatory mediators analyzed, only 5 exhibited significant alterations with THS 2.2 exposure compared with 11 upon CS exposure. Transcriptomic and metabolomic analysis indicated a general reduction of the impact in THS 2.2 aerosol-exposed samples with respect to CS (~79% lower biological impact for the high THS 2.2 aerosol concentration compared to CS, and 13 metabolites significantly perturbed for THS 2.2 vs. 181 for CS). This study indicates that exposure to THS 2.2 aerosol had a lower impact on the pathophysiology of human gingival organotypic cultures than CS.

In vivo studies in rats

Kogel U, Titz B, Schlage WK, Nury C, Martin F, Oviedo A, Lebrun S, Elamin A, Guedj E, Trivedi K, Ivanov NV, Vanscheeuwijck P, Peitsch MC and Hoeng J (2016) Evaluation of the Tobacco Heating System 2.2. Part 7: Systems toxicological assessment of a mentholated version revealed reduced cellular and molecular exposure effects compared with cigarette smoke. Regul. Toxicol. Pharmacol. 81 Suppl 2:S123-S138. (PMID: 27818347).

Abstract: Modified risk tobacco products (MRTPs) are being developed with the aim of reducing smoking-related health risks. The Tobacco Heating System 2.2 (THS 2.2) is a candidate MRTP that uses the heat-not-burn principle. Here, systems toxicology approaches were engaged to assess the respiratory effects of mentholated THS 2.2 (THS 2.2M) in a 90-day rat inhalation study (OECD test guideline 413). The standard endpoints were complemented by transcriptomics and quantitative proteomics analyses of respiratory nasal epithelium and lung tissue and by lipidomics analysis of lung tissue. The adaptive response of the respiratory nasal epithelium to conventional cigarette smoke (CS) included squamous cell metaplasia and an inflammatory response, with high correspondence between the molecular and histopathological results. In contrast to CS exposure, the adaptive tissue and molecular changes to THS 2.2M aerosol exposure were much weaker and were limited mostly to the highest THS 2.2M concentration in female rats. In the lung, CS exposure induced an inflammatory response, triggered cellular stress responses, and affected sphingolipid metabolism. These responses were not observed or were much lower after THS 2.2M aerosol exposure. Overall, this system toxicology analysis complements and reconfirms the results from classical toxicological endpoints and further suggests potentially reduced health risks of THS 2.2M.

Oviedo A, Lebrun S, Kogel U, Ho J, Tan WT, Titz B, Leroy P, Vuillaume G, Bera M, Martin FT, Rodrigo G, Esposito M, Dempsey R, Ivanov NV, Hoeng J, Peitsch MC and Vanscheeuwijck P (2016) Evaluation of the Tobacco Heating System 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with cigarette smoke. Regul. Toxicol. Pharmacol. 81 Suppl 2:S93-S122. (PMID: 27818348).

Abstract: See under *Step 2 of the assessment program.*

Wong E, Kogel U, Veljkovic E, Martin F, Xiang Y, Boue S, Vuillaume G, Leroy P, Guedj E, Rodrigo G, Ivanov NV, Hoeng J, Peitsch MC and Vanscheeuwijck P (2016) Evaluation of the Tobacco Heating System 2.2. Part 4: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects compared with cigarettes

smoke. Regul. Toxicol. Pharmacol. 81 Suppl 2:S59-S81. (PMID: 27793746).

Abstract: See under *Step 2 of the assessment program.*

Sewer A, Kogel U, Talikka M, Wong E, Martin F, Xiang Y, Guedj E, Ivanov NV, Hoeng J and Peitsch MC (2016) Evaluation of the Tobacco Heating System 2.2. Part 5: miRNA expression from a 90-day rat inhalation study indicates reduced effects of the aerosol on lung tissue compared with cigarette smoke exposure. Regul. Toxicol. Pharmacol. 81 Suppl 2:S82-S92. (PMID: 27866933).

Abstract: Modified-risk tobacco products (MRTP) are designed to reduce the individual risk of tobacco-related disease as well as population harm compared to smoking cigarettes. Experimental proof of their benefit needs to be provided at multiple levels in research fields. Here, we examined microRNA (miRNA) levels in the lungs of rats exposed to a candidate modified-risk tobacco product, the Tobacco Heating System 2.2 (THS 2.2) in a 90-day OECD TG-413 inhalation study. Our aim was to assess the miRNA response to THS 2.2 aerosol compared with the response to combustible cigarettes (CC) smoke from the reference cigarette 3R4F. CC smoke exposure, but not THS 2.2 aerosol exposure, caused global miRNA downregulation, which may be explained by the interference of CC smoke constituents with the miRNA processing machinery. Upregulation of specific miRNA species, such as miR-146a/b and miR-182, indicated that they are causal elements in the inflammatory response in CC-exposed lungs, but they were reduced after THS 2.2 aerosol exposure. Transforming transcriptomic data into protein activity based on corresponding downstream gene expression, we identified potential mechanisms for miR-146a/b and miR-182 that were activated by CC smoke but not by THS 2.2 aerosol and possibly involved in the regulation of those miRNAs. The inclusion of miRNA profiling in systems toxicology approaches increases the mechanistic understanding of the complex exposure responses.

In vivo studies in mouse models of disease

Phillips B, Veljkovic E, Boué S, Schlage WK, Vuillaume G, Martin F, Titz B, Leroy P, Buettner A, Elamin A, Oviedo A, Cabanski M, Guedj E, Schneider T, Talikka M, Ivanov NV, Vanscheeuwijck P, Peitsch MC and Hoeng J (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). Corrected *Suppl. Table 1* in Toxicol. Sci., 151:462-4 (PMID: 27225756).

Abstract: Smoking cigarettes is a major risk factor in the development and progression of cardiovascular disease (CVD) and chronic obstructive pulmonary disease (COPD). Modified risk tobacco products (MRTPs) are being developed to reduce smoking-related health risks. The goal of this study was to investigate hallmarks of COPD and CVD over an 8-month period in apolipoprotein E-deficient mice exposed to conventional cigarette smoke (CS) or to the aerosol of a candidate MRTP, tobacco heating system (THS) 2.2. In addition to chronic exposure, cessation or switching to THS 2.2 after 2 months of CS exposure was assessed. Engaging a systems toxicology approach, exposure effects were investigated using physiology and histology combined with transcriptomics, lipidomics, and proteomics. CS induced nasal epithelial hyperplasia and metaplasia, lung inflammation, and emphysematous changes (impaired pulmonary function and alveolar damage). Atherogenic effects of CS exposure included altered lipid profiles and aortic plaque formation. Exposure to THS 2.2 aerosol (nicotine concentration matched to CS, 29.9mg/m(3)) neither induced lung inflammation or emphysema nor did it consistently change the lipid profile or enhance the plaque area. Cessation or switching to THS 2.2 reversed the inflammatory responses and halted progression of initial emphysematous changes and the aortic plaque area. Biological processes, including senescence, inflammation, and proliferation, were significantly impacted by CS but not by THS 2.2 aerosol. Both, cessation and switching to THS 2.2 reduced these perturbations to almost sham exposure levels. In conclusion, in this mouse model cessation or switching to THS 2.2 retarded the progression of CS-induced atherosclerotic and emphysematous changes, while THS 2.2 aerosol alone had minimal adverse effects.

Lo Sasso G, Titz B, Nury C, Boué S, Phillips B, Belcastro V, T, 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 NV, Martin F, Vanscheeuwijck P, Peitsch MC and Hoeng J (2016) Effects of cigarette smoke, cessation and switching to a candidate modified risk tobacco product on the liver of Apoe$^{-/-}$ mice – a systems toxicology analysis. Inhal. Toxicol. 28:226-240. (PMID: 27027324).

Abstract: The liver is one of the most important organs involved in elimination of xenobiotic and potentially toxic substances. Cigarette smoke (CS) contains more than 7000 chemicals, including those that exert biological effects and cause smoking-related diseases. Though CS is not directly hepatotoxic, a growing body of evidence suggests that it may exacerbate pre-existing chronic liver disease. In this study, we integrated toxicological endpoints with molecular measurements and computational analyses to investigate effects of exposures on the livers of Apoe(-/-)mice. Mice were exposed to 3R4F reference CS, to an aerosol from the Tobacco Heating System (THS) 2.2, a candidate modified risk tobacco product (MRTP) or to filtered air (Sham) for up to 8 months. THS 2.2 takes advantage of a "heat-not-burn" technology that, by heating tobacco, avoids pyrogenesis and pyrosynthesis. After CS exposure for 2 months, some groups were either switched to the MRTP or filtered air. While no group showed clear signs of hepatotoxicity, integrative analysis of proteomics and transcriptomics data showed a CS-dependent impairment of specific biological networks. These networks included lipid and xenobiotic metabolism and iron homeostasis that likely contributed synergistically to exacerbating oxidative stress. In contrast, most proteomic and transcriptomic changes were lower in mice exposed to THS 2.2 and in the cessation and switching groups compared to the CS group. Our findings elucidate the complex biological responses of the liver to CS exposure. Furthermore, they provide evidence that THS 2.2 aerosol has reduced biological effects, as compared with CS, on the livers of Apoe(-/-)mice.

Szostak J, Boué S, Talikka M, Guedj E, Martin F, Phillips B, Ivanov NV, Peitsch MC and Hoeng J (2016) Aerosol from Tobacco Heating System 2.2 has reduced impact on mouse heart gene expression compared with cigarette smoke. Food Chem. Toxicol. 101:157-167. (PMID: 28111298).

Abstract: Experimental studies clearly demonstrate a causal effect of cigarette smoking on cardiovascular disease. To reduce the individual risk and population harm caused by smoking, alternative products to cigarettes are being developed. We recently reported on an apolipoprotein E-deficient (Apoe-/-) mouse inhalation study that compared the effects of exposure to aerosol from a candidate modified risk tobacco product, Tobacco Heating System 2.2 (THS 2.2), and smoke from the reference cigarette (3R4F) on pulmonary and vascular biology. Here, we applied a transcriptomics approach to evaluate the impact of the exposure to 3R4F smoke and THS 2.2 aerosol on heart tissues from the same cohort of mice. The systems response profiles demonstrated that 3R4F smoke exposure led to time-dependent transcriptomics changes (False Discovery Rate (FDR) < 0.05; 44 differentially expressed genes at 3-months; 491 at 8-months). Analysis of differentially expressed genes in the heart tissue indicated that 3R4F exposure induced the downregulation of genes involved in cytoskeleton organization and the contractile function of the heart, notably genes that encode beta actin (Actb), actinin alpha 4 (Actn4), and filamin C (Flnc). This was accompanied by the downregulation of genes related to the inflammatory response. None of these effects were observed in the group exposed to THS 2.2 aerosol.

Titz B, Boué S, Phillips B, Talikka M, Vihervaara T, Schneider T, Nury C, Elamin A, Guedj E, Peck MJ, Schlage WK, Cabanski M, Leroy P, Vuillaume G, Martin F, Ivanov NV, Veljkovic E, Ekroos K, Laaksonen R, Vanscheeuwijck P, Peitsch MC and Hoeng J (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).

Abstract: The impact of cigarette smoke (CS), a major cause of lung diseases, on the composition and metabolism of lung lipids is incompletely understood. Here, we integrated quantitative lipidomics and proteomics to investigate exposure effects on lung lipid metabolism in a C57BL/6 and an Apolipoprotein E-deficient (Apoe(-/-)) mouse study. In these studies, mice were exposed to high concentrations of 3R4F reference CS, aerosol from potential modified risk tobacco products (MRTPs) or filtered air (Sham) for up to 8 months. The 2 assessed MRTPs, the prototypical MRTP for C57BL/6 mice and the Tobacco Heating System 2.2 for Apoe(-/-) mice, utilize "heat-not-burn" technologies and were each matched in nicotine concentrations to the 3R4F CS. After 2 months of CS exposure, some groups were either switched to the MRTP or underwent cessation. In both mouse strains, CS strongly affected several categories of lung lipids and lipid-related proteins. Candidate surfactant lipids, surfactant proteins, and surfactant metabolizing proteins were increased. Inflammatory eicosanoids, their metabolic enzymes, and several ceramide classes were elevated. Overall, CS induced a coordinated lipid response controlled by transcription regulators such as SREBP proteins and supported by other metabolic adaptations. In contrast, most of these changes were absent in the mice exposed to the potential MRTPs, in the cessation group, and the switching group. Our findings demonstrate the complex biological response of the lungs to CS exposure and support the benefits of cessation or switching to a heat-not-burn product using a design such as those employed in this study.

Clinical Studies (Step 5)

Brossard P, Weitkunat R, Poux V, Lama N, Haziza C, Picavet P, Baker G and Lüdicke F (2017) Nicotine pharmacokinetic profiles of the Tobacco Heating System 2.2, cigarettes and nicotine gum in Japanese smokers. Regulatory Toxicology and Pharmacology, 89:193-199. (PMID: 28760390).

Abstract: Two open-label randomized cross-over studies in Japanese smokers investigated the single-use nicotine pharmacokinetic profile of the Tobacco Heating System (THS) 2.2, cigarettes (CC) and nicotine replacement therapy (Gum). In each study, one on the regular and one on the menthol variants of the THS and CC, both using Gum as reference, 62 subjects were randomized to four sequences: Sequence 1: THS - CC (n = 22); Sequence 2: CC - THS (n = 22); Sequence 3: THS - Gum (n = 9); Sequence 4: Gum - THS (n = 9). Plasma nicotine concentrations were measured in 16 blood samples collected over 24 h after single use. Maximal nicotine concentration (Cmax) and area under the curve from start of product use to time of last quantifiable concentration ($AUC_{0\text{-}last}$) were similar between THS and CC in both studies, with ratios varying from 88 to 104% for Cmax and from 96 to 98% for $AUC_{0\text{-}last}$. Urge-to-smoke total scores were comparable between THS and CC. The THS nicotine pharmacokinetic profile was close to CC, with similar levels of urge-to-smoke. This suggests that THS can satisfy smokers and be a viable alternative to cigarettes for adult smokers who want to continue using tobacco.

Haziza C, de La Bourdonnaye G, Merlet S, Benzimra M, Ancerewicz J, Donelli A, Baker G, Picavet P and Lüdicke F. (2016) Assessment of the reduction in levels of exposure to harmful and potentially harmful constituents in Japanese subjects using a novel tobacco heating system compared with conventional cigarettes and smoking abstinence: a randomized controlled study in confinement. Regul. Toxicol. Pharmacol. 81:489-499. (PMID: 27693654).

Abstract: Smoking conventional cigarettes (CCs) exposes smokers to harmful and potentially harmful constituents (HPHCs). The Tobacco Heating System 2.2 (THS 2.2), a candidate modified risk tobacco product, was developed to reduce or eliminate the formation of HPHCs, while preserving as much as possible the taste, sensory experience, nicotine delivery profile and ritual characteristics of CC. This randomized, controlled, open-label study in confinement for 5 day exposure aimed to demonstrate the reduction in exposure to selected HPHCs, to assess nicotine uptake and subjective effects, in participants switching to THS 2.2 (n = 80) compared to participants continuing smoking CCs (n = 40) and abstaining from smoking (n = 40). The subjects were randomized according to sex and daily CC consumption. The levels of biomarkers of exposure to HPHCs were significantly reduced in participants switching to THS 2.2, compared to CC use. More importantly, the magnitude of exposure reduction observed was close to that which was seen in participants who abstained from smoking for 5 days, while nicotine uptake was maintained. Reduction in urge-to-smoke was comparable between THS and CC groups, however THS 2.2 was slightly less satisfactory than CCs. The new, alternative tobacco product THS 2.2 was well tolerated.

Haziza C, de La Bourdonnaye G, Skiada D, Ancerewicz J, Baker G, Picavet P and Lüdicke F (2016) Evaluation of the Tobacco Heating System 2.2. Part 8: 5-day randomized reduced exposure clinical trial in Poland. Regul. Toxicol. Pharmacol. 81 Suppl 2:S139-S150. (PMID: 27816672).

Abstract: The Tobacco Heating System (THS) 2.2, a candidate Modified Risk Tobacco Product (MRTP), is designed to heat tobacco without burning it. Tobacco is heated in order to reduce the formation of harmful and potentially harmful constituents (HPHC), and reduce the consequent exposure, compared with combustible cigarettes (CC). In this 5-day exposure, controlled, parallel-group, open-label clinical study, 160 smoking, healthy subjects were randomized to three groups and asked to: (1) switch from CCs to THS 2.2 (THS group; 80 participants); (2) continue to use their own non-menthol CC brand (CC group; 41 participants); or (3) to refrain from smoking (smoking abstinence (SA) group; 39 participants). Biomarkers of exposure, except those associated with nicotine exposure, were significantly reduced in the THS group compared with the CC group, and approached the levels observed in the SA group. Increased product consumption and total puff volume were reported in the THS group. However, exposure to nicotine was similar to CC at the end of the confinement period. Reduction in urge-to-smoke was comparable between the THS and CC groups and THS 2.2 product was well tolerated.

Haziza C, de La Bourdonnaye G, Skiada D, Ancerewicz J, Baker B, Picavet P and Lüdicke F (2017). Biomarker of exposure level data set in smokers switching from conventional cigarettes to Tobacco Heating System 2.2, continuing smoking or abstaining from smoking for 5 days. Data Brief 10:283-293. (PMID: 27995164).

Abstract: Levels of biomarkers of exposure to selected harmful and potentially harmful smoke constituents found in cigarette smoke, in addition to nicotine were measured in 160 smokers randomized for 5 days to continuing smoking conventional cigarettes (41 participants), switching to Tobacco Heating System 2.2 (THS 2.2) (80 participants), or abstaining from smoking (39 participants). The data reported here are descriptive statistics of the levels of each biomarker of exposure expressed as concentrations adjusted to creatinine; at baseline, and at the end of the study, and their relative change from baseline. Reductions in the levels of biomarkers of exposure when expressed as quantity excreted, are also reported. Detailed descriptions of bioanalytical assays used are also provided. The data presented here are related to the article entitled "Evaluation of the Tobacco Heating System 2.2. Part 8: 5-Day randomized reduced exposure clinical study in Poland" (Haziza et al. Regul. Toxicol. Pharmacol. 2016; 81 Suppl 2:S139-S150. PMID: 27816672).

Marchand M, Brossard P, Merdjan H, Lama N, Weitkunat R and Lüdicke F (2017) Nicotine population pharmacokinetics in healthy adult smokers: A retrospective analysis. Eur J Drug Metab Pharmacokinet. *e-pub ahead of print.* (PMID: 28283988).

Abstract:

BACKGROUND AND OBJECTIVE: Characterizing nicotine pharmacokinetics is challenging in the presence of background exposure. We performed a combined retrospective population pharmacokinetic analysis of 8 trials, including exposure to Tobacco Heating System and cigarettes (both inhaled), nicotine nasal spray and oral nicotine gum.

METHOD: Data from 4 single product use trials were used to develop a population pharmacokinetic model with Phoenix® NLME™ and to derive exposure parameters. Data from 4 separate ad libitum use studies were used for external validation. A total of 702 healthy adult smokers (54% males; 21-66 years of age; smoking ≥10 cigarettes/day; from US, Europe and Japan) were eligible for participation.

RESULTS: Two-compartment linear disposition combined with zero-order absorption model was adequate to describe nicotine pharmacokinetics, and a mono-exponentially decreasing background component was utilized to account for nicotine carry-over effects. Apparent nicotine clearance was typically 0.407 L/min in males and 26% higher in females (68% inter-individual variability). Bioavailability was product-specific, decreased with increasing nicotine ISO yield, and increased with increasing body weight. Absorption duration was apparently prolonged with nicotine gum. The typical initial and terminal half-lives were 1.35 and 17 h, respectively. The presence of menthol did not impact the determinants of the area under the curve. The model adequately described the external validation data.

CONCLUSIONS: The population model was able to describe in different populations the nicotine pharmacokinetics after single product use and after 4 days of ad libitum use of Tobacco Heating System, cigarettes, and of different nicotine replacement therapies with various routes of administration.

Martin F, Ivanov NV, Haziza C, Hoeng J and Peitsch MC (2016) Evaluation of the Tobacco Heating System 2.2. Part 9: Application of systems pharmacology to identify exposure response markers in peripheral blood of smokers switching to THS 2.2. Regul. Toxicol. Pharmacol. 81 Suppl 2:S151-S157. (PMID: 27845159).

Abstract: As part of current harm reduction strategies, candidate modified risk tobacco products (MRTP) are developed to offer adult smokers who want to continue using tobacco product an alternative to cigarettes while potentially reducing individual risk and population harm compared to smoking cigarettes. One of these candidate MRTPs is the Tobacco Heating System (THS) 2.2 which does not burn tobacco, but instead heats it, thus producing significantly reduced levels of harmful and potentially harmful constituents (HPHC) compared with combustible cigarettes (CC). A controlled, parallel group, open-label clinical study was conducted with subjects randomized to three monitored groups: (1) switching from CCs to THS 2.2; (2) continuous use of non-menthol CC brand (CC arm); or (3) smoking abstinence (SA arm) for five days. Exposure response was assessed by measuring biomarkers of exposure to selected HPHCs. To complement the classical exposure response measurements, we have used the previously reported whole blood derived gene signature that can distinguish current smokers from either non-smokers or former smokers with high specificity and sensitivity. We tested the small signature consisting of only 11 genes on the blood transcriptome of subjects enrolled in the clinical study and showed a reduced exposure response in subjects that either stopped smoking or switched to a candidate MRTP, the THS 2.2, compared with subjects who continued smoking their regular tobacco product.

Poussin C, Belcastro V, Martin F, Boue S, Peitsch MC and Hoeng J (2017). Crowd-sourced verification of

computational methods and data in systems toxicology: a case study with a heat-not-burn candidate modified risk tobacco product. Chem. Res. Toxicol. 30:934-945. (PMID: 28085253).

Abstract: Systems toxicology intends to quantify the effect of toxic molecules in biological systems and unravel their mechanisms of toxicity. The development of advanced computational methods is required for analyzing and integrating high throughput data generated for this purpose as well as for extrapolating predictive toxicological outcomes and risk estimates. To ensure the performance and reliability of the methods and verify conclusions from systems toxicology data analysis, it is important to conduct unbiased evaluations by independent third parties. As a case study, we report here the results of an independent verification of methods and data in systems toxicology by crowdsourcing. The sbv IMPROVER systems toxicology computational challenge aimed to evaluate computational methods for the development of blood-based gene expression signature classification models with the ability to predict smoking exposure status. Participants created/trained models on blood gene expression data sets including smokers/mice exposed to 3R4F (a reference cigarette) or noncurrent smokers/Sham (mice exposed to air). Participants applied their models on unseen data to predict whether subjects classify closer to smoke-exposed or nonsmoke exposed groups. The data sets also included data from subjects that had been exposed to potential modified risk tobacco products (MRTPs) or that had switched to a MRTP after exposure to conventional cigarette smoke. The scoring of anonymized participants' predictions was done using predefined metrics. The top 3 performers' methods predicted class labels with area under the precision recall scores above 0.9. Furthermore, although various computational approaches were used, the crowd's results confirmed our own data analysis outcomes with regards to the classification of MRTP-related samples. Mice exposed directly to a MRTP were classified closer to the Sham group. After switching to a MRTP, the confidence that subjects belonged to the smoke-exposed group decreased significantly. Smoking exposure gene signatures that contributed to the group separation included a core set of genes highly consistent across teams such as AHRR, LRRN3, SASH1, and P2RY6. In conclusion, crowdsourcing constitutes a pertinent approach, in complement to the classical peer review process, to independently and unbiasedly verify computational methods and data for risk assessment using systems toxicology

Lüdicke F, Picavet P, Baker G, Haziza C, Poux V, Lama N and Weitkunat R (2017a) Effects of switching to the Tobacco Heating System 2.2 menthol, smoking abstinence, or continued cigarette smoking on biomarkers of exposure: a randomized, controlled, open-label, multicenter study in sequential confinement and ambulatory settings (Part 1). Nicotine Tob. Res. *e-pub ahead of print*. (PMID: 28177489).

Abstract:

INTRODUCTION: The menthol Tobacco Heating System 2.2 (mTHS) is a newly developed candidate modified-risk tobacco product intended to reduce exposure to the harmful and potentially harmful constituents (HPHCs) of conventional cigarette (CC) smoke. This study examined the impact of switching to mTHS on biomarkers of exposure to HPHCs relative to menthol CCs (mCCs) and smoking abstinence (SA). Methods: In this three-arm, parallel-group study, 160 Japanese adult smokers (23–65 years; smoking ≥10 mCCs per day) were randomized to mTHS (n=78), mCC (n=42), or SA (n=40) for 5 days in confinement and 85 days in ambulatory settings. Endpoints included biomarkers of exposure to HPHCs, human puffing topography, safety, and subjective effects of smoking measures.

RESULTS: After 5 days of product use, the concentrations of carboxyhemoglobin, 3-hydroxypropylmercapturic acid, monohydroxybutenyl mercapturic acid, and S-phenylmercapturic acid were 55%, 49%, 87%, and 89% lower (P<0.001), respectively, in the mTHS group than in the mCC group. Other biomarkers of exposure (measured as secondary endpoints) were 50% to 94% lower in the mTHS group than in the mCC group on Day 5. These reductions in the mTHS group were maintained at Day 90, similar to the SA group. Switching to mTHS was associated with changes in human puffing topography (shorter puff intervals and more frequent puffs). The urge-to-smoke and smoking satisfaction levels on Day 90 were similar in the mTHS and the mCC groups.

CONCLUSION: Switching from mCCs to mTHS significantly reduced exposure to HPHCs relative to continuing smoking mCCs with concentrations similar to those observed following SA in Japanese adult smokers.

IMPLICATIONS: This randomized study compared the impact of switching to a modified-risk tobacco product candidate (menthol Tobacco Heating System 2.2 [mTHS]) on biomarkers of exposure to harmful and potentially harmful constituents of cigarette smoke relative to continuing smoking cigarettes or abstaining from smoking in sequential confinement and ambulatory settings. The study showed that switching to mTHS was associated with significant biomarker reductions within 5 days in confinement, these reductions being maintained throughout the ambulatory setting up to Day 90. The results provide evidence that switching to mTHS reduces real-life exposure to HPHCs in adult smokers.

Lüdicke F, Picavet P, Baker G, Haziza C, Poux V, Lama N and Weitkunat R (2017b). Effects of switching to the menthol Tobacco Heating System 2.2, smoking abstinence, or continued cigarette smoking on clinically relevant risk markers: a randomized, controlled, open-label, multicenter study in sequential confinement and ambulatory settings (Part 2). Nicotine Tob. Res. *e-pub ahead of print*. (PMID: 28177498).

Abstract:

INTRODUCTION: Modified-risk tobacco products are expected to reduce exposure to harmful and potentially harmful constituents (HPHCs) of cigarette smoke, and ultimately reduce the health burden of smoking-related diseases. Clinically relevant risk markers of smoking-related diseases inform about the risk profile of new tobacco products in the absence of in-market epidemiological data. The menthol Tobacco Heating System 2.2 (mTHS) is a modified-risk tobacco product in development as an alternative to cigarettes (CCs).

METHODS: In this parallel-group study, Japanese adult smokers (23–65 years; ≥10 mCCs/day) were randomized to mTHS, menthol CCs (mCC), or smoking abstinence (SA) for 5 days in confinement and 85 days in ambulatory settings. Endpoints included biomarkers of exposure to HPHCs and clinically relevant risk markers of smoking-related diseases.

RESULTS: One-hundred and sixty participants were randomized to the mTHS (n=78), mCC (n=42), and smoking abstinence (n=40) groups. Switching to the mTHS

was associated with reductions in biomarkers of exposure compared with continuing mCCs. Reductions in 8-epi-prostaglandin F2α (biomarker of oxidative stress), 11-dehydro-thromboxane B2 (biomarker of platelet activation), soluble intracellular adhesion molecule-1 (biomarker of endothelial function), and an increase in high-density lipoprotein cholesterol (biomarker of lipid metabolism) and forced expiratory volume in 1 second (biomarker of lung function) occurred in the mTHS group compared with the mCC group. The changes in the mTHS group approached those in the SA group.

CONCLUSIONS: Switching from mCCs to mTHS was associated with improvements in clinically relevant risk markers linked to mechanistic pathways involved in smoking-related diseases.

IMPLICATIONS: In this three-way randomized study, switching from menthol cigarettes to menthol Tobacco Heating System 2.2 for 5 days in confinement and 85 days in ambulatory settings was associated with reductions in biomarkers of exposure to cigarette smoke, and changes were observed in clinically relevant biomarkers of oxidative stress (8-epi-prostaglandin F2α), platelet activity (11-dehydro-thromboxane B2), endothelial function (soluble intracellular adhesion molecule-1), lipid metabolism (high-density lipoprotein cholesterol) and lung function (forced expiratory volume in 1 second), similar to the smoking abstinence group. The results suggest that switching to the menthol Tobacco Heating System 2.2 has the potential to reduce the adverse health effects of conventional cigarettes.

Population Health Impact Modeling (In Step 6)

Lee PN, Fry JS, Hamling JF, Sponsiello-Wang Z, Baker G and Weitkunat R (2017) Estimating the effect of differing assumptions on the population health impact of introducing a Reduced Risk Tobacco Product in the USA. Regul. Toxicol. Pharmacol. *e-pub ahead of print.* (PMID: 28651854).

Abstract: We use Population Health Impact Modelling to assess effects on tobacco prevalence and mortality of introducing a Reduced Risk Tobacco Product (RRP). Simulated samples start in 1990 with a US-representative smoking prevalence. Individual tobacco histories are updated annually until 2010 using estimated probabilities of switching between never/current/former smoking where the RRP is not introduced, with current users subdivided into cigarette/RRP/dual users where it is. RRP-related mortality reductions from lung cancer, IHD, stroke and COPD are derived from the histories and the assumed relative risks of the RRP.

A basic analysis assumes a hypothetical RRP reduces effective dose 80% in users and 40% in dual users, with an uptake rate generating ~10% RRP and ~6% dual users among current users after 10 years. Sensitivity study changes in tobacco prevalence and mortality from varying effective doses, current smoking risks, quitting half-lives and rates of initiation, switching, re-initiation and cessation. They also study extreme situations (e.g. everyone using RRP), and investigate assumptions which might eliminate the RRP-related mortality reduction. The mortality reduction is proportional to the dose reduction, increasing rapidly with time of follow-up. Plausible increases in re-initiation or dual users' consumption, or decreased quitting by smokers would not eliminate the drop.

II. Key Methods developed by PMI

Aerosol Chemistry (Step 2)

Knorr A, Monge A, Stueber M, Stratmann A, Arndt D, Martin E and Pospíšil Pl (2013) Computer-Assisted Structure Identification (CASI) – An automated platform for high-throughput identification of small molecules by two-dimensional gas chromatography coupled to mass spectrometry. Anal. Chem. 85:11216-11224. (PMID: 24160557).

Abstract: Compound identification is widely recognized as a major bottleneck for modern metabolomic approaches and high-throughput nontargeted characterization of complex matrices. To tackle this challenge, an automated platform entitled computer-assisted structure identification (CASI) was designed and developed in order to accelerate and standardize the identification of compound structures. In the first step of the process, CASI automatically searches mass spectral libraries for matches using a NIST MS Search algorithm, which proposes structural candidates for experimental spectra from two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-TOF-MS) measurements, each with an associated match factor. Next, quantitative structure-property relationship (QSPR) models implemented in CASI predict three specific parameters to enhance the confidence for correct compound identification, which were Kovats Index (KI) for the first dimension (1D) separation, relative retention time for the second dimension separation (2DrelRT) and boiling point (BP). In order to reduce the impact of chromatographic variability on the second dimension retention time, a concept based upon hypothetical reference points from linear regressions of a deuterated n-alkanes reference system was introduced, providing a more stable relative retention time measurement. Predicted values for KI and 2DrelRT were calculated and matched with experimentally derived values. Boiling points derived from 1D separations were matched with predicted boiling points, calculated from the chemical structures of the candidates. As a last step, CASI combines the NIST MS Search match factors (NIST MF) with up to three predicted parameter matches from the QSPR models to generate a combined CASI Score representing the measure of confidence for the identification. Threshold values were applied to the CASI Scores assigned to proposed structures, which improved the accuracy for the classification of true/false positives and true/false negatives. Results for the identification of compounds have been validated, and it has been demonstrated that identification using CASI is more accurate than using NIST MS Search alone. CASI is an easily accessible web-interfaced software platform which represents an innovative, high-throughput system that allows fast and accurate identification of constituents in complex matrices, such as those requiring 2D separation techniques.

Mottier N, Tharin M, Cluse C, Crudo JR, Gomez Lueso M, Goujon-Ginglinger C, Jaquier A, Mitova MI, Rouget EG, Schaller M and Solioz J (2016) Validation of selected analytical methods using accuracy profiles to assess the impact of a Tobacco Heating System on indoor air quality. Talanta 158:165-178. (PMID: 27343591).

Abstract: Studies in environmentally controlled rooms have been used over the years to assess the impact of environmental tobacco smoke on indoor air quality. As new tobacco products are developed, it is important to determine their impact on air quality when used indoors. Before such an assessment can take place it is essential

that the analytical methods used to assess indoor air quality are validated and shown to be fit for their intended purpose. Consequently, for this assessment, an environmentally controlled room was built and seven analytical methods, representing eighteen analytes, were validated. The validations were carried out with smoking machines using a matrix-based approach applying the accuracy profile procedure. The performances of the methods were compared for all three matrices under investigation: background air samples, the environmental aerosol of Tobacco Heating System THS 2.2, a heat-not-burn tobacco product developed by Philip Morris International, and the environmental tobacco smoke of a cigarette. The environmental aerosol generated by the THS 2.2 device did not have any appreciable impact on the performances of the methods. The comparison between the background and THS 2.2 environmental aerosol samples generated by smoking machines showed that only five compounds were higher when THS 2.2 was used in the environmentally controlled room. Regarding environmental tobacco smoke from cigarettes, the yields of all analytes were clearly above those obtained with the other two air sample types.

Pratte P, Cosandey S and Goujon-Ginglinger C (2016) A scattering methodology for droplet sizing of e-cigarette aerosols. Inhal. Toxicol. 28:537-545. (PMID: 27644268).

Abstract:

CONTEXT: Knowledge of the droplet size distribution of inhalable aerosols is important to predict aerosol deposition yield at various respiratory tract locations in human. Optical methodologies are usually preferred over the multi-stage cascade impactor for high-throughput measurements of aerosol particle/droplet size distributions.

OBJECTIVE: Evaluate the Laser Aerosol Spectrometer technology based on Polystyrene Sphere Latex (PSL) calibration curve applied for the experimental determination of droplet size distributions in the diameter range typical of commercial e-cigarette aerosols (147-1361nm).

MATERIALS AND METHODS: This calibration procedure was tested for a TSI Laser Aerosol Spectrometer (LAS) operating at a wavelength of 633nm and assessed against model di-ethyl-hexyl-sebacat (DEHS) droplets and e-cigarette aerosols. The PSL size response was measured, and intra- and between-day standard deviations calculated.

RESULTS: DEHS droplet sizes were underestimated by 15-20% by the LAS when the PSL calibration curve was used; however, the intra- and between-day relative standard deviations were <3%. This bias is attributed to the fact that the index of refraction of PSL calibrated particles is different in comparison to test aerosols. This 15-20% does not include the droplet evaporation component, which may reduce droplet size prior a measurement is performed. Aerosol concentration was measured accurately with a maximum uncertainty of 20%. Count median diameters and mass median aerodynamic diameters of selected e-cigarette aerosols ranged from 130-191nm to 225-293nm, respectively, similar to published values.

DISCUSSION AND CONCLUSION: The LAS instrument can be used to measure e-cigarette aerosol droplet size distributions with a bias underestimating the expected value by 15-20% when using a precise PSL calibration curve. Controlled variability of DEHS size measurements can be achieved with the LAS system; however, this method can only be applied to test aerosols having a refractive index close to that of PSL particles used for calibration.

Pre-clinical Toxicology (Step 3)

Kogel U, Schlage WK, Martin F, Xiang Y, Ansari S, Leroy P, Vanscheeuwijck P, Gebel S, Buettner A, Wyss C, Esposito M, Hoeng J and Peitsch MC (2014) 28-day rat inhalation study with an integrated molecular toxicology endpoint demonstrates reduced exposure effects for a prototypic modified risk tobacco product compared with conventional cigarettes. Food Chem. Toxicol. 68:204-217. (PMID: 24632068).

Abstract: Towards a systems toxicology-based risk assessment, we investigated molecular perturbations accompanying histopathological changes in a 28-day rat inhalation study combining transcriptomics with classical histopathology. We demonstrated reduced biological activity of a prototypic modified risk tobacco product (pMRTP) compared with the reference research cigarette 3R4F. Rats were exposed to filtered air or to three concentrations of mainstream smoke (MS) from 3R4F, or to a high concentration of MS from a pMRTP. Histopathology revealed concentration-dependent changes in response to 3R4F that were irritative stress-related in nasal and bronchial epithelium, and inflammation-related in the lung parenchyma. For pMRTP, significant changes were seen in the nasal epithelium only. Transcriptomics data were obtained from nasal and bronchial epithelium and lung parenchyma. Concentration-dependent gene expression changes were observed following 3R4F exposure, with much smaller changes for pMRTP. A computational-modeling approach based on causal models of tissue-specific biological networks identified cell stress, inflammation, proliferation, and senescence as the most perturbed molecular mechanisms. These perturbations correlated with histopathological observations. Only weak perturbations were observed for pMRTP. In conclusion, a correlative evaluation of classical histopathology together with gene expression-based computational network models may facilitate a systems toxicology-based risk assessment, as shown for a pMRTP.

Roemer E, Lammerich HP, Conroy LL and Weisensee D (2013) Characterization of a gap-junctional intercellular communication (GJIC) assay using cigarette smoke. Toxicol. Lett. 219:248-53. (PMID: 23558295).

Abstract: Inhibition of gap-junctional intercellular communication (GJIC) via exposure to various toxic substances has been implicated in tumor promotion. In the present study, cigarette smoke total particulate matter (TPM), a known inhibitor of GJIC, were used to characterize a new GJIC screening assay in three independent experiments. The main features of this assay were automated fluorescence microscopy combined with non-invasive parachute technique. Rat liver epithelial cells (WB-F344) were stained with the fluorescent dye Calcein AM (acetoxymethyl) and exposed to TPM from the Kentucky Reference Cigarette 2R4F (a blend of Bright and Burley tobaccos) and from two single-tobacco cigarettes (Bright and Burley) for 3h. Phorbol-12-myristate-13-acetate (TPA) was used as positive control and 0.5% dimethyl sulfoxide (DMSO) as solvent control. The transfer of dye to adjacent cells (percentage of stained cells) was used as a measure of cellular communication. A clear and reproducible dose-response of GJIC inhibition following TPM exposure was seen. Reproducibility and repeatability

measurements for the 2R4F cigarette were 3.7% and 6.9%, respectively. The half-maximal effective concentration values were 0.34ng/ml for TPA, 0.050mg/ml for the 2R4F, 0.044mg/ml for the Bright cigarette, and 0.060mg/ml for the Burley cigarette. The assay was able to discriminate between the two single-tobacco cigarettes (P<0.0001), and between the single-tobacco cigarettes and the 2R4F (P=0.0008, 2R4F vs. Burley and P<0.0001, 2R4F vs. Bright). Thus, this assay can be used to determine the activity of complex mixtures such as cigarette smoke with high throughput and high precision.

Weber S, Hebestreit M, Wilms T, Conroy LL and Rodrigo G (2013) Comet assay and air-liquid interface exposure system: A new combination to evaluate genotoxic effects of cigarette whole smoke in human lung cell lines. Toxicol. In Vitro 27:1987-1991. (PMID: 23845897).

Abstract: Over the past three decades, the genotoxic effects of cigarette smoke have generally been evaluated in non-human cell models after exposure to particulate phase, gas phase, or cigarette smoke condensate, rather than the whole smoke aerosol itself. In vitro setups using human cell lines and whole smoke exposure to mimic actual aerosol exposure should more accurately reflect human cigarette smoke exposure. We investigated the VITROCELL® 24 air-liquid interface exposure system in combination with the comet assay to assess DNA damage in two different human lung epithelial cell lines exposed to whole smoke. Results showed a repeatable and reproducible dose-response relationship between DNA damage and increased whole smoke dose in both cell lines. Thus, the combination of the comet assay with the VITROCELL® 24 represents a valuable new in vitro test system to screen and assess DNA damage in human lung cells exposed to whole smoke.

Weisensee D, Poth A, Roemer E, Conroy LL and Schlage WK (2013) Cigarette smoke-induced morphological transformation of Bhas 42 cells in vitro. Altern. Lab. Anim. 41:181-189. (PMID: 23781935).

Abstract: In vitro cell transformation assays detect transformed cells that have acquired the distinct characteristics of malignant cells and thus model one stage of in vivo carcinogenesis. These assays have been proposed as surrogate models for predicting the non-genotoxic carcinogenic potential of chemicals. The Bhas 42 cell transformation assay, a short-term assay that uses v-Ha-ras-transfected Balb/c 3T3 cells, can detect the tumour promoter-like activities of chemicals, but has not previously been used with cigarette smoke. The particulate phase of cigarette smoke (total particulate matter [TPM]) is known to induce tumours in vivo in the mouse skin painting assay. Therefore, we investigated the ability of this Bhas cell assay to form morphologically transformed foci in vitro when repeatedly challenged with TPM from a standard research cigarette. TPM induced a dose-dependent increase in Type III foci, and a significant increase (up to 20-fold) in focus formation at moderately toxic concentrations between 5 and 60µg TPM/ml, with a peak at 20µg/ml. Three batches of TPM were tested in three independent experiments. Precision (repeatability and reproducibility) was calculated by using 0, 5, 10, and 20µg TPM/ml. Repeatability and reproducibility, expressed as the relative standard deviation obtained from the normalised slopes of the dose-response curves, were 17.2% and 19.6%, respectively; the slopes were 0.7402 ± 0.1247, 0.9347 ± 0.1316, and 0.8772 ± 0.1767 (increase factor∗ml/mg TPM; mean ± SD) ; and the goodness of fit (r2) of the mean slopes, each derived from n = 6 repeats, was 0.9449, 0.8198, and 0.8344, respectively. This in vitro assay with Bhas 42 cells, which are regarded as already initiated in the two-stage paradigm of carcinogenesis (initiation and promotion), is able to detect cell transformation induced by cigarette smoke in a dose-dependent manner with a high sensitivity and good precision. Because this assay is fast and yields reliable results, it may be useful in product assessment, as well as for further investigation of the non-genotoxic carcinogenic activity of tobacco smoke-related test substances.

Systems Toxicology (Step 4)

Concepts, tools and algorithms

Boue S, Talikka M, Westra JW, Hayes W, Di Fabio A, Park JS, Schlage WK, Sewer A, Fields BR, Ansari S, Martin F, Veljkovic E, Kenney RD, Peitsch MC and Hoeng J (2015) Causal Biological Network (CBN) database: a comprehensive platform of causal biological network models focused on the pulmonary and vascular systems. Database 2015: bav030; 1-14. (PMID: 25887162).

Abstract: With the wealth of publications and data available, powerful and transparent computational approaches are required to represent measured data and scientific knowledge in a computable and searchable format. We developed a set of biological network models, scripted in the Biological Expression Language, that reflect causal signaling pathways across a wide range of biological processes, including cell fate, cell stress, cell proliferation, inflammation, tissue repair and angiogenesis in the pulmonary and cardiovascular context. This comprehensive collection of networks is now freely available to the scientific community in a centralized web-based repository, the Causal Biological Network database, which is composed of over 120 manually curated and well annotated biological network models and can be accessed at http://causalbionet.com. The website accesses a MongoDB, which stores all versions of the networks as JSON objects and allows users to search for genes, proteins, biological processes, small molecules and keywords in the network descriptions to retrieve biological networks of interest. The content of the networks can be visualized and browsed. Nodes and edges can be filtered and all supporting evidence for the edges can be browsed and is linked to the original articles in PubMed. Moreover, networks may be downloaded for further visualization and evaluation. Database URL: http://causalbionet.com

Fluck J, Madan S, Ansari S, Hoeng J, Zimmermann M, Hofmann-Apitius M and Peitsch MC (2014) BELIEF - A semiautomatic workflow for BEL network creation. In: Proceedings of the 6th International Symposium on Semantic Mining in Biomedicine (SMBM 2014). Edited by: Bodenreider, Olivier; Oliveira, José Luis; Rinaldi, Fabio. Aveiro, 2014. http://dx.doi.org/10.5167/uzh-98982. (link)

Abstract: In order to build networks for systems biology from the literature an UIMA based extraction workflow using various named entity recognition processes and different relation extraction methods has been composed. The Unstructured Information Management architecture (UIMA) is a Java-based framework that allows assembling complicated workflows from a set of NLP components. The new system is processing scientific articles and is writing the open-access biological expression language (BEL) as output. BEL is a machine and human readable

language with defined knowledge statements that can be used for knowledge representation, causal reasoning, and hypothesis generation. In order to curate the automatically derived BEL statements, our workflow integrates a curation interface that provides access to BEL statements generated by text mining and that integrates supporting information to facilitate manual curation. By using the semi-automated curation pipeline, expert time to model relevant causal relationships in BEL could be significantly reduced. In this paper the UIMA workflow and key features of the curation interface are described.

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 Discov. Today 17:413-418. (PMID: 22155224). No abstract available.

Hoeng J, Talikka M, Martin F, Sewer A, Yang X, Iskandar A, Schlage W and Peitsch MC (2013) Case study: The role of mechanistic network models in systems toxicology. Drug Discov. Today 19:183-192. (PMID: 23933191).

Abstract: Twenty first century systems toxicology approaches enable the discovery of biological pathways affected in response to active substances. Here, we briefly summarize current network approaches that facilitate the detailed mechanistic understanding of the impact of a given stimulus on a biological system. We also introduce our network-based method with two use cases and show how causal biological network models combined with computational methods provide quantitative mechanistic insights. Our approach provides a robust comparison of the transcriptional responses in different experimental systems and enables the identification of network-based biomarkers modulated in response to exposure. These advances can also be applied to pharmacology, where the understanding of disease mechanisms and adverse drug effects is imperative for the development of efficient and safe treatment options.

Iskandar AR, Gonzalez-Suarez I, Majeed S, Marescotti D, Sewer A, Xiang Y, Leroy P, Guedj E, Mathis C, Schaller J-P, Vanscheeuwijck P, Frentzel S, Martin F, Ivanov NV, Peitsch MC and Hoeng J (2016) A framework for in vitro systems toxicology assessment of e-liquids. Toxicol. Mech. Methods 26:389-413. (PMID: 27117495).

Abstract: Various electronic nicotine delivery systems (ENDS), of which electronic cigarettes (e-cigs) are the most recognized prototype, have been quickly gaining ground on conventional cigarettes because they are perceived as less harmful. Research assessing the potential effects of ENDS exposure in humans is currently limited and inconclusive. New products are emerging with numerous variations in designs and performance parameters within and across brands. Acknowledging these challenges, we present here a proposed framework for an in vitro systems toxicology assessment of e-liquids and their aerosols, intended to complement the battery of assays for standard toxicity assessments. The proposed framework utilizes high-throughput toxicity assessments of e-liquids and their aerosols, in which the device-to-device variability is minimized, and a systems-level investigation of the cellular mechanisms of toxicity is an integral part. An analytical chemistry investigation is also included as a part of the framework to provide accurate and reliable chemistry data solidifying the toxicological assessment. In its simplest form, the framework comprises of three main layers: (1) high-throughput toxicity screening of e-liquids using primary human cell culture systems; (2) toxicity-related mechanistic assessment of selected e-liquids, and (3) toxicity-related mechanistic assessment of their aerosols using organotypic air-liquid interface airway culture systems. A systems toxicology assessment approach is leveraged to enable in-depth analyses of the toxicity-related cellular mechanisms of e-liquids and their aerosols. We present example use cases to demonstrate the suitability of the framework for a robust in vitro assessment of e-liquids and their aerosols.

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

Abstract:

BACKGROUND: High-throughput measurement technologies such as microarrays provide complex datasets reflecting mechanisms perturbed in an experiment, typically a treatment vs. control design. Analysis of these information rich data can be guided based on a priori knowledge, such as networks or set of related proteins or genes. Among those, cause-and-effect network models are becoming increasingly popular and more than eighty such models, describing processes involved in cell proliferation, cell fate, cell stress, and inflammation have already been published. A meaningful systems toxicology approach to study the response of a cell system, or organism, exposed to bio-active substances requires a quantitative measure of dose-response at network level, to go beyond the differential expression of single genes.

RESULTS: We developed a method that quantifies network response in an interpretable manner. It fully exploits the (signed graph) structure of cause-and-effect networks models to integrate and mine transcriptomics measurements. The presented approach also enables the extraction of network-based signatures for predicting a phenotype of interest. The obtained signatures are coherent with the underlying network perturbation and can lead to more robust predictions across independent studies. The value of the various components of our mathematically coherent approach is substantiated using several in vivo and in vitro transcriptomics datasets. As a proof-of-principle, our methodology was applied to unravel mechanisms related to the efficacy of a specific anti-inflammatory drug in patients suffering from ulcerative colitis. A plausible mechanistic explanation of the unequal efficacy of the drug is provided. Moreover, by utilizing the underlying mechanisms, an accurate and robust network-based diagnosis was built to predict the response to the treatment.

CONCLUSION: The presented framework efficiently integrates transcriptomics data and "cause and effect" network models to enable a mathematically coherent framework from quantitative impact assessment and data interpretation to patient stratification for diagnosis purposes.

Sturla SJ, Boobis AR, FitzGerald RE, Hoeng J, Kavlock RJ, Schirmer K, Whelan M, Wilks MF and Peitsch MC (2014) Systems Toxicology: from basic research to risk assessment. Chem. Res. Toxicol. 27:314-329. (PMID: 24446777).

Abstract: Systems Toxicology is the integration of classical toxicology with quantitative analysis of large networks of molecular and functional changes occurring across

multiple levels of biological organization. Society demands increasingly close scrutiny of the potential health risks associated with exposure to chemicals present in our everyday life, leading to an increasing need for more predictive and accurate risk-assessment approaches. Developing such approaches requires a detailed mechanistic understanding of the ways in which xenobiotic substances perturb biological systems and lead to adverse outcomes. Thus, Systems Toxicology approaches offer modern strategies for gaining such mechanistic knowledge by combining advanced analytical and computational tools. Furthermore, Systems Toxicology is a means for the identification and application of biomarkers for improved safety assessments. In Systems Toxicology, quantitative systems-wide molecular changes in the context of an exposure are measured, and a causal chain of molecular events linking exposures with adverse outcomes (i.e., functional and apical end points) is deciphered. Mathematical models are then built to describe these processes in a quantitative manner. The integrated data analysis leads to the identification of how biological networks are perturbed by the exposure and enables the development of predictive mathematical models of toxicological processes. This perspective integrates current knowledge regarding bioanalytical approaches, computational analysis, and the potential for improved risk assessment.

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

Abstract: Exposure to biologically active substances such as therapeutic drugs or environmental toxicants can impact biological systems at various levels, affecting individual molecules, signaling pathways, and overall cellular processes. The ability to derive mechanistic insights from the resulting system responses requires the integration of experimental measures with a priori knowledge about the system and the interacting molecules therein. We developed a novel systems biology-based methodology that leverages mechanistic network models and transcriptomic data to quantitatively assess the biological impact of exposures to active substances. Hierarchically organized network models were first constructed to provide a coherent framework for investigating the impact of exposures at the molecular, pathway and process levels. We then validated our methodology using novel and previously published experiments. For both in vitro systems with simple exposure and in vivo systems with complex exposures, our methodology was able to recapitulate known biological responses matching expected or measured phenotypes. In addition, the quantitative results were in agreement with experimental endpoint data for many of the mechanistic effects that were assessed, providing further objective confirmation of the approach. We conclude that our methodology evaluates the biological impact of exposures in an objective, systematic, and quantifiable manner, enabling the computation of a systems-wide and pan-mechanistic biological impact measure for a given active substance or mixture. Our results suggest that various fields of human disease research, from drug development to consumer product testing and environmental impact analysis, could benefit from using this methodology.

Experimental approaches

Ansari S, Baumer K, Boue S, Dijon S, Dulize R, Ekroos K, Elamin A, Foong C, Guedj E, Ivanov N, Krishnan S, Leroy P, Martin F, Merg C, Peck M, Peitsch MC, Phillips B, Schlage W, Schneider T, Talikka M, Titz B, Vanscheeuwijck P, Veljkovic E, Vihervaara T, Vuillaume G, Woon CQ (2016) Comprehensive systems biology analysis of a 7-month cigarette smoke inhalation study in C57BL/6 mice. Sci. Data 3:150077. (PMID: 26731301).

Abstract: Smoking of combustible cigarettes has a major impact on human health. Using a systems toxicology approach in a model of chronic obstructive pulmonary disease (C57BL/6 mice), we assessed the health consequences in mice of an aerosol derived from a prototype modified risk tobacco product (pMRTP) as compared to conventional cigarettes. We investigated physiological and histological endpoints in parallel with transcriptomics, lipidomics, and proteomics profiles in mice exposed to a reference cigarette (3R4F) smoke or a pMRTP aerosol for up to 7 months. We also included a cessation group and a switching-to-pMRTP group (after 2 months of 3R4F exposure) in addition to the control (fresh air-exposed) group, to understand the potential risk reduction of switching to pMRTP compared with continuous 3R4F exposure and cessation. The present manuscript describes the study design, setup, and implementation, as well as the generation, processing, and quality control analysis of the toxicology and 'omics' datasets that are accessible in public repositories for further analyses.

Gonzalez Suarez I, Sewer A, Walker P, Mathis C, Ellis S, Woodhouse H, Guedj E, Dulize R, Marescotti D, Acali S, Martin F, Ivanov NV, Hoeng J and Peitsch MC (2014) A systems biology approach for evaluating the biological impact of environmental toxicants in vitro. Chem. Res. Toxicol. 27:367-376. (PMID: 2442867).

Abstract: Exposure to cigarette smoke is a leading cause of lung diseases including chronic obstructive pulmonary disease and cancer. Cigarette smoke is a complex aerosol containing over 6000 chemicals and thus it is difficult to determine individual contributions to overall toxicity as well as the molecular mechanisms by which smoke constituents exert their effects. We selected three well-known harmful and potentially harmful constituents (HPHCs) in tobacco smoke, acrolein, formaldehyde and catechol, and established a high-content screening method using normal human bronchial epithelial cells, which are the first bronchial cells in contact with cigarette smoke. The impact of each HPHC was investigated using 13 indicators of cellular toxicity complemented with a microarray-based whole-transcriptome analysis followed by a computational approach leveraging mechanistic network models to identify and quantify perturbed molecular pathways. HPHCs were evaluated over a wide range of concentrations and at different exposure time points (4, 8, and 24 h). By high-content screening, the toxic effects of the three HPHCs could be observed only at the highest doses. Whole-genome transcriptomics unraveled toxicity mechanisms at lower doses and earlier time points. The most prevalent toxicity mechanisms observed were DNA damage/growth arrest, oxidative stress, mitochondrial stress, and apoptosis/necrosis. A combination of multiple toxicological end points with a systems-based impact assessment allows for a more robust scientific basis for the toxicological assessment of HPHCs, allowing insight into time- and dose-dependent

molecular perturbations of specific biological pathways. This approach allowed us to establish an in vitro systems toxicology platform that can be applied to a broader selection of HPHCs and their mixtures and can serve more generally as the basis for testing the impact of other environmental toxicants on normal bronchial epithelial cells.

Hoeng J, Talikka M, Martin F, Ansari S, Drubin D, Elamin A, Gebel S, Ivanov NV, Deehan R, Koegel U, Mathis C, Schlage WK, Sewer A, Sierro N, Thomson T and Peitsch MC (2014) Toxicopanomics: applications of genomics, transcriptomics, proteomics and lipidomics in predictive mechanistic toxicology. In: Hayes' Principles and Methods on Toxicology, Sixth Edition, Chapter 7, pp. 295-332. Edited by Edited by A. Wallace Hayes and Claire L. Kruger. CRC Press.

Kuehn D, Majeed S, Guedj E, Dulize R, Baumer K, Iskandar A, Boue S, Martin F, Kostadinova R, Mathis C, Ivanov N, Frentzel S, Hoeng J and Peitsch MC (2015) Impact assessment of repeated exposure of organotypic 3D bronchial and nasal tissue culture models to whole cigarette smoke. J. Vis. Exp. 96:e52325. (PMID: 25741927).

Abstract: Cigarette smoke (CS) has a major impact on lung biology and may result in the development of lung diseases such as chronic obstructive pulmonary disease or lung cancer. To understand the underlying mechanisms of disease development, it would be important to examine the impact of CS exposure directly on lung tissues. However, this approach is difficult to implement in epidemiological studies because lung tissue sampling is complex and invasive. Alternatively, tissue culture models can facilitate the assessment of exposure impacts on the lung tissue. Submerged 2D cell cultures, such as normal human bronchial epithelial (NHBE) cell cultures, have traditionally been used for this purpose. However, they cannot be exposed directly to smoke in a similar manner to the in vivo exposure situation. Recently developed 3D tissue culture models better reflect the in vivo situation because they can be cultured at the air-liquid interface (ALI). Their basal sides are immersed in the culture medium; whereas, their apical sides are exposed to air. Moreover, organotypic tissue cultures that contain different type of cells, better represent the physiology of the tissue in vivo. In this work, the utilization of an in vitro exposure system to expose human organotypic bronchial and nasal tissue models to mainstream CS is demonstrated. Ciliary beating frequency and the activity of cytochrome P450s (CYP) 1A1/1B1 were measured to assess functional impacts of CS on the tissues. Furthermore, to examine CS-induced alterations at the molecular level, gene expression profiles were generated from the tissues following exposure. A slight increase in CYP1A1/1B1 activity was observed in CS-exposed tissues compared with air-exposed tissues. A network-and transcriptomics-based systems biology approach was sufficiently robust to demonstrate CS-induced alterations of xenobiotic metabolism that were similar to those observed in the bronchial and nasal epithelial cells obtained from smokers.

Lo Sasso G, Schlage WK, Boué S, Veljkovic E, Peitsch MC and Hoeng J (2016) The Apoe$^{-/-}$ mouse model: a suitable model to study cigarette smoke-induced cardiovascular and respiratory diseases. J. Transl. Med. 14:146. (PMID: 27207171).

Abstract: Atherosclerosis-prone apolipoprotein E-deficient (Apoe(-/-)) mice display poor lipoprotein clearance with subsequent accumulation of cholesterol ester-enriched particles in the blood, which promote the development of atherosclerotic plaques. Therefore, the Apoe(-/-) mouse model is well established for the study of human atherosclerosis. The systemic proinflammatory status of Apoe(-/-) mice also makes them good candidates for studying chronic obstructive pulmonary disease, characterized by pulmonary inflammation, airway obstruction, and emphysema, and which shares several risk factors with cardiovascular diseases, including smoking. Herein, we review the results from published studies using Apoe(-/-) mice, with a particular focus on work conducted in the context of cigarette smoke inhalation studies. The findings from these studies highlight the suitability of this animal model for researching the effects of cigarette smoking on atherosclerosis and emphysema.

Majeed S, Frentzel S, Wagner S, Kuehn D, Leroy P, Guy PA, Knorr A, Hoeng J and Peitsch MC (2014) Characterization of an in vitro aerosol exposure system (VITROCELL® 24/48) using mainstream cigarettes smoke (3R4F). Chem. Cent. J. 8:62. (PMID: 25411580).

Abstract:

BACKGROUND: Only a few exposure systems are presently available that enable cigarette smoke exposure of living cells at the air-liquid interface, of which one of the most versatile is the Vitrocell® system (Vitrocell® Systems GmbH). To assess its performance and optimize the exposure conditions, we characterized a Vitrocell® 24/48 system connected to a 30-port carousel smoking machine. The Vitrocell® 24/48 system allows for simultaneous exposure of 48 cell culture inserts using dilution airflow rates of 0-3.0 L/min and exposes six inserts per dilution. These flow rates represent cigarette smoke concentrations of 7-100%.

RESULTS: By characterizing the exposure inside the Vitrocell® 24/48, we verified that (I) the cigarette smoke aerosol distribution is uniform across all inserts, (II) the utility of Vitrocell® crystal quartz microbalances for determining the online deposition of particle mass on the inserts, and (III) the amount of particles deposited per surface area and the amounts of trapped carbonyls and nicotine were concentration dependent. At a fixed dilution airflow of 0.5 L/min, the results showed a coefficient of variation of 12.2% between inserts of the Vitrocell® 24/48 module, excluding variations caused by different runs. Although nicotine and carbonyl concentrations were linear over the tested dilution range, particle mass deposition increased nonlinearly. The observed effect on cell viability was well-correlated with increasing concentration of cigarette smoke.

CONCLUSIONS: Overall, the obtained results highlight the suitability of the Vitrocell® 24/48 system to assess the effect of cigarette smoke on cells under air-liquid interface exposure conditions, which is closely related to the conditions occurring in human airways.

Marescotti D, Gonzalez-Suarez I, Acali S, Johne S, Laurent A, Frentzel S, Hoeng J and Peitsch MC (2016) High content screening analysis to evaluate the toxicological effects of harmful and potentially harmful constituents (HPHC). J. Vis. Exp. 111:e53987. (PMID: 27228213).

Abstract: Cigarette smoke (CS) is a major risk factor for cardiovascular and lung diseases. Because CS is a complex aerosol containing more than 7,000 chemicals it is challenging to assess the contributions of individual constituents to its overall toxicity. Toxicological profiles of

individual constituents as well as mixtures can be however established in vitro, by applying high through-put screening tools, which enable the profiling of Harmful and Potentially Harmful Constituents (HPHCs) of tobacco smoke, as defined by the U.S. Food and Drug Administration (FDA). For an initial assessment, an impedance-based instrument was used for a real-time, label-free assessment of the compound's toxicity. The instrument readout relies on cell adhesion, viability and morphology that all together provide an overview of the cell status. A dimensionless parameter, named cell index, is used for quantification. A set of different staining protocols was developed for a fluorescence imaging-based investigation and a HCS platform was used to gain more in-depth information on the kind of cytotoxicity elicited by each HPHC. Of the 15 constituents tested, only five were selected for HCS-based analysis as they registered a computable LD50 (< 20 mM). These included 1-aminonaphtalene, Arsenic (V), Chromium (VI), Crotonaldehyde and Phenol. Based on their effect in the HCS, 1-aminonaphtalene and Phenol could be identified to induce mitochondrial dysfunction, and, together with Chromium (VI) as genotoxic based on the increased histone H2AX phosphorylation. Crotonaldehyde was identified as an oxidative stress inducer and Arsenic as a stress kinase pathway activator. This study demonstrates that a combination of impedance-based and HCS technologies provides a robust tool for in vitro assessment of CS constituents.

Mathis C, Poussin C, Weisensee D, Gebel S, Hengstermann A, Sewer A, Belcastro V, Xiang Y, Ansari S, Wagner S, Hoeng J and Peitsch MC (2013) Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers. Am. J. Physiol. Lung Cell. Mol. Physiol. 304:L489-503. (PMID: 23355383).

Abstract: Organotypic culture of human primary bronchial epithelial cells is a useful in vitro system to study normal biological processes and lung disease mechanisms, to develop new therapies, and to assess the biological perturbations induced by environmental pollutants. Herein, we investigate whether the perturbations induced by cigarette smoke (CS) and observed in the epithelium of smokers' airways are reproducible in this in vitro system (AIR-100 tissue), which has been shown to recapitulate most of the characteristics of the human bronchial epithelium. Human AIR-100 tissues were exposed to mainstream CS for 7, 14, 21, or 28 min at the air-liquid interface, and we investigated various biological endpoints [e.g., gene expression and microRNA profiles, matrix metalloproteinase 1 (MMP-1) release] at multiple postexposure time points (0.5, 2, 4, 24, 48 h). By performing a Gene Set Enrichment Analysis, we observed a significant enrichment of human smokers' bronchial epithelium gene signatures derived from different public transcriptomics datasets in CS-exposed AIR-100 tissue. Comparison of in vitro microRNA profiles with microRNA data from healthy smokers highlighted various highly translatable microRNAs associated with inflammation or with cell cycle processes that are known to be perturbed by CS in lung tissue. We also found a dose-dependent increase of MMP-1 release by AIR-100 tissue 48 h after CS exposure in agreement with the known effect of CS on this collagenase expression in smokers' tissues. In conclusion, a similar biological perturbation than the one observed in vivo in smokers' airway epithelium could be induced after a single CS exposure of a human organotypic bronchial epithelium-like tissue culture.

Stinn W, Berges A, Meurrens K, Buettner A, Gebel S, Lichtner RB, Janssens K, Veljkovic E, Xiang Y, Roemer E and Hausmann HJ (2013) Towards the validation of a lung tumorigenesis model with mainstream cigarette smoke inhalation using the A/J mouse. Toxicology 305:49-64. (PMID: 23357402).

Abstract: A generally accepted and validated laboratory model for smoking-associated pulmonary tumorigenesis would be useful for both basic and applied research applications, such as the development of early diagnostic endpoints or the evaluation of modified risk tobacco products, respectively. The A/J mouse is susceptible for developing both spontaneous and induced lung adenomas and adenocarcinomas, and increased lung tumor multiplicities were also observed in previous cigarette smoke inhalation studies. The present study was designed to collect data useful towards the validation of an 18-month mainstream smoke (MS) inhalation model. Male and female A/J mice were exposed whole-body at three MS concentration levels for 6h/day, and the results were compared to a previous study in the same laboratory and with a similar design. A linear MS concentration-dependent increase in lung tumorigenesis was observed with similar slopes for both sexes and both studies and a maximal 5-fold increase in multiplicity beyond sham control. The minimal detectable difference in lung tumor multiplicity for the current study was 37%. In the larynx, papillomas were detectable in all MS-exposed groups in a non-concentration dependent manner. No other extra-pulmonary MS-dependent neoplastic lesions were found. Gene expression signatures of lung tumor tissues allowed a clear differentiation of sham- and high dose MS-exposed mice. In combination with data from previous smoke inhalation studies with A/J mice, the current data suggest that this model for MS inhalation-induced pulmonary tumorigenesis is reliable and relevant, two crucial requirements towards validation of such a model.

Talikka M, Kostadinova R, Xiang Y, Mathis C, Sewer A, Majeed S, Kuehn D, Frentzel S, Geertz M, Martin F, Ivanov N, Peitsch MC and Hoeng J (2014) The response of human nasal and bronchial organotypic tissue cultures to repeated whole cigarette smoke exposure. Int. J. Toxicol. 33:506-517. (PMID: 25297719).

Abstract: Exposure to cigarette smoke (CS) is linked to the development of respiratory diseases, and there is a need to understand the mechanisms whereby CS causes damage. Although animal models have provided valuable insights into smoking-related respiratory tract damage, modern toxicity testing calls for reliable in vitro models as alternatives for animal experimentation. We report on a repeated whole mainstream CS exposure of nasal and bronchial organotypic tissue cultures that mimic the morphological, physiological, and molecular attributes of the human respiratory tract. Despite the similar cellular staining and cytokine secretion in both tissue types, the transcriptomic analyses in the context of biological network models identified similar and diverse biological processes that were impacted by CS-exposed nasal and bronchial cultures. Our results demonstrate that nasal and bronchial tissue cultures are appropriate in vitro models for the assessment of CS-induced adverse effects in the respiratory system and promising alternative to animal experimentation.

Titz B, Elamin A, Martin F, Schneider T, Dijon S, Ivanov NV, Hoeng J and Peitsch MC (2014) Proteomics in Systems Toxicology. Comput. Struct. Biotechnol. J. 11:73-90. (PMID: 25379146).

Abstract: Current toxicology studies frequently lack measurements at molecular resolution to enable a more mechanism-based and predictive toxicological assessment. Recently, a systems toxicology assessment framework has been proposed, which combines conventional toxicological assessment strategies with system-wide measurement methods and computational analysis approaches from the field of systems biology. Proteomic measurements are an integral component of this integrative strategy because protein alterations closely mirror biological effects, such as biological stress responses or global tissue alterations. Here, we provide an overview of the technical foundations and highlight select applications of proteomics for systems toxicology studies. With a focus on mass spectrometry-based proteomics, we summarize the experimental methods for quantitative proteomics and describe the computational approaches used to derive biological/mechanistic insights from these datasets. To illustrate how proteomics has been successfully employed to address mechanistic questions in toxicology, we summarized several case studies. Overall, we provide the technical and conceptual foundation for the integration of proteomic measurements in a more comprehensive systems toxicology assessment framework. We conclude that, owing to the critical importance of protein-level measurements and recent technological advances, proteomics will be an integral part of integrative systems toxicology approaches in the future.

Verification and data sharing

Belcastro V, Poussin C, Xiang Y, Giordano M, Parijat Tripathi K, Boda A, Tugrul Balci A, Bilgen I, Kumar Dhand S, Duan Z, Gong X, Kumar R, Romero R, Sinan Sarac O, Tarca AL, Wang P, Yang H, Yang W, Zhang C, Boué S, Guarracino MR, Martin F, Peitsch MC and Hoeng J (2017) The sbv IMPROVER Systems Toxicology Computational Challenge: Identification of Human and Species-Independent Blood Response Markers as Predictors of Smoking Exposure and Cessation Status. Computational Toxicology, *in press*. doi: 10.1016/j.comtox.2017.07.004. → Verification of the blood-based gene expression signature of smoking exposure described in Martin *et al.*, 2015 and used in Martin *et al.*, 2016. Article linked to Poussin *et al., 2017.*

Abstract: Cigarette smoking entails chronic exposure to a mixture of harmful chemicals that trigger molecular changes over time, and is known to increase the risk of developing diseases. Risk assessment in the context of 21st century toxicology relies on the elucidation of mechanisms of toxicity and the identification of exposure response markers, usually from high-throughput data, using advanced computational methodologies. The sbv IMPROVER Systems Toxicology computational challenge (Fall 2015-Spring 2016) aimed to evaluate whether robust and sparse (≤40 genes) human (sub-challenge 1, SC1) and species-independent (sub-challenge 2, SC2) exposure response markers (so called gene signatures) could be extracted from human and mouse blood transcriptomics data of current (S), former (FS) and never (NS) smoke-exposed subjects as predictors of smoking and cessation status. Best-performing computational methods were identified by scoring anonymized participants' predictions. Worldwide participation resulted in 12 (SC1) and six (SC2) final submissions qualified for scoring. The results showed that blood gene expression data were informative to predict smoking exposure (i.e. discriminating smoker versus never or former smokers) status in human and across species with a high level of accuracy. By contrast, the prediction of cessation status (i.e. distinguishing FS from NS) remained challenging, as reflected by lower classification performances. Participants successfully developed inductive predictive models and extracted human and species-independent gene signatures, including genes with high consensus across teams. Post-challenge analyses highlighted "feature selection" as a key step in the process of building a classifier and confirmed the importance of testing a gene signature in independent cohorts to ensure the generalized applicability of a predictive model at a population-based level. In conclusion, the Systems Toxicology challenge demonstrated the feasibility of extracting a consistent blood-based smoke exposure response gene signature and further stressed the importance of independent and unbiased data and method evaluations to provide confidence in systems toxicology-based scientific conclusions.

Boué S, Exner T, Ghosh S, Belcastro V, Dokler J, Page D, Boda A, Bonjour F, Hardy B, Vanscheeuwijck P, Hoeng J and Peitsch MC (2017) Supporting evidence-based analysis for modified risk tobacco products through a toxicology data-sharing infrastructure. F1000Research 2017, 6:12. [version 1; referees: awaiting peer review]. doi: 10.12688/f1000research.10493.1.

Abstract: The US FDA defines modified risk tobacco products (MRTPs) as products that aim to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products. Establishing a product's potential as an MRTP requires scientific substantiation including toxicity studies and measures of disease risk relative to those of cigarette smoking. Best practices encourage verification of the data from such studies through sharing and open standards. Building on the experience gained from the OpenTox project, a proof-of-concept database and website (INTERVALS) has been developed to share results from both in vivo inhalation studies and in vitro studies conducted by Philip Morris International R&D to assess candidate MRTPs. As datasets are often generated by diverse methods and standards, they need to be traceable, curated, and the methods used well described so that knowledge can be gained using data science principles and tools. The data-management framework described here accounts for the latest standards of data sharing and research reproducibility. Curated data and methods descriptions have been prepared in ISA-Tab format and stored in a database accessible via a search portal on the INTERVALS website. The portal allows users to browse the data by study or mechanism (e.g., inflammation, oxidative stress) and obtain information relevant to study design, methods, and the most important results. Given the successful development of the initial infrastructure, the goal is to grow this initiative and establish a public repository for 21st-century preclinical systems toxicology MRTP assessment data and results that supports open data principles.

Hoeng J, Stolovitzky G and Peitsch MC (2013) sbv IMPROVER Diagnostic Signature Challenge. Syst. Biomed. 1:193-195. (Link).

Abstract: The task of predicting disease phenotype from gene expression data has been addressed hundreds if not thousands of times in the recent literature. This expanding body of work is not only an indication that the problem is of great importance and general interest, but it also reveals that neither the experimental nor the

computational limitations of translating data to disease information have been satisfactorily understood. To contribute to the advancement of the field, promote collaborative thinking and enable a fair and unbiased comparison of methods, IMPRO VER revisited the problem of gene-expression to phenotype prediction using a collaborative-competition paradigm. This special issue of Systems Biomedicine reports the results of the sbvIMPROVER Diagnostic Signature Challenge designed to identify best analytic approaches to predict phenotype from gene expression data.

Meyer P, Alexopoulos LG, Bonk T, Cho C, Califano A, de Graaf D, de la Fuente A, Hartemink A, Hoeng J, Ivanov NV, Koeppl H, Linding R, Marbach D, Norel R, Peitsch MC, Rice JJ, Royyuru A, Schacherer F, Sprengel J, Stolle K, Vitkup D and Stolovitzky G (2011) Verification of systems biology research in the age of collaborative-competition. Nature Biotechnol. 29:811-815. (PMID: 21904331). No abstract available.

Meyer P, Hoeng J, Rice JJ, Norel R, Sprengel J, Stolle K, Bonk T, Corthesy S, Royyuru A, Peitsch MC and Stolovitzky G (2012) Industrial Methodology for Process Verification in Research (IMProVeR): Towards systems biology verification. Bioinformatics 28:1193-1201. (PMID: 22423044).

Abstract:

MOTIVATION: Analyses and algorithmic predictions based on high-throughput data are essential for the success of systems biology in academic and industrial settings. Organizations, such as companies and academic consortia, conduct large multi-year scientific studies that entail the collection and analysis of thousands of individual experiments, often over many physical sites and with internal and outsourced components. To extract maximum value, the interested parties need to verify the accuracy and reproducibility of data and methods before the initiation of such large multi-year studies. However, systematic and well-established verification procedures do not exist for automated collection and analysis workflows in systems biology which could lead to inaccurate conclusions.

RESULTS: We present here, a review of the current state of systems biology verification and a detailed methodology to address its shortcomings. This methodology named 'Industrial Methodology for Process Verification in Research' or IMPROVER, consists on evaluating a research program by dividing a workflow into smaller building blocks that are individually verified. The verification of each building block can be done internally by members of the research program or externally by 'crowd-sourcing' to an interested community. www.sbvimprover.com

IMPLEMENTATION: This methodology could become the preferred choice to verify systems biology research workflows that are becoming increasingly complex and sophisticated in industrial and academic settings.

Poussin C, Belcastro V, Martin F, Boué S, Peitsch MC and Hoeng J. (2017) Crowd-sourced verification of computational methods and data in systems toxicology: a case study with a heat-not-burn candidate modified risk tobacco product. Chem. Res. Toxicol. 30:934-945. (PMID: 28085253). → Also includes the verification of the blood signature-based classification of REXC participants.

Abstract: Systems toxicology intends to quantify the effect of toxic molecules in biological systems and unravel their mechanisms of toxicity. The development of advanced computational methods is required for analyzing and integrating high throughput data generated for this purpose as well as for extrapolating predictive toxicological outcomes and risk estimates. To ensure the performance and reliability of the methods and verify conclusions from systems toxicology data analysis, it is important to conduct unbiased evaluations by independent third parties. As a case study, we report here the results of an independent verification of methods and data in systems toxicology by crowdsourcing. The sbv IMPROVER systems toxicology computational challenge aimed to evaluate computational methods for the development of blood-based gene expression signature classification models with the ability to predict smoking exposure status. Participants created/trained models on blood gene expression data sets including smokers/mice exposed to 3R4F (a reference cigarette) or noncurrent smokers/Sham (mice exposed to air). Participants applied their models on unseen data to predict whether subjects classify closer to smoke-exposed or nonsmoke exposed groups. The data sets also included data from subjects that had been exposed to potential modified risk tobacco products (MRTPs) or that had switched to a MRTP after exposure to conventional cigarette smoke. The scoring of anonymized participants' predictions was done using predefined metrics. The top 3 performers' methods predicted class labels with area under the precision recall scores above 0.9. Furthermore, although various computational approaches were used, the crowd's results confirmed our own data analysis outcomes with regards to the classification of MRTP-related samples. Mice exposed directly to a MRTP were classified closer to the Sham group. After switching to a MRTP, the confidence that subjects belonged to the smoke-exposed group decreased significantly. Smoking exposure gene signatures that contributed to the group separation included a core set of genes highly consistent across teams such as AHRR, LRRN3, SASH1, and P2RY6. In conclusion, crowdsourcing constitutes a pertinent approach, in complement to the classical peer review process, to independently and unbiasedly verify computational methods and data for risk assessment using systems toxicology.

Rhrissorrakrai K, Rice JJ, Boue S, Talikka M, Bilal E, Martin F, Meyer P, Norel R, Xiang Y, Stolovitzky G, Hoeng J and Peitsch MC (2013) Diagnostic signature challenge. Design and results. Syst. Biomed. 1:196-207. (Link).

Abstract: The sbvIMPROVER (systems biology verification—Industrial Methodology for Process Verification in Research) process aims to help companies verify component steps or tasks in larger research workflows for industrial applications. IMPROVER is built on challenges posed to the community that draws on the wisdom of crowds to assess the most suitable methods for a given research task. The Diagnostic Signature Challenge, open to the public from Mar. 5 to Jun. 21, 2012, was the first instantiation of the IMPROVER methodology and evaluated a fundamental biological question, specifically, if there is sufficient information in gene expression data to diagnose diseases. Fifty-four teams used publically available data to develop prediction models in four disease areas: multiple sclerosis, lung cancer, psoriasis, and chronic obstructive pulmonary disease. The predictions were scored against unpublished, blinded data provided by the organizers, and the results, including methods of the top performers, presented at a

conference in Boston on Oct. 2–3, 2012. This paper offers an overview of the Diagnostic Signature Challenge and the accompanying symposium, and is the first article in a special issue of Systems Biomedicine, providing focused reviews of the submitted methods and general conclusions from the challenge. Overall, it was observed that optimal method choice and performance appeared largely dependent on endpoint, and results indicate the psoriasis and lung cancer subtypes subchallenges were more accurately predicted, while the remaining classification tasks were much more challenging. Though no one approach was superior for every sub-challenge, there were methods, like linear discriminant analysis, that were found to perform consistently well in all.

Tarca AL, Lauria M, Unger M, Bilal E, Boue S, Dey KK, Hoeng J, Koeppl H, Martin F, Meyer P, Nandy P, Norel R, Peitsch MC, Rice JJ, Romero R, Stolovitzky G, Talikka M, Xiang Y, Zechner C and IMPROVER DSC Collaborators (2013) Strengths and limitations of microarray-based phenotype prediction: Lessons learned from the IMPROVER Diagnostic Signature Challenge. Bioinformatics 29:2892-2899. (PMID: 23966112).

Abstract:

MOTIVATION: After more than a decade since microarrays were used to predict phenotype of biological samples, real-life applications for disease screening and identification of patients who would best benefit from treatment are still emerging. The interest of the scientific community in identifying best approaches to develop such prediction models was reaffirmed in a competition style international collaboration called IMPROVER Diagnostic Signature Challenge whose results we describe herein.

RESULTS: Fifty-four teams used public data to develop prediction models in four disease areas including multiple sclerosis, lung cancer, psoriasis and chronic obstructive pulmonary disease, and made predictions on blinded new data that we generated. Teams were scored using three metrics that captured various aspects of the quality of predictions, and best performers were awarded. This article presents the challenge results and introduces to the community the approaches of the best overall three performers, as well as an R package that implements the approach of the best overall team. The analyses of model performance data submitted in the challenge as well as additional simulations that we have performed revealed that (i) the quality of predictions depends more on the disease endpoint than on the particular approaches used in the challenge; (ii) the most important modeling factor (e.g. data preprocessing, feature selection and classifier type) is problem dependent; and (iii) for optimal results datasets and methods have to be carefully matched. Biomedical factors such as the disease severity and confidence in diagnostic were found to be associated with the misclassification rates across the different teams.

AVAILABILITY: The lung cancer dataset is available from Gene Expression Omnibus (accession, GSE43580). The maPredictDSC R package implementing the approach of the best overall team is available at www.bioconductor.org or http://bioinformaticsprb.med.wayne.edu/.

Biomarkers

Martin F, Talikka M, Hoeng J and Peitsch MC (2015) Identification of gene expression signature for cigarette smoke exposure response - from man to mouse. Hum. Exp. Toxicol. 34:1200-1211. (PMID: 26614807).

Abstract: Gene expression profiling data can be used in toxicology to assess both the level and impact of toxicant exposure, aligned with a vision of 21st century toxicology. Here, we present a whole blood-derived gene signature that can distinguish current smokers from either nonsmokers or former smokers with high specificity and sensitivity. Such a signature that can be measured in a surrogate tissue (whole blood) may help in monitoring smoking exposure as well as discontinuation of exposure when the primarily impacted tissue (e.g., lung) is not readily accessible. The signature consisted of LRRN3, SASH1, PALLD, RGL1, TNFRSF17, CDKN1C, IGJ, RRM2, ID3, SERPING1, and FUCA1. Several members of this signature have been previously described in the context of smoking. The signature translated well across species and could distinguish mice that were exposed to cigarette smoke from ones exposed to air only or had been withdrawn from cigarette smoke exposure. Finally, the small signature of only 11 genes could be converted into a polymerase chain reaction-based assay that could serve as a marker to monitor compliance with a smoking abstinence protocol.

Clinical Research

Weitkunat R, Baker G and Lüdicke F (2016) Intention-to-Treat Analysis but for Treatment Intention: How should consumer product randomized controlled trials be analyzed? Int. J. Stats. Med. Res. 5:90-98. (link).

Abstract:

BACKGROUND: Experimental study design, randomization, blinding, control, and the analysis of such data according to the intention-to-treat (ITT) principle are de-facto “gold standards” in pharmacotherapy research. While external treatment allocation under conditions of medical practice is conceptually reflected by in-study randomization in randomized controlled trials (RCTs) of therapeutic drugs, actual product use is based on self-selection in a consumer product setting.

DISCUSSION: With in-market product allocation being consumer-internal, there is no standard against which protocol adherence can be attuned, and the question arises, as to whether compliance-based analysis concepts reflect the real-world effects of consumer products.

SUMMARY: The lack of correspondence between RCTs and consumer market conditions becomes evident by the fact that even if, theoretically, all data would be available from all members of the real-world target population, it would be impossible to calculate either an ITT or a per-protocol effect. This renders the calculation of such estimates meaningless in consumer product research contexts.

Epidemiology

Weitkunat R, Lee PN, Baker G, Sponsiello-Wang Z, Gonzalez-Zuloeta Ladd AM and Lüdicke F (2015) A novel approach to assess the population health impact of introducing a Modified Risk Tobacco Product. Regul. Toxicol. Pharmacol. 72: 87-93. (PMID: 25819932).

Abstract: Based on the Food and Drug Administration's Modified Risk Tobacco Product (MRTP) Application draft guideline, Philip Morris International (PMI) has developed a Population Health Impact Model to estimate the reduction in the number of deaths over a period following

the introduction of an MRTP. Such a model is necessary to assess the effect that its introduction would have on population health, given the lack of epidemiological data available prior to marketing authorization on any risks from MRTPs. The model is based on publicly available data on smoking prevalence and on the relationships between smoking-related disease-specific mortality and various aspects of the smoking of conventional cigarettes (CCs), together with an estimate of exposure from the MRTP relative to that from CCs, and allows the exploration of possible scenarios regarding the effect of MRTP introduction on the prevalence of CC and MRTP use, individually and in combination. By comparing mortality attributable in a scenario where the MRTP is introduced with one where it is not, the model can estimate the mortality attributable to CCs and the MRTP, as well as the reduction in the deaths attributable to the introduction of the MRTP.

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PMI SCIENCE
PHILIP MORRIS INTERNATIONAL

Appendix Z: Quality Management System

Standards by Assessment Step

Philip Morris International R&D

At each step of the RRP assessment, we apply rigorous scientific standards during data generation. A risk-based Quality Management System (QMS) has been developed to coordinate and guide activities with the aim of ensuring quality and integrity of data and product during its complete lifecycle, from the conception through to commercialization. This QMS comprises the following elements:

Post-market studies	IEA GEP[1]; Applicable National Regulations
Perception and Behavior Assessment	GEP-DGEpi[2]; FDA Guidance on PRO[3]; ISPOR PGP for the TCA[4]; Applicable National Regulations
Clinical Assessment	WMA Declaration of Helsinki[5]; ICH GCP E6 (R1)[6]; Applicable National Regulations
Toxicological Assessment	The standard toxicology studies are conducted following OECD GLP[7], INVITTOX 3A/ERGATT/FRAME; OECD[8] Test Guidelines 412, 413, 471, 487, 451, 453, 490. The systems toxicology studies are conducted under a GLP-like quality system.
Platform Development	Product Design and Control: Quality by Design (QbD)[9] Aerosol Chemistry: OECD GLP; ISO[10] 17025; ICH Q2 (R1)[11]; ISO 3308*, 3402, 4387*, 8454, 10315:2013, 10362-1*, 13110, 19290; CORESTA CRM81[12] Indoor Air Quality: ISO 17025; EN 15251[13]; ISO 15593, 18144, 18144, 16814, 16000-6, 11454

* With slight modifications needed to adapt to RRPs.

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[1] IEA Guidelines for proper conduct in epidemiologic research (2007). Available at: http://ieaweb.org/good-epidemiological-practice-gep/.
[2] Good Epidemiologic Practice (GEP) - German Society for Epidemiology (DGEpi). Available at: http://www.dgepi.de/short-english-summary.html.
[3] Food and Drug Administration (2009) Guidance for Industry - Patient-Reported Outcome Measures: Use in Medical Product Development to Support Labeling Claims. Available at: https://www.fda.gov/downloads/drugs/guidances/ucm193282.pdf.
[4] Wild D, Grove A, Martin M, Eremenco S, McElroy S, Verjee-Lorenz A and Erikson P; ISPOR Task Force for Translation and Cultural Adaptation (2005) Principles of good practice for the translation and cultural adaptation process for patient-reported outcomes (PRO) measures: report of the ISPOR task force for translation and cultural adaptation. Value Health 2:94-104. (PMID: 15804318).
[5] World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. Available at: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/.
[6] ICH - Guideline for Good Clinical Practice. Available at: http://www.ich.org/products/guidelines/efficacy/efficacy-single/article/good-clinical-practice.html.
[7] OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring. Available at: http://www.oecd.org/chemicalsafety/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm.
[8] OECD testing guidelines can be found at: http://www.oecd.org/
[9] Juran JM (1992) Juran on Quality by Design: The New Steps for Planning Quality into Goods and Services. Free Press. ISBN 9780029166833.
[10] International Organization for Standardization. All standards can be found at: https://www.iso.org/standards.html
[11] ICH - Validation of Analytical Procedures: Text and Methodology. Available at: http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html.
[12] CORESTA Recommended Method 81. Available at: https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf.
[13] European Committee for Standardization, 2006. CEN European Standard EN 15251. Indoor Environmental Input Parameters for Design and Assessment of Energy Performance of Buildings Addressing Indoor Air Quality, Thermal Environment, Lighting and Acoustics. European Committee for Standardization, Brussels.

Assessment Steps	Study Type / Test Guideline[a]	QMS[c]	Study Name	THS Version	Comparator Groups	Completed / End date	Related Publications[k]
Aerosol Chemistry and Physics	Aerosol Chemistry	GLP / ISO 17025	Aerosol composition	THS 2.2[d] THS 2.2M	3R4F[e]	Completed Completed	Schaller et al. (2016a) Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47. Schaller et al. (2016b) Regul. Toxicol. Pharmacol. 81 Suppl 2:S48-S58.
			Aerosol composition	THS 2.2	Commercial Products	Completed	Jaccard et al. (2017) Regul. Toxicol. Pharmacol. 90:1-8.
			Solid particle analysis	THS 2.2	3R4F	Completed	Pratte et al. (2016) Hum. Exp. Toxicol. In press.
			Indoor air quality	THS 2.2	Commercial Product	Completed	Mitova et al. (2016) Regul. Toxicol. Pharmacol. 80:91-101.
Standard Toxicology *in vitro*	INVITTOX 3a	GLP	*In vitro* cytotoxicity	THS 2.2M THS 2.2	3R4F	Completed Completed	Schaller et al. (2016a) Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47.
	OECD TG471	GLP	*In vitro* mutagenicity	THS 2.2 THS 2.2M	3R4F	Completed Completed	Schaller et al. (2016a) Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47.
	OECD TG490	GLP	*In vitro* mutagenicity	THS 2.2M THS 2.2	3R4F	Completed Completed	Schaller et al. (2016a) Regul. Toxicol. Pharmacol. 81 Suppl 2:S27-S47.
Standard Toxicology *in vivo*	OECD TG413 Plus[b]	GLP / SysTox QMS	90-day inhalation studies	THS 2.2	3R4F, Sham (Fresh Air)	Completed	Wong et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S59-S81. Oviedo et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S93-S122. Sewer et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S82-S92. Kogel et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S123-S138.
				THS 2.2M		Completed	
	OECD TG453 Plus	GLP / SysTox QMS	A/J mouse 18-month inhalation study	THS 2.2	3R4F, Sham (Fresh Air)	Second Quarter 2018	

[a,c] Complete list provided under *Quality Management System*.
[b] Refers to systems toxicology-augmented OECD testing guidelines defined by PMI approach.
[d] THS 2.2 is the regular version; THS 2.2M is the mentholated version.
[e] 3R4F refers to the reference combustible cigarette.
[f,g] Own brand cigarette (CC) and mentholated cigarette (mCC).
[h] Nasal nicotine spray.
[i] Smoking abstinence for the duration of the study.
[k] Publications with abstracts and links to www.PubMed.gov are available in *Appendix C*.

Assessment Steps	Study Type / Test Guideline[a]	QMS[c]	Study Name	THS Version	Comparator Groups	Completed / End date	Related Publications[k]
Systems Toxicology *in vitro*	Whole Aerosol Exposure of human organotypic tissue cultures	SysTox QMS	Bronchial Nasal Oral Gingival	THS 2.2	3R4F	Completed Completed Completed Completed	Iskandar et al. (2017b) Toxicol. In Vitro 39:29-51. Iskandar et al. (2017a) Altex 34:23-48. Zanetti et al. (2016) Chem. Res. Toxicol. 29:1252-1269. Zanetti et al. (2017) Food Chem. Toxicol. 101:15-35.
	Human monocyte adhesion to human endothelial cells	Research Study	In vitro cell adhesion assay	THS 2.2	3R4F	Completed	Poussin et al. (2016) Toxicology 339:73-86.
	Human monocyte transmigration and chemotaxis	Research Study	In vitro chemotaxis and transendothelial migration assay	THS 2.2	3R4F	Completed	van der Toorn et al. (2015) Food Chem. Toxicol. 86:81-87.
	High Content Screening of human primary cells exposed to aerosol fractions	Research Study	In vitro Normal Bronchial Epithelial Cell assay	THS 2.2	3R4F	Completed	Gonzalez Suarez et al. (2016) Chem. Res. Toxicol. 29:3-18.
Systems Toxicology *in vivo*	Switching study in animal model of disease	SysTox QMS	ApoE mouse 8-month inhalation study	THS 2.2	3R4F, Sham (Fresh Air), Cessation, Switching	Completed	Phillips et al. (2016) Toxicol. Sci. 149:411-432. Lo Sasso et al. (2016) Inhal. Toxicol. 28:226-240. Szostak et al. (2017) Food Chem. Toxicol. 101:157-167. Titz et al. (2016) Toxicol. Sci. 149:441-457.

Assessment Steps	Study Type / Test Guideline[a]	QMS[c]	Study Name	THS Version	Comparator Groups	Completed / End date	Related Publications[k]
Clinical Trials	PK/PD with single stick use	GCP	ZRHR-PK-01-EU / NCT01967732	THS 2.2	CC[f], NRT (NNS[h])	Completed	Brossard et al. (2017) Regul. Toxicol. Pharmacol. 89:193-199. Marchand et al. (2017) Eur. J. Drug Metab. Pharmacokinet. In press.
			ZRHR-PK-02-JP / NCT01959607	THS 2.2	CC, NRT (nicotine gum)	Completed	
			ZRHM-PK-05-JP / NCT01967706	THS 2.2M	mCC[g], NRT (nicotine gum)	Completed	
			ZRHM-PK-06-US / NCT01967719	THS 2.2M	mCC, NRT (NNS)	Completed	
	Reduced Exposure Study, 5-day, confinement, ad libitum use	GCP	ZRHR-REXC-03-EU / NCT01959932	THS 2.2	CC, SA[i]	Completed	Haziza et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S139-S150. Haziza et al. (2017) Data Brief 10:283-293. Martin et al. (2016) Regul. Toxicol. Pharmacol. 81 Suppl 2:S151-S157. Poussin et al. (2017) Chem. Res. Toxicol. 30:934-945.
			ZRHR-REXC-04-JP / NCT01970982	THS 2.2	CC, SA	Completed	Haziza et al. (2016). Regul. Toxicol. Pharmacol. 81:489-499.
	Reduced Exposure Study, 90-day, ambulatory, ad libitum use	GCP	ZRHM-REXA-07-JP / NCT01970995	THS 2.2 Menthol	mCC, SA	Completed	Lüdicke et al. (2017a) Nicotine Tob. Res. in press. Lüdicke et al. (2017b) Nicotine Tob. Res. in press.
			ZRHM-REXA-08-US / NCT01989156	THS 2.2 Menthol	mCC, SA	Completed	Manuscript in preparation
	Clinical Risk Marker study, ambulatory, ad libitum use, 6+6 months	GCP	ZRHR-ERS-09-US / NCT02396381	THS 2.2	CC	End 2017	
			ZRHR-ERS-09-EXT-US / NCT02649556	THS 2.2	CC	End 2017	