Tobacco Heating System (THS 2.2): Scientific References, Abstracts, and Key Methods Classified by Assessment Step

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Description of the assessment program

Figure 1: PMI's assessment program for candidate reduced-risk products. 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 reduced-risk products 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 candidate reduced-risk products.

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)

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 (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: 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 (2016) 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. Under revision.

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)

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. (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 (2017) 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 (2017). 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.

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

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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