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Investigating a toxic risk (self-inflicted) the example of conventional and advanced studies of a novel Tobacco Heating System

Keywords:
Modified risk tobacco product
Novel toxicity testing
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A B S T R A C T

This special issue of *Regulatory Toxicology and Pharmacology* contains 9 scientific papers from Philip Morris International about the laboratory and 1 about early clinical investigation of a novel 'Tobacco Heating System'. The studies have employed conventional and a wide range of newer 'omics and bioinformatics techniques to seek and explore potential toxic actions of the inhalable vapour it generates. The methods of study and display of results employed are considered to be a valuable guide and model for wider application in other toxicological investigations because they are directed more to proximal causes of effects than to the cruder distal end points revealed by conventional, empirical procedures. As such they should be regarded as a paradigm for the applicability and accuracy of the testing and prediction of toxic risks.

1. Introduction

Cigarette smoking has been known as a risk to human health for many years and investigation of diseases in active and passive smokers, the substances involved and the mechanisms of their effects have led to considerable advances in epidemiology, medicine and in *vivo* toxicity testing and in *vitro* studies of causal mechanisms. In a perverse way there has been some benefit from the grave harms produced to offset against the societal, financial and political impacts of smoking.

In the past fifteen years or so potential 'Modified Risk Tobacco Products' have been introduced in the expectation that those whose addiction to nicotine to the diseases if they inhaled vapours containing less or even none of the harmful substances generated in conventional cigarettes reliant on the combustion of tobacco. These materials have been developed by ingenious engineering to minimise or avoid known risks and to comply with the requirements of various official agencies governing the availability and nature of current and future tobacco products; see, for example, the US Family Smoking Prevention and Tobacco Control Act. Public Law No. 111–131 (June 22, 2009) and the Tobacco Products Directive of the EU (2014/40/EU). The best known type of new product has been the *e-cigarette* in which various materials are heated in such a way that there is no combustion, thus minimising production of many dangerous substances, whilst still providing the physical experience and sufficient nicotine to satisfy smokers who 'vape' with them.

The development and availability of the *e-cigarette* has led to a major difference of opinion between official agencies and medical academies in Europe and the USA, the former accepting that they offer real benefits to smokers and the latter being opposed to them and favouring only complete abstinence from cigarettes; see, for example, Green et al. (2016), McNeill et al. (2015) and Nutt et al. (2016). The controversy is more about overall benefit to smokers or its absence and there has been less direct consideration of the potential harmfulness of particular substances or groups of compounds and their effects to which users would be exposed apart from some uncertainty about the position of nicotine in the debate.

Consider how such a product – a tobacco product or some other material and heating device – might be investigated in advance to demonstrate whether it would deliver little if any of the complex mixture of known harmful products whilst still providing enough nicotine for customer satisfaction. A classical programme of testing would involve many chemical and biochemical analyses, studies of respiratory and other physiological effects, extensive inhalation toxicity testing with detailed studies of the respiratory tract and clinical evidence of tolerance and the absence of exposure to potentially harmful substances. If those experiments were done in the right way the results would be extrapolated to the human population as a whole, including the passive smoker, and some more or less conventional biomonitoring schemes could be devised to explore exposure in the real world. In the more distant future epidemiological surveys would be expected to demonstrate the expected fall in smoking–associated diseases but that would be a long way ahead and the picture would be confused by the serious problem of the air pollution our life style has caused. All that programme would depend on the availability and adequate extent of multiple, sophisticated analytical and other experimental technique, many equally applicable to *in vitro* and in *vivo* experiments

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and human samples to show the level of exposure to a very large number of substances and to demonstrate the degree or absence of relevant biological end points, such as DNA damage, reactive oxygen species and their effects, inflammatory changes and mutations in cells of the respiratory tree and elsewhere. That is a paradigm of classical toxicological methodologies based on studying diverse final end points, which often cannot be correlated with each other, and probably of having to try to extrapolate different end-points to common proximal but remote causal mechanisms.

Very considerable advances beyond this position are offered by recent developments in cellular and molecular biology, such the 'omics technologies and the broad data analytical possibilities of *bioinformatics*. The 10 papers in this special issue of *Regulatory Toxicology and Pharmacology* provide detailed reports of modern and conventional experimental investigations and a brief clinical study of a 'Tobacco Heating System' developed by Philp Morris International that display the power and additional value of the newer over more conventional toxicological techniques. The company deserves thanks for its openness in publishing the work as it can be a guide and stimulus to improvements in other areas of toxicological investigations.

Their scientists have shown how diverse 'omics techniques supplemented by sophisticated data analyses and displays make it possible to detect and follow not only complex secondary end point effects due to toxic exposures, such as *inflammation* seen simplistically as infiltrating cells or levels of a marker in a body fluid, but also to assess the primary causal mechanisms and processes in considerable detail. The breadth of the newer techniques makes broad surveys possible that can detect and reveal the detailed nature of many toxic actions and cellular responses and so to show what would be worth measuring to provide the earliest indication of a toxic action. As many of the samples and procedures are as applicable to laboratory experiments as to samples from humans they offer the possibility of developing very sensitive and quantitative monitoring of exposed people. Given quantitative results of these types dose-response analysis should become feasible based on real life circumstances rather than indirect extrapolations and that should support more rigorous prediction of toxic risks than can now be done based on current pragmatic observations.

2. Conclusions

The basic importance in toxicology of the newer procedures and analyses is that they can reveal primary effects and their mechanisms instead of the more conventional end-points, which can only represent the net balance of complex distal or even terminal actions and reactions involving different cellular mechanisms at different times and stages. It is true that over the past 150 years we have learnt ways in which empirical observations of end effects, such as tumour formation or altered metabolic pathways, can pragmatically be related to overall measures of exposure and dose, but how much safer and more reliable it will be to be able to relate exposures to primary effects and consequential lesions. And, the more accurate and focused information about pathogenic processes should support more precise quantification of harms and hence more accurate prediction of risk.

The programme of studies published here by Philip Morris International may not yet go quite so far but it does display the potential and indicates how it might be achieved. The body of their work may not be unique but it is very unusual to be able to follow such a comprehensive account of investigations and results. As such it can properly be regarded as a guide and goad to stimulate toxicologists and others and a presentation of real value to the toxicological community.

Acknowledgements

I am grateful to Philip Morris International for the invitation and support to write this foreword and for giving me early access to the final manuscripts of their publications. At all times I alone have been responsible for the text and opinions in this foreword.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.07.020.

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Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program

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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 (THS2.2). 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. THS2.2 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 THS2.2 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 THS2.2, 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 THS2.2 as an MRTP is demonstrated by the assessment and additional publications cited in this series.

1. Introduction

1.1. General

The U.S. Family Smoking Prevention and Tobacco Control Act defines a Modified Risk Tobacco Product (MRTP) as any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products (Family Smoking Prevention and Tobacco Control Act). This publication is part of a series of nine publications describing the nonclinical and part of the clinical assessment of a candidate MRTP, THS2.2 regular and a mentholated version (THS2.2M). The series of publications provides part of the overall scientific program to assess the potential for THS2.2 to be a reduced risk product. This first publication in this series describes THS2.2 and the assessment program for MRTPs. This is followed by six publications that describe the nonclinical assessment of THS2.2 regular and THS2.2M (Kogel et al., 2016; Oviedo et al., 2016; Schaller et al., 2016a; Schaller et al., 2016b; Sewer et al., 2016; Wong et al., 2016). The eighth publication in the series describes a clinical study to assess whether the reduced formation of Harmful and Potentially Harmful Constituents (HPHC) for THS2.2 regular also leads to reduced exposure to HPHCs when the product is used in a clinical setting (Haziza, 2016). A final publication utilizes data gathered from the reduced exposure clinical study on THS2.2 regular to determine if a systems pharmacology approach can identify exposure response markers in peripheral blood of smokers switching to THS2.2 (Martin et al., 2016).

Abbreviations: MRTP, Modified Risk Tobacco Product; THS2.2, Tobacco Heating System version 2.2 regular; THS2.2M, Tobacco Heating System version 2.2 menthol version; HPHC, Harmful and potentially harmful constituents; PMI, Philip Morris International; FSPTCA, Family Smoking Prevention and Tobacco Control Act; FDA, Food and Drug Administration; CTP, Center for Tobacco Products; CDER, Center for Drug Evaluation and Research; CC, Combustible Cigarette; CVD, Cardiovascular disease; COPD, Chronic obstructive lung disease; NPA, Network perturbation amplitude; BIF, Biological Impact Factor; OECD, Organization for Economic Cooperation and Development; PHIM, Population health impact model; HCI, Health Canada intense smoking regime; 3R4F, University of Kentucky Reference Cigarette; miRNA, Micro-ribonucleic acid; MRC, Mentholated reference cigarettes; SA, Smoking abstinence.

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1.2. Tobacco harm reduction

Cigarette smoking is one of the leading causes of preventable death both in the United States and globally. For many decades, the foundational principles of reducing this harm have been focused on preventing smoking initiation and promoting smoking cessation. In recent years, a third opportunity to reduce the harm from combusted tobacco products has emerged, based on switching consumers to less harmful products that have significantly reduced levels of toxic and harmful compounds. The United States Surgeon General (US Department of Health and Human Services (2010)) concluded that '*Inhaling the complex chemical mixture of combustion compounds in tobacco smoke causes adverse health outcomes, particularly cancer and cardiovascular and pulmonary diseases, through mechanisms that include DNA damage, inflammation and oxidative stress.*' It has long been known that the best way for smokers to reduce the adverse health consequences of smoking is to quit. However, though many smokers are interested in and attempt to quit, it can be very difficult to quit smoking cigarettes and hence the rates of long-term smoking cessation remain low. For example, according to the United States Surgeon General (US Department of Health and Human Services (2010)) although about 45% of smokers quit for a day, only approximately 5% succeed in achieving long-term abstinence for one year or longer.

As outlined by the U.K. Royal College of Physicians (Royal College of Physicians (2016)), '*Smoking is completely preventable, yet, more than half a century after the health harm of smoking first became widely known, almost 1 billion people worldwide still smoke. They do so primarily because they are addicted to the nicotine in tobacco smoke and, as this addiction can be extremely difficult to overcome, many will continue to smoke until they die.*'

Referring to an earlier report (Royal College of Physicians (2007)) that promoted the principle of harm reduction in nicotine addiction, the Tobacco Advisory Group of the U.K. Royal College of Physicians (Royal College of Physicians (2016)) stated that '*as most of the harm caused by smoking arises not from nicotine but from other components of tobacco smoke, the health and life expectancy of today's smokers could be radically improved by encouraging as many as possible to switch to a smoke-free source of nicotine. While recognizing the primacy of complete cessation of all tobacco and nicotine use as the ultimate goal to prevent harm from smoking, the report argued that promoting widespread substitution of cigarettes and other tobacco combustion products would, for smokers who made the change, achieve much the same thing. Harm reduction, as a complement to conventional tobacco control policies, could therefore offer a means to prevent millions of deaths among tobacco smokers in the UK alone.*'

As noted by McNeil (McNeil, 2012) '*Since nicotine itself is not a highly hazardous drug, encouraging smokers to obtain nicotine from sources that do not involve tobacco combustion is a potential means to reduce the morbidity and mortality they sustain, without the need to overcome their addiction to nicotine.*'

The harm reduction approach can be used to complement the existing strategies of reducing smoking related harm (i.e., preventing initiation and promoting cessation of smoking), to provide smokers with novel tobacco or nicotine containing products that are substantially less toxic than cigarettes. However, the potential public health benefit of such an approach will only be achieved if these novel nicotine products are scientifically substantiated to reduce risk and are acceptable alternatives that allow smokers to switch to the reduced-risk products.

Philip Morris International (PMI) is developing a portfolio of such novel nicotine products to address a wide range of adult smoker preferences where each product type is designed to significantly reduce or eliminate the formation of HPHCs in the inhaled aerosol while preserving as much as possible the taste, sensory experience, nicotine delivery profile and ritual characteristics of cigarettes.

The novel nicotine product described in this series of papers is a '*heat-not-burn*' tobacco product, which heats tobacco at a temperature below that required to initiate combustion. Different classes of tobacco constituents decompose at different temperatures, releasing chemical compounds into the aerosol. Heating at much lower temperatures than those found at the tip of a burning cigarette generates fewer and lower levels of HPHCs. The resulting aerosol contains nicotine but has significantly reduced levels of HPHCs compared with cigarette smoke.

The development of *heat-not-burn* tobacco products is not new and earlier efforts to develop such products (notably Premier and Eclipse products from R.J. Reynolds and Accord from Philip Morris) have been reviewed (Baker, 2006). Baker concluded that consumer acceptance of these products was low primarily because of sensory and usability issues, explaining their lack of commercial success. Consumer acceptance of reduced-risk products is crucially important if they are to be used in place of cigarettes and realize the potential to reduce risk for the individual smoker and for harm reduction at the population level (Fig. 1).

The studies presented in this series of papers form part of an assessment strategy to characterize a potentially reduced-risk product that generates an inhalable aerosol by heating tobacco instead of burning it. A description of this Tobacco Heating System (THS) version 2.2 is provided below, followed by an overview of our MRTP assessment strategy.

2. Product characteristics of THS2.2

THS 2.2 is a novel tobacco product type. It has three distinct components that perform different functions (Fig. 2): (i) a *tobacco stick* - a novel patent-pending tobacco product with processed tobacco made from tobacco powder, (ii) a *holder* into which the *tobacco stick* is inserted and which heats the tobacco material by means of an electronically controlled heating blade, and (iii) a *charger* that is used to recharge the *holder* after each use.

The THS2.2 product differs from a cigarette in significant ways. First, the *tobacco stick* does not contain tobacco cut-filler (tobacco leaf cut in small pieces found in cigarettes). Instead, the tobacco is ground and reconstituted into sheets (termed cast-leaf) following the addition of water, glycerin, guar gum and cellulose fibers. Second, the *tobacco stick* (Fig. 3) contains much smaller amounts of tobacco compared with a cigarette. The weight of the tobacco plug in the *tobacco stick* is approximately 320 mg compared with the 550–700 mg cut-filler found in conventional cigarettes. The reconstituted tobacco cast-leaf is fashioned into a small plug through a proprietary process known as '*crimping*'. Third, unlike a cigarette, the *tobacco stick* contains two unique and independent filters: (i) a polymer-film filter to cool the aerosol and (ii) a low-density cellulose acetate mouthpiece filter to mimic the sensory aspects of a cigarette. Furthermore, a hollow acetate tube separates the tobacco plug and the polymer-film filter.

To operate the THS2.2 product, the user inserts a *tobacco stick*

Fig. 1. The Harm Reduction Equation. Harm reduction at the population level is the result of the availability of a scientifically substantiated reduced-risk product that is an acceptable alternative to adult smokers and is not likely to attract non-smokers.

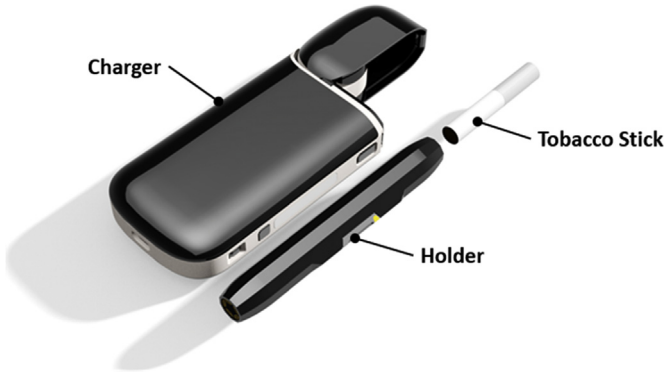

Fig. 2. The three components of the THS 2.2 product.

analysis of the inhalable aerosol delivered by the THS2.2 product in comparison with the smoke of a 3R4F reference cigarette (Schaller et al., 2016a).

3. The MRTP assessment strategy

In 2009, the United States Congress passed the Family Smoking Prevention and Tobacco Control Act (FSPTCA) granting the United States Food and Drug Administration (FDA) authority to regulate tobacco products, which, among other things, established the first regulatory procedure for reviewing an application and authorizing to market a reduced-risk tobacco product, (referred to in the U.S. law as a 'Modified Risk Tobacco Product,' or MRTP) (Family Smoking Prevention and Tobacco Control Act). An MRTP is defined by the FSPTCA as '*any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products.*' The FDA stated in its MRTP Draft Guidance document (Food and Drug Administration, 2012) that the MRTP provisions of the FSPTCA '*may be valuable tools in the effort to promote public health by reducing the morbidity and mortality associated with tobacco use, particularly if companies take advantage of these provisions by making bold, innovative product changes …* '

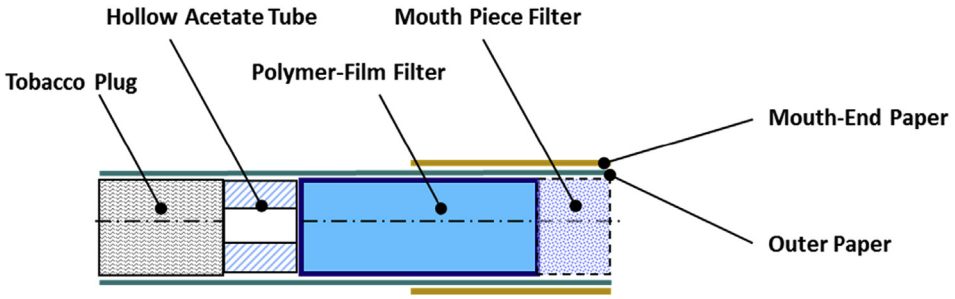

Fig. 3. Cross-sectional view of the *tobacco stick*.

into the *holder* and turns on the device by means of a switch. This initiates the heating of the tobacco via the heating blade inserted into the tobacco plug. The tobacco neither ignites nor burns. The electronically controlled heating, in combination with the uniquely processed tobacco, prevents combustion from occurring. Heat is supplied to the *tobacco stick* for a fixed period of approximately 6 min and allows up to 14 puffs to be taken during that time. The temperature of the heating blade is carefully controlled and the energy supply to the blade is cut if its operating temperature exceeds 350 °C.

When a cigarette is lit, the combination of tobacco (fuel) and oxygen in the air generates a self-sustaining combustion process that consumes the tobacco. During the period between puffs, the tobacco smolders at temperatures ranging from 600 to 800 °C in the center of the combustion zone. During a puff, the temperature increases to more than 900 °C at the periphery of the combustion zone (Baker, 1975). The combustion of tobacco results in formation of heat, smoke and ash. The smoke formed is a complex aerosol mixture estimated to contain more than 8000 compounds (Rodgman and Perfetti, 2013).

In contrast, the operating temperature of the THS 2.2 product is substantially lower than that required to cause ignition and combustion of tobacco and the temperature measured in the tobacco does not exceed 300 °C. When a puff is taken from the *tobacco stick* the tobacco temperature drops as ambient air is drawn through the *tobacco stick*. Since combustion does not occur, the structural integrity of the *tobacco stick* is retained after use. The tobacco is not consumed as in a cigarette and no ash is formed. The experimental confirmation that no combustion takes place during use of the THS 2.2 product as intended have been presented recently (Cozzani et al., 2016.) (and a separate publication is in preparation). This absence of combustion, because of controlled heating, is designed to significantly reduce formation of HPHCs by the THS2.2 product compared with cigarettes. This is confirmed by the chemical

The FSPTCA provides for the authorization of an MRTP when reduced exposure or reduced risk has been substantiated by applying a rigorous scientific assessment. Different levels of evidence are required for these respective authorizations, with ability for communicating product attributes with a reduced risk versus a modified exposure order. The FSPTCA requires applicants to demonstrate that the product, as actually used, will (i) significantly reduce harm and the risk of tobacco-related disease to individual tobacco users, and (ii) benefit the health of the population as a whole, taking into account both the users of tobacco products and persons who do not currently use tobacco products.

In contrast, the approach adopted by the European Union Tobacco Products Directive (European Parliament and Council Directive 2014/40/EU, 2014) is less detailed in its requirements than the approach adopted by FDA. The EU requires the submission of available product-specific studies and information in its notification process, but currently has no mechanism to authorize consumer information relating to reduced exposure or reduced risk.

Since the European Union Tobacco Products Directive (European Parliament and Council Directive 2014/40/EU, 2014) was implemented in May 2016, manufacturers and importers are required to submit a notification to the competent authorities of Member States for any novel tobacco product they intend to market. A novel tobacco product is defined as one that does not fall into any of the existing categories of tobacco products and is placed on the market after 19 May 2014. The notification should include:

a) Available scientific studies on toxicity, addictiveness and attractiveness of the novel tobacco product, in particular as regards its ingredients and emissions,
b) Available studies, executive summaries thereof and market research on the preferences of various consumer groups, including young people and current smokers,
c) Other available and relevant information, including a risk/benefit analysis of the product, its expected effects on cessation of tobacco consumption, its expected effects on initiation of tobacco consumption and predicted consumer perception.

The reduced exposure evaluation of an early *heat-not-burn* product (electrically heated cigarette smoking system – 'Heatbar') developed and assessed by PMI prior to the enactment of the FSPTCA, has been described previously (Schorp et al., 2012); it was recognized that '*a comprehensive assessment of reduced exposure is necessary, but is not sufficient for determining a modified tobacco product's potential to reduce risk.*'

The approaches to assess the risk of MRTPs relative to cigarette products have been described by the Institute of Medicine (Institute of Medicine (2012)) and reviewed recently (Berman et al., 2015). In this context, we have developed an assessment strategy designed to meet the more stringent requirements of the FDA's draft MRTP guidance that would also be applicable for the assessment of candidate MRTPs to be marketed in other jurisdictions.

The draft guidance from the FDA Center for Tobacco Products (CTP) indicates that the basis for authorizing an MRTP is somewhat different to the criteria applied by the Center for Drug Evaluation and Research (CDER) to the approval of a drug product. In the case of a drug, the general approach is to focus on a single or a very limited number of well-substantiated clinically relevant endpoints as indicators of a therapeutic effect. In the case of an MRTP, however, where product-specific epidemiological evidence is not available and clinical experience is limited, a different approach to product assessment has to be developed. It is well understood that the relative risk of smoking is not defined by a single endpoint or even endpoints reflective of a single disease or biological mechanism. Therefore, the evaluation of relative risk must take into account the complex nature of the whole organism and the many biological mechanisms that are affected by smoking. The approach to assess a candidate MRTP therefore needs to address this complexity by exploring a broad array of disease indicators to demonstrate that the use of the candidate MRTP has a reduced impact – compared with cigarettes – on mechanisms leading to tobacco-related diseases. This approach needs to be based on the best available science short of long-term epidemiological studies, which can be initiated once the product is on the market and under actual use conditions. The CTP has acknowledged this limitation in the draft guidance (Family Smoking Prevention and Tobacco Control Act; Food and Drug Administration, 2012). We understand this to mean that initial authorization of an MRTP will be based on non-clinical and clinical data which will be supplemented with post-marketing data. Evaluation of the relative level of risk of long-term use of an MRTP can begin once the product is authorized based on a weight-of-evidence approach confirming the potential for risk reduction relative to cigarette use.

3.1. The MRTP assessment framework

In this context, we have formulated a framework that utilizes what is known about combustible cigarette (CC) smoking and incorporates both epidemiological and mechanistic evidence to define our assessment approach. Epidemiological studies inform about the causal relationship between CC smoking and disease risk, as well as the benefits of smoking cessation. Ongoing exposure to cigarette smoke leads to both a time- and dose-dependent increase in the risk of developing smoking-related diseases, such as cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD), and lung cancer. The accrued health risk over time can be reduced gradually by smoking cessation. The cause-and-effect relationships between smoking and these diseases are based on sound epidemiological evidence, conceptually depicted in Fig. 4 (red and green lines). The United States Institute of Medicine (Institute of Medicine (2012)) states that cessation is the '*gold standard*' for the assessment of an MRTP, providing '*an aspirational goal for risk and exposure*.' This sets the fundamental objective of an MRTP: '*switching to an MRTP must reduce the risk of developing smoking-related diseases with a risk profile approaching that of cessation*' (Fig. 4, orange lines). This MRTP assessment framework lays out the foundations for the assessment approach. In brief, if the changes observed in adult smokers who switch from cigarettes to an MRTP consistently approach the changes observed following smoking cessation, and those changes are further supported by coherent findings from non-clinical research, it is reasonable to conclude that the product will reduce risk.

Smoking-related diseases have a complex etiology. Broadly accepted mechanisms underlying many smoking-related diseases are related to impaired organ function from progression of pathological changes and comorbidity. Exposure to cigarette smoke induces molecular changes in the exposed organism and disrupts various biological processes. This in turn causes alterations at the cell and tissue level that result in physiological changes that eventually manifest themselves as diseases (Fig. 5).

Recent advances in molecular measurement and imaging technologies, mathematical modeling, and computational biology enable the integrative analysis of large data sets to quantify the biological impact of exposure to toxicants (Hoeng et al., 2012, 2014; Sturla et al., 2014). The integration of these methods with standard toxicological endpoints defines our systems toxicology-informed risk assessment approach. Using computable biological network models (Boue et al., 2015) of the key mechanisms affected by toxicants, systems toxicology enables the quantification of the biological network perturbation amplitudes (NPA) (Martin et al., 2012) caused by exposure to such toxicants and their numerical aggregation into an overall biological impact factor (BIF) (Thomson et al., 2013). This approach permits a systematic and quantitative mechanism-based comparison of the biological impact of switching to a candidate MRTP with continued smoking of CC as well as the

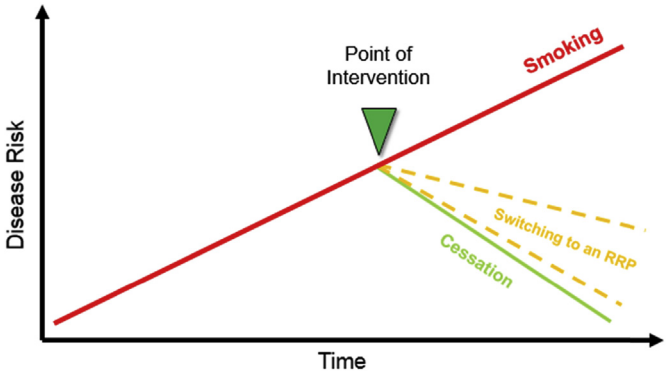

Fig. 4. Risk framework for MRTP assessment. Conceptual depiction of the cumulated risk of smoking and the effect of cessation over time. These represent the two boundaries for the assessment of an MRTP: 1) comparing switching to an MRTP with continued smoking and 2) benchmarking switching against smoking cessation (gold standard). Note that the straight lines used in this figure are for illustration purposes only as the accumulation of disease risk and the reduction upon cessation and switching to an MRTP follow different trajectories for specific diseases.

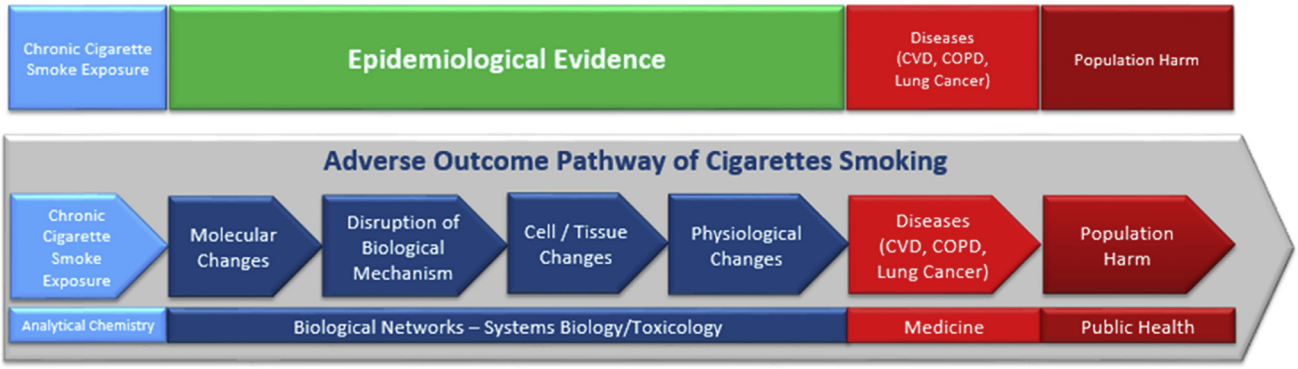

Fig. 5. Chronic exposure to cigarette smoke affects a number of biological networks associated with smoking-related diseases in a causal chain of events known as Adverse Outcome Pathways (Sturla et al., 2014 and references therein).

benchmarking of the impact of switching against that of smoking cessation in animal models of disease. We can therefore emulate disease progression and reversibility in a short timeframe.

3.2. The MRTP assessment program

We have developed a generally applicable assessment program which integrates seven assessment steps (Fig. 6) necessary to demonstrate that a candidate MRTP i) significantly reduces harm and the risk of tobacco-related disease to individual tobacco users, and ii) benefits the health of the population as a whole, taking into account both users of tobacco products and persons who do not currently use tobacco products. These assessment steps are designed to provide five levels of evidence as the assessment program is completed (Fig. 6):

1. The first step of the assessment is designed to ensure that the product is manufactured to appropriate quality standards and is sufficiently characterized to document product performance parameters. This product characterization enables the establishment of a product specification. All products entering the subsequent assessment steps must meet the established specification, as must the products that are introduced to the market. To ensure this consistency, a well-controlled Change Management process has been established. This process ensures that the established specifications are met for any proposed change to the product before it is implemented by determining any potential impact on product performance and aerosol composition. For instance, this process was utilized following changes to the tobacco blends used in the *tobacco stick* based on feedback from taste panels and to ensure sustainability of tobacco sources used to formulate the blend. The aerosol generated from *tobacco sticks* reformulated with different tobacco blends (with blend code D2 for the regular version and D1 for the menthol version) was compared with the aerosol from *tobacco sticks* using the previous tobacco blends (blend code FR1). There was no change in HPHC yields or *in vitro* toxicology findings for the different blends – further details provided in: (Schaller et al., 2016b).
2. The second assessment step is designed to analyze the chemical composition of the aerosol generated by the candidate MRTP and quantify the reduction in HPHCs formation in comparison with a cigarette. This analysis is also needed to evaluate whether new potentially harmful constituents are generated by the MRTP. During this step, it is also necessary to assess the influence of usage patterns and *puffing regimens* on aerosol composition, to ensure that the candidate MRTP delivers a stable reduction in HPHCs, as designed. Furthermore, the analysis of the aerosol chemistry data, collected under various conditions, including in the absence of oxygen, confirms the absence of the involvement of combustion in aerosol generation. The aerosol particles are also analyzed to verify i) that the aerosol contains a similar particle size distributions as the one found in CC smoke, to ensure a similar delivery of nicotine in the aerosol and ii) the significant reduction or absence of the insoluble solid particles found in cigarette smoke. Finally, during this step we also analyze the effects of MRTP use on indoor air chemistry to evaluate its impact on air quality in comparison with CC use (Mitova et al., 2016) and benchmark against national and international standards for exposure to environmental toxicants e.g.(European Agency for Safety and Health at Work, 2006). These environmental studies are conducted under well-controlled and realistic conditions based on accepted building standards (European Committee for Standardization, 2006).
3. The third step of the assessment determines whether the *reduced formation of HPHCs* leads to *reduced toxicity in laboratory models.* This step also contributes to the evaluation of any new hazards. This second level of evidence is based on toxicological studies conducted both *in vitro* and *in vivo.* First, we selected a battery of *in vitro* assays designed to assess the cytotoxicity and the genotoxicity of candidate MRTP aerosols in comparison with

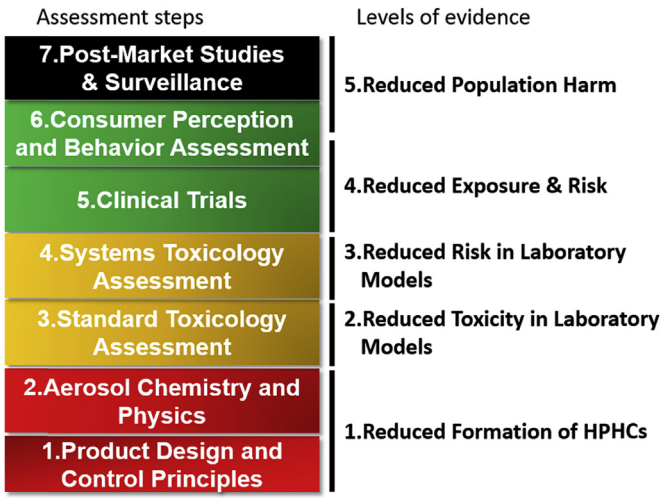

Fig. 6. The MRTP assessment program. Seven steps of assessment lead to five levels of evidence. Taken together, these levels of evidence provide the scientific evidence to demonstrate that a candidate MRTP is indeed a reduced risk product.

CC smoke (Schaller et al., 2016a). Second, the direct inhalation toxicity of candidate MRTP aerosols is analyzed in animal inhalation studies according to the guidelines from the Organization for Economic Cooperation and Development (Organization for Economic Co-operation and Development (OECD), 2009; Oviedo et al., 2016; Wong et al., 2016). In these studies we rigorously monitor the test atmosphere composition and measure biomarkers of exposure in the urine and blood of the exposed animals. This methodology allows us to assess the degree of *reduced exposure in laboratory models*, which provides an indication of what can be achieved independent of human behavior and use patterns. This evidence level also provides support to the substantiation of *reduced exposure and risk.*

4. The fourth assessment step is used to determine whether *reduced formation of HPHCs* and *reduced toxicity in laboratory models* lead to *reduced risk in laboratory models.* This evidence is based on systems toxicology studies (Sturla et al., 2014) conducted both *in vitro* and *in vivo*. The approach adopted for these *in vitro* assays is to use primary human cells and organotypic tissue cultures of human origin, as they are deemed to be more relevant than immortalized cell lines (Iskandar et al., 2016). The initial step in this assessment compares the effects of MRTP aerosol and CC smoke extracts on primary normal human bronchial epithelial cells using high-content screening (Marescotti et al., 2016) as well as a detailed mechanistic analysis of the changes occurring at the transcriptome level (Gonzalez-Suarez et al., 2014; Kogel et al., 2015). This type of study provides an initial assessment of an MRTP's impact on key pathways of toxicity. Second, we assess the impact of the whole MRTP aerosol in comparison with whole cigarette smoke on disease mechanisms using *in vitro* assay systems designed to recapitulate the structure of the human epithelial tissues exposed to aerosol/smoke (Kuehn et al., 2015; Mathis et al., 2013; Schlage et al., 2014; Talikka et al., 2014) and/or key events in disease pathways (Poussin et al., 2014, 2015; van der Toorn et al., 2015a). Third, we have complemented *in vivo* studies conducted under OECD guidelines (Kogel et al., 2014; Phillips et al., 2015a) with systems toxicology methods to quantify the impact of candidate MRTP aerosols on biological mechanisms perturbed by CC smoke, in conjunction with the physiological and toxicological endpoints measured under the OECD guidelines. Fourth, we employ animal models of disease with a study design that mimics the MRTP Assessment Framework. Briefly, these studies allow us to compare the effects of initial exposure to CC smoke followed by switching to a candidate MRTP aerosol with those caused by continuous exposure to CC smoke and benchmark them against those of cessation (Ansari et al., 2016; Phillips et al., 2015b). To enable a comprehensive analysis of these effects, these studies leverage the principles of systems toxicology through a very broad array of measurements, ranging from comprehensive molecular quantifications through transcriptomics, proteomics, and lipidomics, to full histopathological evaluation and disease risk markers, such as measurement of lung emphysema and atherosclerotic plaque formation. Furthermore, the use of an animal model such as the Apoe$^{-/-}$ mouse enables the concomitant analysis and quantification of both pulmonary and vascular effects of smoking, cessation and switching (Boue et al., 2013; Lietz et al., 2013; Lo Sasso et al., 2016a). Similarly, life-time exposure studies in the A/J mouse enable the concomitant analysis and quantification of both emphysema and cancer-related endpoints (Stinn et al., 2013) while leveraging the systems toxicology approach. Studies conducted in this assessment step provide the evidence that a candidate MRTP presents a *reduced risk in laboratory models.* It is essential to establish the performance of the product in laboratory models prior to performing clinical studies.
5. The fifth step of the assessment utilizes clinical studies to assess whether *reduced formation of HPHCs* leads to *reduced exposure and risk* in humans who use the candidate MRTP. These studies are conducted with adult smokers who are randomized into three groups reflecting the MRTP Assessment Framework: i) continued smoking, ii) cessation, or iii) switching to the candidate MRTP. Studies conducted in clinical confinement for a week can be used to quantify the maximum possible reduction in HPHC exposure compared with ongoing smoking and cessation, examples of these studies on a previous product are provided: (Tricker et al., 2012a, 2012b, 2012c, 2012d). This type of study is then complemented with studies conducted in ambulatory mode e.g. (Martin et al., 2012), to assess whether the reductions in HPHC exposure observed in short-term confinement can be sustained for a longer period in a near to real-world setting. Furthermore, with study periods ranging from three months to one year, it is possible to assess whether *reduced exposure* leads to a favorable change in smoking-related clinical risk endpoints, and hence provide assurance that *reduced exposure* leads to *reduced risk.* The endpoints to assess *reduced exposure* must be selected to reflect the range of molecular entities contained in CC smoke for which appropriate biomarkers of exposure can be reliably measured. Similarly, the endpoints to assess *reduced risk* must be selected to reflect the effects of CC smoke on different organ systems and biological mechanisms. These disease risk markers, or biomarkers of effect, must also be responsive to smoking cessation within the duration of the study and measurable using validated methods.

   As outlined in the introduction (Fig. 1), for effective harm reduction at the population level, an alternative tobacco product must not only be scientifically substantiated to reduce risk, but also be acceptable to cigarette consumers. It is unlikely that new products that do not satisfy smokers will enable them to switch. The nicotine delivery profile and the rewarding subjective effects of tobacco products are critical components of product satisfaction and their actual use. Lack of adoption of alternative nicotine delivery systems may therefore be related to ineffective nicotine delivery and/or a low level of satisfaction. To assess whether a candidate MRTP delivers satisfying levels of nicotine, with a delivery profile similar to cigarettes, we also conduct pharmacokinetic studies in the fifth step of our assessment program. These studies are conducted along the lines of a previous report (Picavet et al., 2016).
6. It is important that accurate, non-misleading, scientifically substantiated product information and benefits are communicated to adult smokers to provide them with an incentive to switch from cigarettes to an MRTP. The sixth step of the assessment involves studies that measure, prior to market introduction, the likely effect of introducing a new tobacco product with its associated communication materials on tobacco use behavior among adult smokers and non-smokers. This step assesses the likelihood that adult smokers will switch from cigarettes to an MRTP, and that former smokers, smokers who are motivated to quit, and non-smokers are not likely to use the product. Integral to the above is the assessment of consumer understanding and risk perceptions that any product communication would generate. The objective is to ensure that the intended product communication enables the public to correctly comprehend the modified exposure/risk claims and form the correct perception of the health risks of using an MRTP in comparison with cigarettes, nicotine replacement therapies, and cessation.

7. Once the product is on the market, it will be necessary to conduct post-market studies and surveillance to understand how the product is used in real-world settings. Passive surveillance measures are used to gather spontaneous reports of any adverse events related to product use. Longer-term assessment of exposure and health outcomes will be carried out, together with an ongoing assessment of consumer perception and tobacco use behavior.

To be effective, an MRTP must benefit the health of the population as a whole, accounting for current, former, and never-smokers (Family Smoking Prevention and Tobacco Control Act; Food and Drug Administration, 2012). However, population-level data regarding the risks and uptake of an MRTP prior to its market introduction are clearly lacking. To gain an understanding of the potential impact of an MRTP market introduction on population-level mortality, one can employ a mathematical model (Vugrin et al., 2015). Towards this end, PMI has developed a population health impact model (PHIM) for MRTPs (Weitkunat et al., 2015) that leverages publicly available epidemiology data. PMI's PHIM is designed to estimate the impact of the introduction of an MRTP on mortality based on assumptions about the fractional residual risk of an MRTP relative to that of cigarettes, and possible scenarios for the uptake of the MRTP in the population. The model estimates the impact on mortality in a population that survives until a specific time after the introduction of the MRTP on the market. Such a model can be used to predict the potential impact from the introduction of an MRTP based on the exploration of a wide range of scenarios describing realistically the prevalence of cigarette and MRTP use, individually and in combination.

The publications in this series are outlined below and report the results obtained having completed part of the overall assessment approach with the candidate MRTP THS2.2 in the context of the second to the fifth step of the MRTP assessment program.

4. Outline of the publications that follow in this series

4.1. Publications of studies conducted in the 2nd step of the MRTP assessment program

In Part 2 of this series of papers (Schaller et al., 2016a), the mainstream aerosol composition of both regular and mentholated THS2.2 is compared with that of the mainstream smoke from a reference cigarette (3R4F). The criteria for selection and the results for 58 HPHCs and analytes determined are provided and demonstrate that the majority of HPHCs measured in THS2.2 aerosol are reduced by more than 90% when compared with reference cigarette smoke. *In vitro* toxicological assessment of THS2.2 aerosol fractions is also described, revealing a >90% reduction in cytotoxicity, as determined by the neutral red uptake (NRU) assay, and a similar reduction in mutagenic potency in the mouse lymphoma assay (MLA). The THS2.2 aerosol fraction was not mutagenic in the Ames mutagenicity assay.

The performance of THS2.2 operated under simulated extreme climatic conditions (desert and tropical conditions) was assessed by monitoring aerosol composition to show no significant modification with climatic condition. The aerosol composition was also measured when using puffing regimens that were more intense than the standard Health Canada Intense (HCI) conditions, to confirm that HPHC levels remained lower than the levels formed in reference cigarette smoke generated with the HCI regimen.

In part 3 of this series of papers (Schaller et al., 2016b), the influence of 43 different tobacco blends (from a large range of tobacco types) on the formation of HPHCs in THS2.2 aerosol was determined. The aerosols produced by these blends in the THS2.2 contained significantly lower concentrations of HPHCs than did 3R4F mainstream smoke. For most HPHCs, the blend composition had a minimal impact on the yields in the resulting aerosols. However, some HPHCs presented significant variability across the different blends, likely resulting from the distillation of endogenous preformed compounds present in certain tobacco types. This approach provided the information required to intelligently blend tobaccos to meet consumer needs while maintaining low HPHC delivery.

4.2. Publications of studies conducted in the 3rd and 4th step of the MRTP assessment program

In part 4 of this series of papers (Wong et al., 2016), a 90-day nose-only inhalation study in rats was performed according to OECD Test Guideline 413 (Organization for Economic Co-operation and Development (OECD), 2009). The approach was modified to combine classical and systems toxicology approaches, transcriptomic analysis and miRNA expression (the latter results included in part 5 below). The effects of exposure of respiratory tract organs in THS2.2-exposed animals were much lower than those in rats exposed to 3R4F cigarette smoke. The results also confirmed that for the THS2.2 aerosol, there was no apparent new toxicity effects, compared with 3R4F cigarette smoke.

Part 5 of this series of papers (Sewer et al., 2016) demonstrated that 3R4F cigarette smoke, but not THS2.2 aerosol, caused global miRNA downregulation. Certain miRNA species, notably those associated with the inflammatory response, were upregulated in 3R4F cigarette smoke-exposed lung, but reduced following THS2.2 aerosol exposure. This work contributed to an increase in mechanistic understanding of the complex exposure responses.

In part 6 of this series of papers (Oviedo et al., 2016), a 90-day nose-only inhalation study in rats was performed according to OECD Test Guidelines 413 (OECD, 2009) on a mentholated variant of THS2.2 (THS2.2M) assessing both classical endpoints (described in the OECD guidelines such as histopathology, etc.), and complemented with transcriptomics and quantitative proteomics analyses of respiratory nasal epithelium and lung tissue, together with lipidomic analysis of lung tissue. Rats were exposed to either filtered air (sham), THS2.2M, two mentholated reference cigarettes (MRC, designed to meet 3R4F specifications with menthol added at different levels), or the 3R4F reference cigarette. The study results show that systemic toxicity and alterations in the respiratory tract were significantly lower in THS2.2M-exposed rats than in MRC and 3R4F.

In part 7 of this series of papers (Kogel et al., 2016), the systems toxicological assessment results from the study described in part 6 are discussed. The results demonstrated adaptive responses in the respiratory nasal epithelium to 3R4F cigarette smoke; these adaptations included squamous cell metaplasia and inflammatory response, with a close correspondence of the molecular and histopathological findings. In contrast, the adaptive tissue and molecular changes to THS2.2M aerosol exposure were much weaker, and limited mostly to the highest THS2.2M concentration in female rats. 3R4F smoke exposure induced an inflammatory response, triggered cellular stress responses, and affected sphingolipid metabolism. These responses were not observed or were much lower after THS2.2M aerosol exposure.

4.3. Publications of studies conducted in the 5th step of the MRTP assessment program

Part 8 of this series of papers (Haziza et al., 2016) describes a 5-day, controlled, parallel group, open-label clinical study where 160 smoking, healthy adult subjects were randomized to three groups

and asked to: (1) switch from CC to THS2.2 (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). All biomarkers of HPHC 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. Greater product consumption and total puff volume were reported in the THS group, but exposure to nicotine was similar to CC at the end of the confinement period in the clinic. Reduction in the urge to smoke was comparable between the THS and CC groups, and the THS was well tolerated with few adverse events.

Part 9 of this series of papers (Martin et al., 2016) reports the results from gene expression profiling of whole blood collected during the clinical study referred to in part 8 (Haziza et al., 2016). A whole-blood-derived gene signature that can distinguish smokers from either non-smokers or former smokers with a high degree of specificity and sensitivity has been described previously (Martin et al., 2015). The small signature, consisting of only 11 genes, was tested on the blood transcriptome of subjects enrolled in the clinical study as a complementary measure of exposure response. The signature performed remarkably well in predicting significant reduction in exposure response within just 5 days after subjects switched to THS2.2 or abstained from smoking. The blood transcriptomics profiling can therefore serve as a complementary measure of exposure response.

5. Conclusions

The data presented in this series of papers demonstrates that, compared with the 3R4F reference cigarette, both regular and mentholated versions of THS2.2 yield significantly reduced levels of HPHCs. This reduced formation of HPHCs by both versions of THS2.2 leads to a reduced toxicity, assessed both *in vitro* using assays for cytotoxicity and mutagenicity and *in vivo* in two distinct 90-day inhalation studies in rats. An important finding is that the reduced formation of HPHCs (measured under standardized machine smoking conditions) also leads to the reduced exposure when used *ad libitum* in a short-term clinical study conducted in adult smokers in a controlled environment.

We have previously reported on a systems toxicology study (step four of the MRTP assessment program) conducted in an animal model assessing the impact of THS2.2 on disease mechanisms (Lo Sasso et al., 2016b; Phillips et al., 2016; Titz et al., 2016). This study has shown that exposure to THS2.2, in comparison with 3R4F exposure, leads to a reduced exposure to HPHCs, which in turn leads to a reduced perturbation amplitude of disease-associated mechanisms as well as a reduced severity of disease endpoints *in vivo*. In addition, the effects of switching from 3R4F to THS2.2 were approaching those of cessation. Furthermore, we have previously reported on five *in vitro* systems toxicology studies conducted with THS2.2 in human primary cells. These studies were designed to compare the effects of 3R4F smoke with those of THS2.2 aerosol on key cellular toxicity endpoints (Gonzalez-Suarez et al., 2016), organotypic airway epithelium (Iskander et al., 2016b; Zanetti et al., 2016), as well as on mechanisms involved in vascular inflammation (Poussin et al., 2016)and endothelial dysfunction (van der Toorn et al., 2015b). The results of these studies showed that THS2.2 aerosol is less toxic than 3R4F smoke. Taken together, all these results show that THS2.2 has the potential to be an MRTP.

6. Outlook

The data presented in this series of papers and the previously published studies (Poussin et al., 2016; van der Toorn et al., 2015b; Gonzalez-Suarez et al., 2016; Phillips et al., 2016; Titz et al., 2016; Lo Sasso et al., 2016b; Iskandar et al., 2016b; Zanetti et al., 2016) are an essential component of our MRTP assessment program applied to THS2.2. The converging lines of evidence emerging from these study results show that THS2.2 has the potential to be a reduced-risk product. However, to confirm that THS2.2 is indeed a reduced-risk product we are conducting longer-term clinical studies, designed to quantify disease risk markers in addition to biomarkers of exposure. Future publications describing in more details the absence of combustion during THS2.2 use (i.e. that the aerosol produced is not smoke), further clinical studies, perception and behavior studies, and population impact modeling will be published elsewhere.

Conflict of interest statement

The work reported in all nine parts of this supplement involved a candidate Modified Risk Tobacco Product developed by Philip Morris International (PMI) and was solely funded by PMI. All authors are (or were) employees of PMI R&D or worked for PMI R&D under contractual agreements.

Transparency document

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Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol

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ABSTRACT

The chemical composition, *in vitro* genotoxicity, and cytotoxicity of the mainstream aerosol from the Tobacco Heating System 2.2 (THS2.2) were compared with those of the mainstream smoke from the 3R4F reference cigarette. In contrast to the 3R4F, the tobacco plug in the THS2.2 is not burnt. The low operating temperature of THS2.2 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 THS2.2 aerosol was not mutagenic in the Ames assay. The chemical composition of the THS2.2 aerosol was also evaluated under extreme climatic and puffing conditions. When generating the THS2.2 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.

1. Introduction

The U.S. Family Smoking Prevention and Tobacco Control Act (FSPTCA) defines a Modified Risk Tobacco Product (MRTP) as "any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products" (Family Smoking Prevention and Tobacco Control Act, 2009). This publication is part of a series of nine publications describing the nonclinical and part of the clinical assessment of a candidate MRTP, THS2.2 regular and a mentholated version (THS2.2M). The series of publications provides part of the overall scientific program to assess the potential for THS2.2 to be a reduced risk product. The first publication in this series describes THS2.2 and the assessment program for MRTPs (Smith et al., 2016). This is followed by six publications, including this one, that describe the nonclinical assessment of THS2.2 regular and THS2.2M (Kogel et al., 2016; Oviedo et al., 2016; Schaller et al., 2016a; Schaller et al., 2016b; Sewer et al., 2016; Wong et al., 2016). The eighth publication in the series describes a clinical study to assess whether the reduced formation of Harmful and Potentially Harmful Constituents (HPHCs) for THS2.2 regular also leads to reduced exposure to HPHCs when the product is used in a clinical setting (Haziza et al., 2016). A final publication utilizes data gathered from the reduced exposure clinical study on THS2.2 regular to determine if a systems pharmacology approach can identify exposure response markers in peripheral blood of

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Abbreviations

(NNK) 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
(EHCSS) Electrically Heated Cigarette Smoking System
(FID) flame ionization detection
(GC/MS) gas chromatograph-mass spectrometer
(GC) gas chromatography
(GVP) gas-vapor phase
(GEF) global evaluation factor
(HPHC) harmful and potentially harmful constituent
(HCI) Health Canada Intense
(ICP-MS) inductively coupled plasma mass spectrometry
(IARC) International Agency for Research on Cancer
(LOQ) limit of quantification
(LC-MS/MS) liquid chromatograph tandem mass spectrometer
(LC) liquid chromatography
(LB) lower boundaries
(LOGEL) lowest observed genotoxic effect level
(MMAD) mass median aerodynamic diameter
(MRTP) Modified Risk Tobacco Product
(MLA) mouse lymphoma assay
(MF) mutation frequency
(NRU) neutral red uptake
(NFDPM) nicotine-free dry particulate matter
(NO_x) nitrogen oxides
(NAB) *N*-nitrosoanabasine
(NAT) *N*-nitrosoanatabine
(NNN) *N*-nitrosonornicotine
(PBS) phosphate-buffered saline
(PDSP) programmable dual syringe pump
(RH) relative humidity
(RTG) relative total growth
(IP) Intermediate Precision
(THS2.2) Tobacco Heating System 2.2
(TPM) total particulate matter
(UB) upper boundaries

smokers switching to THS2.2 (Martin et al., 2016). This publication is the second of the series and presents the chemical analyses, the physical characterization, and the *in vitro* genotoxicity and cytotoxicity assessments of the mainstream aerosol of the THS2.2.

The smoke produced by the combustion of tobacco in a combustible cigarette (CC) is a complex and dynamic chemical mixture which contains more than 8000 identified chemical compounds (Rodgman and Perfetti, 2013). Tobacco smoke from CC consists of an aerosol containing liquid droplets ('particulate phase') suspended in a carrier gas and surrounded by its own gas-vapor phase. It is generated by complex and overlapping burning, pyrolysis, pyrosynthesis, distillation, sublimation, and condensation processes (Borgerding and Klus, 2005). With minor exceptions, both pyrogenesis and pyrosynthesis of HPHCs result from the thermal decomposition of organic compounds present in tobacco occurring at temperatures up to 900 °C observed in cigarettes (Torikaiu et al., 2005; Baker, 2006); thus, a reduction of these toxicants may be achieved by heating rather than burning tobacco to produce an aerosol. The development of *heat-not-burn* tobacco products is not new and earlier efforts to develop such products (notably Premier and Eclipse products from The R.J. Reynolds Tobacco Company and Accord from Philip Morris) have been reviewed by Baker (Baker, 2006). The Electrically Heated Cigarette Smoking System (EHCSS) was the first generation of tobacco heated products commercialized by Philip Morris. The EHCSS series-E has been subject to extensive analytical and toxicological evaluation, demonstrating simplified smoke chemistry compared with the 1R4F reference cigarette of the University of Kentucky (Patskan and Reininghaus, 2003). Notably there was a significant reduction in carbon monoxide (CO) and an increased yield of formaldehyde in EHCSS-E mainstream smoke, compared with the 1R4F cigarette. On a per-milligram total particulate matter (TPM) basis, the concentration of formaldehyde was increased approximately sevenfold (Stabbert et al., 2003). In later developments of EHCSS (series-JLI and series-K), in order to reduce these excessive levels of formaldehyde, ammonium magnesium phosphate (AMP) was used in the cigarette paper to replace calcium carbonate. It was anticipated that ammonia released during the pyrolysis of AMP would condense with formaldehyde to form hexamethylenetetramine (HMT) (Schorp et al., 2012). Chemical analysis of smoke from the EHCSS-JLI and EHCSS-K cigarettes containing AMP showed lower yields of formaldehyde and several HPHCs, a further decrease in CO yield, and increased yields of ammonia and HMT (Roemer et al., 2008; Werley et al., 2008; Zenzen et al., 2012). The THS2.2 is the latest generation of *heat-not-burn* products from Philip Morris International. It produces an aerosol by carefully heating the tobacco with a heater blade reaching a maximum temperature of 350 °C. This system enables a careful control of the energy applied to the tobacco plug (Smith et al., 2016) and limits the thermal physico-chemical processes while producing an aerosol capable of satisfying adult smokers enabling them to switch from cigarettes.

Although the causal relationship between smoking and several diseases is well established (Doll et al., 2004), there is still very little understanding of the underlying mechanisms by which smoking causes disease. Among the more than 8000 chemical compounds that have been identified in cigarette tobacco smoke (Rodgman and Perfetti, 2013), public health authorities and others have proposed some 100 HPHCs as possible causes of smoking-related diseases such as lung cancer, heart disease, and emphysema (Health Canada, 2000; World Health Organisation, 2008; U.S. Food and Drug Administration, 2012). For the US Food and Drug Administration, the notion of "harmful and potentially harmful constituent" includes any chemical or chemical compound in a tobacco product or in tobacco smoke that is, or potentially is, inhaled, ingested, or absorbed into the body, including as an aerosol (vapor) or any other emission; and causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products (U.S. Food and Drug Administration, 2016). However, there is no consensus, that lowering or eliminating any single compound (or even a combination of compounds) in smoke would have a significant impact on risk. The current approach, which eliminates direct tobacco combustion and limits tobacco pyrolysis by heating at significantly lower temperatures than encountered in CC, has the potential to reduce a broad range of HPHCs in the THS2.2 aerosol. Consequently, criteria were established to develop a list of relevant analytes, including HPHCs to assess their reductions in the THS2.2 aerosol, compared to the mainstream smoke of the University of Kentucky reference cigarette 3R4F, as follows:

- **Criterion 1**: Smoke constituents determined by International Organization for Standardization (ISO) methods. This list includes total particulate matter (TPM) (International Organisation for Standardization, 2011), water in TPM (International Organisation for Standardization, 2011), nicotine (International Organisation for Standardization, 2013), nicotine-free dry particulate matter (NFDPM) (International Organisation for Standardization, 2011); carbon monoxide (CO) (International Organisation for Standardization, 2010b) and benzo[*a*]pyrene (International Organisation for Standardization, 2012).
- **Criterion 2**: Priority toxicants in tobacco smoke selected from the lists issued by regulatory bodies, or proposed by cognizant authorities (Health Canada, 2000; World Health Organisation, 2008; U.S. Food and Drug Administration, 2012). This list includes the analytes recommended by ISO under Criterion 1. In addition, the following HPHCs are also included: 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde, acrylonitrile, 1,3-butadiene, benzene, isoprene, pyridine, quinoline, styrene, toluene, catechol, *m*-cresol, *p*-cresol, *o*-cresol, hydroquinone, phenol, resorcinol, *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosonornicotine (NNN), ammonia, hydrogen cyanide, nitric oxide (NO), nitrogen oxides (NO_x), arsenic, cadmium, chromium, lead, mercury, nickel, and selenium.
- **Criterion 3**: Toxicants with an established biomarker of exposure, i.e., for use in a clinical study to determine exposure to the parent toxicant (Haziza et al., 2016). The toxicants include some analytes already listed under "Criterion 1" and "Criterion 2": CO (biomarker: blood carboxyhemoglobin (COHb) (Pojer et al., 1984), nicotine (biomarker: serum cotinine (Benowitz and Iii, 1984) or total nicotine equivalents in urine (Benowitz and Jacob, 1994)), 2-aminonaphthalene, 4-aminobiphenyl and *o*-toluidine (biomarker: parent amines in urine (Riedel et al., 2006)), acrolein (biomarker: 3-hydroxypropylmercapturic acid (3-HPMA) in urine (Mascher et al., 2001)), crotonaldehyde (biomarker: 3-hydroxy-2-methylpropyl mercapturic acid (HMPMA) in urine (Scherer et al., 2007)), acrylonitrile (biomarker: 2-cyanoethylmercapturic acid (CEMA) in urine (Minet et al., 2011)), acrylamide (biomarker: acrylamide mercapturic acid (AAMA) in urine (Urban et al., 2006)), 1,3-butadiene (biomarker: 1-hydroxy-2-(N-acetyl-cysteineyl)-3-butene (MHBMA) in urine (van Sittert et al., 2000)), benzene (biomarker: S-phenylmercapturic acid (S-PMA) in urine (Medeiros et al., 1997)), NNK (biomarker: total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine (Carmella et al., 2003)), NNN (total NNN in urine (Kavvadias et al., 2009)), benzo[*a*]pyrene and pyrene (biomarker: total 1-hydroxypyrene (1-OHP) in urine (Strickland et al., 1996)).
- **Criterion 4:** Toxicants which are predominantly formed below 400 °C, and which are not included under "Criterion 2": acrylamide (possibly formed from asparagine and reducing sugars through a Maillard type of reaction occurring between 120 and 200 °C (Stadler et al., 2002; Blank et al., 2005; Becalski et al., 2011)), acetamide (possibly formed from the pyrolysis of Amadori compounds (formed by the reaction of amino acids and sugars) and from the decomposition of ammonium acetate at around 250 °C (Moldoveanu, 2010)), propylene oxide (possibly formed by dehydration of propylene glycol which is used as a humectant in CC and for application of flavors to tobacco (Diekmann et al., 2006; Laino et al., 2012)), nitrobenzene, ethylene oxide and vinyl chloride (the source of the 3 last compounds is less clear but ethylene oxide and vinyl chloride are both classified by the International Agency for Research on Cancer (IARC) as Group 1 (carcinogenic to humans), and nitrobenzene as Group 2B (possibly carcinogenic to humans)).
- **Criterion 5**: Toxicants which are predominantly formed above 400 °C, and which are not included under "Criterion 1" and "Criterion 2": dibenz[*a,h*]anthracene and benz[*a*]anthracene (McGrath et al., 2007).

In addition, glycerin (a humectant used during CC and *Tobacco stick* manufacturing) and menthol (for mentholated products only) were also quantified. This results in a total of 59 analytes for THS2.2 regular products (60 for mentholated products) that were quantified to perform the chemical assessment of the THS2.2 aerosol. Among them, 54 are HPHCs targets for reduction compared to 3R4F when developing *heat-not-burn* products: carbon monoxide, benzo[*a*]pyrene, 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde, acrylonitrile, 1,3-butadiene, benzene, isoprene, pyridine, quinoline, styrene, toluene, catechol, *o*-cresol, *m*-cresol, *p*-cresol, hydroquinone, phenol, resorcinol, NAB, NAT, NNK, NNN, ammonia, hydrogen cyanide, nitric oxide, nitrogen oxides, arsenic, cadmium, chromium, lead, mercury, nickel, selenium, pyrene, *o*-toluidine, acetamide, acrylamide, ethylene oxide, nitrobenzene, propylene oxide, vinyl chloride, benz[*a*]anthracene and dibenz[*a,h*]anthracene. As previously mentioned, this list was mainly based on analytes proposed by public health authorities and it covers a large range of potential toxicants identified in cigarette smoke. However, the scientific literature continues to describe new compounds with a potential toxicity mainly in aerosols from new products. For instance, only recently has glycidol been identified as a potential toxic compound in aerosols from electronic cigarettes (Sleiman et al., 2016). The present list of HPHCs was based on knowledge available at the time of designing the studies and glycidol, which was not included in the FDA list (U.S. Food and Drug Administration, 2012), was not identified at the time as a potential target to be included.

In addition to the 54 HPHCs listed above, TPM, water, nicotine, NFDPM, glycerin and menthol (for mentholated products only) are used to assess product performance but are not targets for reduction compared to the 3R4F. These analytes are major aerosol constituents for which the level has to be maintained in order to provide satisfactory sensory properties. For instance, nicotine is addictive and has toxic properties. However, at the levels nicotine is consumed from tobacco products, it is not considered to be a primary cause of smoking related disease (The Royal College of Physicians, 2016). As noted by the Royal College of Physicians (The Royal College of Physicians, 2016) "The ideal harm-reduction device should therefore deliver nicotine in a manner as similar as possible to cigarettes, while at the same time maximizing palatability and nicotine delivery to approximate the experience of cigarette smoking more closely." Following this logic, the nicotine content in the delivered aerosol was carefully monitored and the nicotine level was by design maintained at an appropriate level. It is important to note that, although TPM and NFDPM are analytes that were originally defined in the context of CC smoke, they can also be used in the context of aerosol produced by heating tobacco, as they characterize the mass of aerosol delivered. However, TPM and NFDPM yields obtained from heated products should not be directly compared to the values obtained for cigarettes, as the smoke produced by combustion and the aerosol produced by heating tobacco have substantially different chemical compositions.

The exposure to HPHCs may also be affected by the physical properties of the inhaled aerosol. The particle/droplet size

distribution determines the fraction of aerosol or smoke that is able to pass through the upper respiratory tract to reach the lungs and the fraction that is retained in the respiratory system (Robinson and Yu, 2001; Bernstein, 2004; Kane et al., 2010). An aerosol is considered respirable when the mass median aerodynamic diameter (MMAD) calculated from the measured size distribution is below 2.5 μm (Hinds, 1999). In the past decades, several instruments have been used to determine the size distributions of CC mainstream smoke, and more recently, of e-cigarette aerosols as well (Singh et al., 2006; Ingebrethsen et al., 2012; Fuoco et al., 2014; Geiss et al., 2015). These analytical techniques have been shown to have both advantages and drawbacks in measuring the aerosol physical parameters. Past studies revealed that different methodologies led to size distributions ranging from 0.2 to 0.9 μm for CC smoke (Ishizu et al., 1978; Davies, 1988). This wide range of droplet sizes obtained for CC was presumably due to particular aerosol sampling and dilution methodologies. The methodologies enabling a real-time aerosol physical characterization used the measured aerosol number size distribution to calculate the related mass concentration distribution and the MMAD, assuming spherical particles or droplets with a chosen arbitrary density. Consequently, the conversion from number to mass distribution could lead to the overestimation or underestimation of the MMAD. To minimize these problems, the selected physical characterization of the aerosol was based on multistage cascade impactor technology, allowing a determination of the MMAD. The impactor technique enabled the gravimetrical classification of aerosol droplets in distinct size classes, and was often associated with the related aerosol deposition behavior in lungs (Hinds, 1999). Additionally, the technique allowed the determination of the MMAD assuming equivalent unit density and spherical droplet shapes.

The development of a new product such as the THS2.2 should be accompanied not only by the analysis of its chemical composition but also by *in vitro* and *in vivo* toxicological assays, because current knowledge is insufficient for predicting the effect of complex mixtures based solely on the chemical composition (Carchman, 1988; Institute of Medicine, 2001; Tewes et al., 2003). To further extend the characterization of the THS2.2 aerosol, both the cytotoxic and mutagenic activity of the aerosol were compared to the activities of mainstream smoke of the reference cigarette 3R4F. Furthermore, two 90-day rodent inhalation studies are also reported in this issue (Sewer et al., 2016; Wong et al., 2016).

The current studies report the aerosol characterization from 4 different versions of the THS2.2 Tobacco Sticks: two THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M). These studies include the cytotoxic and genotoxic activities of the mainstream THS2.2 aerosol compared to mainstream smoke of the reference cigarette 3R4F and a comprehensive physical and chemical characterization of the produced aerosols. For both types of aerosols, the cytotoxicity of the gas-vapor phase (GVP) and the particulate phase (TPM) was assessed using the neutral red uptake (NRU) assay (Borenfreund and Puerner, 1985) and the genotoxicity was assessed using both the *Salmonella typhimurium* reverse mutation assay (TPM only) (Ames (Ames et al., 1973)) and the mouse lymphoma assay (MLA) (Clive et al., 1972). The physicochemical characterization was based on the comparison of the droplet diameter of both aerosols and the presence of 54 HPHCs in each aerosol using the Health Canada Intense (HCI) machine-smoking protocol (Health Canada, 2000). In addition, the THS2.2 aerosol chemical composition was assessed under different climatic conditions (temperature and relative humidity) and with different machine-smoking regimens to simulate different use than described in the HCI machine-smoking protocol.

2. Materials and methods

2.1. Reference cigarette

The 3R4F reference cigarette was obtained from the University of Kentucky (Lexington, KY, USA; http://www.ca.uky.edu/refcig/).

2.2. THS2.2 tobacco stick

The *tobacco stick* was designed to be used with the THS2.2 holder (Smith et al., 2016). Its construction is shown in Fig. 1.

A *tobacco stick* is constructed by the sequential assembly of the following components:

1. Tobacco plug wrapped in a paper over-wrap.
2. Hollow acetate tube wrapped in a paper over-wrap.
3. Polymer-film filter, wrapped in a paper over-wrap.
4. Mouthpiece filter wrapped in a paper over-wrap.

All these elements are wrapped in an outer paper, and a tipping paper is added on the mouth end (Fig. 1).

Unlike CC, the THS *tobacco stick* does not burn when used, and thus its length remains unchanged. The tobacco plug is made of reconstituted cast leaf tobacco containing various tobacco types from different origins, as well as binders and humectants. The humectants were added to prevent the cast leaves becoming too brittle. Heating the humectants caused them to evaporate and recondense to form small droplets, generating a visible aerosol. Four different *tobacco stick* variants were used for aerosol characterization: two versions of the THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M). These four THS2.2 tobacco sticks contain flavor ingredients. THS2.2 FR1 M and THS2.2 D1 M contain natural *l*-menthol applied to a cellulose acetate yarn included in the polymer-film filter and to the inner liner paper included in the tobacco stick pack. FR1, D2 and D1 are different tobacco blends but the tobacco sticks were of the same design.

2.3. Cambridge glass-fiber filter pad, cigarette, and tobacco stick conditioning

The reference cigarette 3R4F and THS2.2 *tobacco sticks* were stored at 5 ± 3 °C with uncontrolled humidity conditions in the original packaging, before conditioning. For the ISO/Health Canada conditioning, test articles were conditioned for at least 48 h at 22 ± 1 °C and 60± 2% relative humidity (RH), according to the International Organization for Standardization (ISO) method 3402 (International Organisation for Standardization, 2010a). Cambridge glass-fiber filter pads were conditioned under the same conditions.

Prior to the analyses performed in “tropical“ and “desert“ conditions, test articles were either conditioned for at least 48 h at 30 ± 1 °C and 75± 2% RH (“tropical“ conditions), or at 30 ± 1 °C and 35± 2% RH (“desert“ conditions). The corresponding Cambridge glass-fiber filter pads were conditioned under the same climatic conditions.

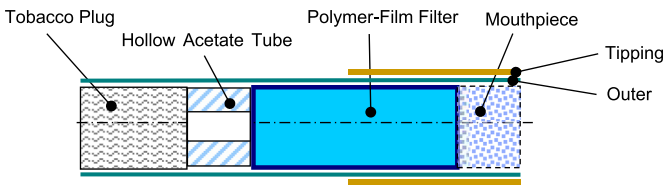

Fig. 1. THS2.2 *tobacco stick.*

2.4. Generation of THS2.2 aerosol and mainstream smoke of 3R4F according to the HCI machine-smoking regimen

The mainstream aerosol of THS2.2 and the smoke of the reference cigarette 3R4F were generated on a Borgwaldt linear smoking machine type LM20X (Borgwaldt KC GmbH, Hamburg, Germany) for the determination of all analytes (except elements) according to the HCI machine-smoking regimen (Health Canada, 2000). For the elements, the mainstream aerosol of THS2.2 and the smoke of the reference cigarette 3R4F were generated from a Burghart rotary smoking machine type RMB 20 (Burghart Tabaktechnik GmbH, Wedel, Germany) with the same smoking regimen.

For the *in vitro* biological assays test battery (Ames, MLA, and NRU), the 3R4F mainstream smoke and THS2.2 aerosols were generated using a Burghart rotary smoking machine type RMB 20 (Burghart Tabaktechnik GmbH, Wedel, Germany) according to the Health Canada Intense (HCI) smoking regimen (Health Canada, 2000). The generated aerosol and smoke were trapped to analyze the aerosols. After trapping, the samples were analyzed and processed (section 2.7). Processing describes the conversion from a primary result, e.g., a peak area or counts to a value per cigarette or per stick, taking the number of accumulations, the trapping or extraction volume or dilution into account. For *in vitro* assessments, the aerosol was fractionated into two parts, TPM and GVP, during the same aerosol collection (except for the Ames assay, where only TPM was tested). At the end of the aerosol generation, the collected TPM aerosol fraction on the Cambridge glass-fiber filter pad was solubilized in dimethyl sulfoxide (DMSO), and the water-soluble GVP fraction was immobilized into an impinger of ice-cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) solution.

The reference cigarette 3R4F was smoked to a butt length of 35 mm using a bell-shaped puff profile and 100% blocking of ventilation holes. THS2.2 Regular and Menthol *tobacco sticks* were 'smoked' using a bell-shaped puff profile to a defined puff count of 12 puffs. The limitation to 12 puffs is based on the fixed settings of the THS2.2 system, which is programmed to finish heating after a maximum period of 6 min, and the puff interval of the HCI regimen (30 s).

2.5. Generation of THS 2.2 aerosol under different climatic conditions

THS2.2 aerosol was generated under different ambient temperature and RH conditions of 22 ± 2 °C and 60± 5% RH, 30 ± 2 °C and 75± 5% RH, and 30 ± 2 °C and 35± 5% RH to simulate "Mediterranean", "Tropical" and "Desert" climates, respectively (Table 1), using a linear smoking machine prototype SM405XR (Cerulean Molins PLC, Milton Keynes, UK) and the HCI machine-smoking regimen (Health Canada, 2000). The smoking machine was housed in a conditioned air cabinet (temperature range: 10 °C–35 °C; humidity range: 10%–80% RH) fitted with a Delta 335 air conditioning unit (Design Environmental Ltd, Ebbw Vale, UK). The atmosphere of the cabinet was constantly refreshed with conditioned air. The temperature and RH in the cabinet was monitored using a TH1 dataloger (ELPRO-Buchs AG, Buchs, Switzerland).

Table 1
Climatic conditions.

Conditions	Temperature [°C]	Relative Humidity [% RH]
Mediterranean	22 ± 2	60± 5%
Tropical	30 ± 2	75± 5%
Desert	30 ± 2	35± 5%

2.6. Generation of THS2.2 aerosol under alternative puffing regimens

THS2.2 aerosol was generated according to the alternative puffing regimen presented in Table 2. A Cerulean SM450RH smoking machine (Cerulean Molins PLC, Milton Keynes, UK) was used to generate aerosols for the analysis of all analytes except nitric oxide (NO) and nitrogen oxides (NO_x). The NO and NO_x measurements were performed on a Borgwaldt linear smoking machine type LM20X (Borgwaldt KC GmbH, Hamburg, Germany). Since this smoking machine is limited to puffs of 100 ml, NO and NO_x measurement were not performed for the LR-3 regimen (Table 2).

The alternative puffing regimens (SR-1, SR-4, SR-5, SR-6 and LR-3) were selected according to human puffing behavior observed with THS2.2 users (Campelos et al., 2016).

2.7. Chemical analyses

All analytes were determined using 15 separate aerosol and smoke generations. The 15 separate analyte groups and corresponding individual analytes are shown in Table 3.

The description of the analytical methods used to quantify the analytes in the THS2.2 aerosol and in the smoke of the 3R4F reference cigarette are presented in the supplementary material section.

2.8. Physical measurements

The droplet size distribution measurements were conducted using a PIXE multistage cascade impactor (PIXE International Corp., Tallahassee, FL USA) using a sampling flow rate of 1 l/min. During this study, the PIXE cascade impactor was composed of nine impactor stages. For both 3R4F and THS2.2 Tobacco Sticks, the average mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were estimated from 10 replicate aerosol samples. The average GSD was determined as the square root of the average GSD^2. The test items were connected to the inlet of a programmable dual syringe pump (PDSP) (Burghart Messtechnik GmbH, Wedel, Germany). The outlet of the PDSP was connected to a glass T-junction that allowed aerosol transfer before it entered the PIXE cascade impactor. The outlet of the PIXE cascade impactor was connected to a pump (Vacuubrand GmbH + CO KG, Wertheim, Germany) (Fig. 2).

Table 2
Smoking machine settings for the generation of THS2.2 aerosol generation under alternative puffing regimens.

Regimen	Puff volume [ml]	Puff duration [s]	Puff interval [s]	Number of puffs[a] [n]
ISO	35	2.0	60	6
SR-1	40	2.4	30	8
SR-5	80	2.4	30	8
HCI	55	2.0	30	12
SR-4	60	2.4	25	14
SR-6	80	2.4	25	14
LR-3	110	4.5	22	14

[a] The number of puffs results from puff intervals of the different smoking regimens and the fixed settings of the THS2.2 system, which is programmed to finish heating after a maximum period of 6 min and allows up to 14 puffs to be taken during that time.

Table 3
Analyte groups and corresponding individual analytes.

Analyte Group	Individual Analytes
ISO parameters and product-specific constituents	Total particulate matter (TPM), water, nicotine, nicotine-free dry particulate matter (NFDPM), carbon monoxide (CO), glycerol
Volatiles- and semi-volatiles	1,3-butadiene, isoprene, benzene, toluene, styrene, pyridine, quinoline, acrylonitrile
Carbonyls	acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde
Aromatic amines	1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, *o*-toluidine
Nitrogen oxides	nitric oxide (NO), oxides of nitrogen (NO_x)
Hydrogen cyanide	hydrogen cyanide
Ammonia	Ammonia
Epoxides and vinyl chloride	ethylene oxide, propylene oxide, vinyl chloride
Tobacco-specific nitrosamines	*N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), *N*-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)
Phenols and acid derivatives	catechol, *o*-cresol, *m*-cresol, *p*-cresol, hydroquinone, phenol, resorcinol, acetamide, acrylamide
Polycyclic aromatic hydrocarbons	benzo[*a*]pyrene, benz[*a*]anthracene, dibenz[*a*,*h*]anthracene, pyrene
Nitrobenzene	Nitrobenzene
Elements (except mercury)	arsenic, cadmium, chromium, lead, nickel, selenium
Mercury	Mercury
Menthol	Menthol

The calculation of MMAD and GSD was done separately for each measurement. The following steps were performed:

1. Calculation of the net weight for each impactor stage of the cascade impactor: ΔM_i = [Weight loaded (g)]$_i$ − [Weight empty (g)]$_i$ (i = 1, …, i).
2. Calculation of the total mass by summing up all of the i net weights: TM = $\sum \Delta M_i$.
3. Calculation of the mass fraction for each stage: $Mf_i = \Delta M_i/TM$ (i = 1, …, i).
4. Normalization of the mass fraction for each stage by the width of successive cutoff diameter [ΔD50%]: The cutoff diameter represents the smaller size that can be captured on the related stage, whereas the maximum size collected on that stage is related to the next larger cutoff diameter.
   $nMf_i = Mf_i/[\Delta D50\%]_i$ (i = 1, …, i)
   The values for the cutoff diameters $[\Delta D50\%]_i$ (i = 1, …, i) are given in Table 4.
5. The calculation of the MMAD and GSD was performed using Igor Pro version 6.3.2.3 (WaveMetrics, Inc., Lake Oswego, OR, USA) using a lognormal mono-modal fitting distribution (Equation (1)) of the normalized mass fraction nMf_i vs. the respective mid-point diameter D_i

$$f(d_d) = \frac{1}{d_d \ln(GSD)\sqrt{2\pi}} e^{-\frac{\left(\ln(d_d)-\ln(MMAD)\right)^2}{2(\ln(GSD))^2}} \quad (1)$$

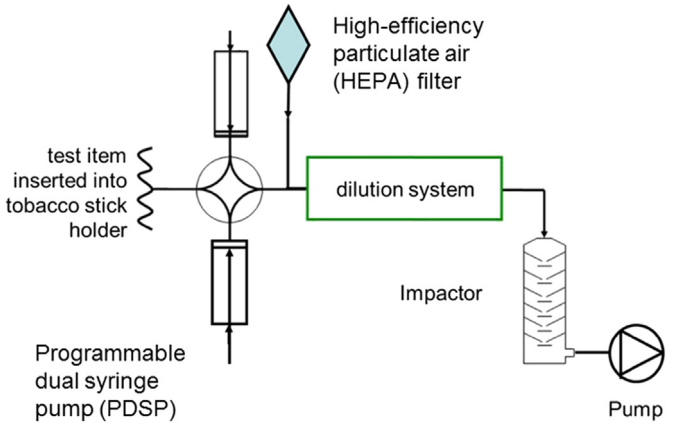

Fig. 2. Experimental setup for aerosol physical measurement.

where d_d is the size diameter in micrometers, GSD is the geometric standard deviation, MMAD is the mass median aerodynamic diameter, and $f(d_d)$ represents the normalized mass fraction data.

The mass of each impactor stage was recorded prior to and after each aerosol collection. Subsequently, the mass fraction deposited on the different impactor stages was calculated, whereas the size bins were normalized by dividing the mass fractions with their respective widths. This process permitted the transformation of discrete data points into density functions that could be fitted with a continuous lognormal distribution function, from which both the MMAD and the GSD were calculated. The negative values were not replaced by zero. The lower boundaries (LB) and upper boundaries (UB) were calculated at the 95% confidence interval using the following equation:

$$LB = MMAD/GSD^2;\ UB = MMAD \times GSD^2$$

2.9. In vitro toxicology

All *in vitro* studies were performed in full accordance with the principles of Good Laboratory Practice.

2.9.1. Neutral RED uptake (NRU) assay

The mouse embryonic fibroblast cell line Balb/c 3T3 (clone A31) was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK), and was used to perform the NRU assay according to INVITTOX protocol 3a (INVITTOX, 1990), with some modifications (Borenfreund and Puerner, 1985). Sodium dodecyl

Table 4
Cutoff diameters and mid-point diameters for each stage of the PIXE cascade impactor.

Stage	Cutoff diameter (μm)	Mid-point (μm)
	ΔD50%	D_i
7	16	16
6	8	12
5	4	6
4	2	3
3	1	1.5
2	0.5	0.75
1	0.25	0.375
L2	0.12	0.185
L1	0.06	0.09

sulfate was used as the positive control.

In brief, 20–28 h prior to aerosol fraction generation, cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (10% v/v) (Thermo Fisher Scientific, Waltham, MA, USA), 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Subsequently, 4.75–5.25 $\times$ 10^3 viable cells were seeded in each well of a 96-well plate and cultured at 37 °C (5% CO_2 and 70% RH). Cells were exposed to eight concentrations of each test substance for 23 ± 1 h in 96-well plates with six wells used per concentration. The exposure plates were sealed with a CO_2-permeable plastic film to prevent potential carry-over of volatile substances. Following the exposure phase, the cell culture medium was replaced with cell culture medium containing neutral red dye at 50 μg/ml, and incubated at 37 °C (5% CO_2 and 70% RH) for an additional 3 ± 0.5 h. Subsequently, the cells were washed with PBS, and the neutral red dye taken up by cells was extracted by the addition of destaining solution (ethanol, water, and acetic acid, mixed in a 50:49:1 ratio). The plates were mechanically shaken using a vibrating platform shaker (Titramax 1000, Heidolph Instruments, Schwabach, Germany) for 10 min at approximately 450 strokes/min. Neutral red absorbance was measured at 540 nm with a microplate reader (Safire 2, Tecan GmbH, Grödig, Austria). The measured absorbance for each concentration was normalized against the appropriate solvent control and converted to a percentage value. Cytotoxicity was expressed as $1/EC_{50}$, and expressed as a function of the mass of TPM trapped on the Cambridge glass-fiber filter pads (TPM basis) and on a per-mg nicotine basis. For consistency and to compare GVP fractions, data were calculated and expressed on a per-mg TPM basis and on a per-mg nicotine basis (Roemer et al., 2014). The EC_{50} endpoint measurement corresponds to the concentration of test substance for which a decrease of 50% in the uptake of the neutral red dye is observed, and was determined with the SAS® Enterprise guide® 4.3 (SAS 9.2) software program (SAS, Cary, NC, USA).

The relationship between the concentration of the substance and the decrease in the uptake of neutral red dye has a sigmoid shape and is described by the Hill function, which is a four parameter non-linear function. Statistical analysis was performed using SAS. Unless mentioned otherwise in the text, all reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.9.2. Ames assay

Mutagenic activity was evaluated by using the *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 with and without an S9 enzymatic metabolizing fraction, by following a pre-incubation method (Maron and Ames, 1983) and the OECD 471 test guideline (Organisation for Economic Co-operation and Development, 1997). The S9 enzymatic metabolizing fraction was obtained from Aroclor 1254-induced male Sprague–Dawley rat liver (Moltox, NC, USA). The TPM mainstream smoke fraction from the 3R4F reference cigarette was generated and tested in parallel with the THS2.2 aerosol fraction. The strains were grown overnight in a shaking incubator at 37 °C for approximately 10 h in Oxoid Nutrient Broth No. 2 (Fisher Scientific, Reinach, Switzerland). To determine mutagenic activity, seven different concentrations of THS2.2 TPM diluted in DMSO were tested. The bacteria (approximately 1 $\times$ 10^9 in 100 μl) were combined with 50 μl of either the test item, the solvent, or the positive control, and 500 μl of the cofactor buffer (pH 7.4) supplemented with S9 as appropriate and pre-incubated at 37 °C for 20 min prior to adding 2 ml of histidine (50 μM final concentration) supplemented soft top agar and plating the entire mixture onto histidine-deficient 90 mm minimal glucose agar base plates for 2 days at 37 °C. Revertant colonies were counted using an automatic colony counter (Sorcerer, Perceptive Instruments, Bury Saint Edmunds, UK). All experiments were performed in triplicate. Toxicity was detected as either a reduction in the number of histidine revertants or as a thinning of the auxotrophic background lawn. The mutagens used as positive controls, i.e. substances known to induce a mutagenic response to demonstrate the assay is working efficiently, in experiments without the S9 mix were 4-nitrophenylenediamine (10 μg/plate) for TA98 and TA100, sodium azide (1.25 μg/plate) for TA1535 and TA1537, and cumene hydroperoxide (3 μg/plate) for TA102. In the experiments that included the S9 fraction, benzo[*a*]pyrene (1 μg/plate) was used for TA98, and 2-aminoanthracene (2.5 μg/plate) was used for TA100, TA102, TA1535, and TA1537. DMSO (50 μl/plate) served as the solvent control. All positive control chemicals were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Moltox (Boone, NC, USA). The biological activity after 1 mg of TPM exposure is reported as a means to permit a rapid assessment of the impact of the 3R4F aerosol vs. the THS2.2 aerosol. One milligram was the maximum dose tested of the 3R4F aerosol (for toxicity reasons) and thus comparison at 5 mg or higher concentrations was not technically possible.

2.9.3. Mouse lymphoma assay (MLA)

The L5178Y *tk* $^{\pm}$ cell line (sub-clone 3.7.2C (IVGT), Public Health England, UK) was used in the MLA. Spontaneously-occurring $tk^{-/-}$ mutants were purged from working stocks using methotrexate to select against *tk*-deficient cells and thymidine, hypoxanthine, and guanine to ensure optimal growth of *tk*-proficient cells as previously described (Chen and Moore, 2004). Cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with heat-inactivated horse serum (10% v/v) (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (200 U/ml), streptomycin (200 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and pluronic acid F68 (0.1% v/v) (Sigma–Aldrich, St. Louis, MO, USA). Short-term treatments (4 h) were carried out in reduced serum medium (3% v/v), while 10% v/v-containing medium was utilized for longer-term exposures (24 h). Cloning was carried out in 20% v/v-containing serum medium in the absence of pluronic acid F68. Aroclor 1254-induced male Sprague–Dawley rat liver S9 in 0.15 M KCl (Moltox, USA), in combination with a cofactor mix of glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (both from Roche Applied Science, Basel, Switzerland), were used as the exogenous metabolic activation system in the assay. The final concentration of S9 in cell cultures was 0.95 mg protein/ml (2% v/v). The controls used were methyl methanesulfonate (15 and 20 μg/ml) for the 4 h S9- test arm, methyl methanesulfonate (5 and 7.5 μg/ml) for the 24 h S9- arm, and 7,12-dimethylbenz[a]anthracene (1 and 1.5 μg/ml) for the 4 h S9+ arm.

The microwell version of the MLA was performed according to the OECD TG 490 guideline (Organisation for Economic Co-operation and Development, 2015). Briefly, on two independent test occasions, L5178Y cells in single replicate cultures were seeded at a density of 5 $\times$ 10^5 or 2 $\times$ 10^5 cells/ml (at least 6 $\times$ 10^6 cells), and exposed to 14 concentrations of TPM and GVP derived from THS2.2 and the 3R4F reference cigarette for 4 h in the presence (+S9) and absence (-S9) of metabolic activation and 24 h in the absence of metabolic activation (−S9) treatment conditions, respectively. For each treatment condition, cells were exposed for 4 or 24 h at 37 °C in the presence of 5% CO_2 and RH $\geq$ 65%. Following treatment, cells were washed and sub-cultured at a maximum density of 2 $\times$ 10^5 cells/ml (at most 6 $\times$ 10^6 cells) for two further days to allow phenotypic expression of the *tk* gene prior to mutant selection. Cells at 8 cells/ml or 1 $\times$ 10^4 cells/ml were then distributed into 96-microwell plates (200 μl per well) to determine final levels of TPM- and GVP-induced cytotoxicity and mutagenicity, respectively. Cytotoxicity was determined from the relative total growth (RTG) of

the cell cultures following treatment and sub-culture periods in non-selective growth medium (typically 10–11 days). TPM- and GVP-treated cell cultures which underwent excessive cytotoxicity were discarded through the assay procedure as mutagenicity data derived from these cells are difficult to interpret because of their questionable biological relevance. *Tk* mutants were detected following culture in trifluorothymidine (TFT)-containing growth medium (Sigma–Aldrich, St. Louis, MO, USA) for typically 14 days. Mutant colonies were enumerated visually; colonies with a size less than a quarter of the microwell's surface area were defined as small colonies, while ones covering more than a quarter of the microwell's surface area were defined as large colonies. Mutation frequencies were calculated according to published method (Clements, 2000). The controls used were methyl methanesulfonate (15 and 20 μg/ml) for the 4 h S9- test arm, methyl methanesulfonate (5 and 7.5 μg/ml) for the 24 h S9- arm and finally 7,12-dimethylbenz[*a*]anthracene (1 and 1.5 μg/ml) for the 4 h S9+ arm.

The data generated from solvent-treated and positive controls in each treatment condition on the separate test occasions were evaluated for acceptability according to OECD TG 490 guideline (Organisation for Economic Co-operation and Development, 2015) and the laboratory's historical control database. Furthermore, a response to an aerosol fraction was considered positive, i.e. mutagenic, in the MLA if there was a concentration-related increase in mutation frequency (MF) with a corresponding RTG not lower than 10%, and if an MF exceeded the sum of the microwell global evaluation factor (GEF) of 126 plus the mean MF of the solvent-treated controls (Moore et al., 2006, 2007). The microwell GEF of 126 mutants per 10^6 viable cells was previously defined as the mean of the negative/solvent control MF distribution plus one standard deviation in a multi-laboratory microwell MLA study (Moore et al., 2006). Mutagenic potencies were evaluated using the lowest observed genotoxic effect level (LOGEL) (Guo et al., 2015). A LOGEL in this study was defined as the lowest concentration of TPM or GVP (expressed on a per-mg nicotine basis) tested that induced a mutagenic response which exceeded the GEF.

2.10. Statistical analyses

The results of the analyte quantifications are expressed in two different ways: On a per-Tobacco Stick/cigarette basis, and on a per-mg nicotine basis. For all analyte, the number of values (N), the arithmetic mean (M), and the confidence interval of the mean at 95% ($CI_{95\%}$) are given. For groups including at least one measured value below the limit of quantification (LOQ) of the analytical method, only the median or the LOQ was given, depending on whether the median was above or below the LOQ. The mean on a per-mg nicotine basis and the respective confidence interval of the mean were calculated as follows:

$$M = \frac{M(A)}{M(B)}$$

where A denotes the value of an analyte on a cigarette basis, and B denotes the mean value for nicotine on a cigarette basis.

and

$$\pm CI_{95\%}\left(\frac{M(A)}{M(B)}\right) = \pm t(0.975, N-1) * \frac{\frac{M(A)}{M(B)} * \sqrt{\frac{S^2_{M(A)}}{M(A)^2} + \frac{S^2_{M(B)}}{M(B)^2}}}{\sqrt{N}}$$

where A denotes the mean of an analyte on a cigarette basis, B denotes the mean for nicotine on a cigarette basis, S is the standard deviation, N is the number of measurements, t(p,df) is the percentile of Student's distribution, and df is degrees of freedom.

For the NRU assay, the cytoxicity ($1/EC_{50}$) of the 3R4F reference cigarette and the THS2.2 FR1 and THS2.2 FR1 M Tobacco Sticks, expressed on a per-TPM basis and on a per-mg nicotine basis, was compared using one-way analysis of variance followed by Dunnett's multiple comparison procedure, with the 3R4F reference cigarette as the reference group. This statistical approach was used because 2 THS2.2 test items were compared with 3R4F within one study. However, the cytotoxicity of the THS2.2 D2 and THS2.2 D1 M *tobacco sticks* was compared against the 3R4F reference cigarette using Student's *t*-test. In this case the *t-test* was used because 2 studies were performed, each with one test item and the reference cigarette 3R4F.

3. Results

3.1. Chemical composition of the THS2.2 aerosol, and comparison with the mainstream smoke from the 3R4F reference cigarette

Two versions of the THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M) were compared with the 3R4F reference cigarette under HCI machine-smoking conditions. Fifty-nine analytes (60 for menthol products) were determined, covering various chemical classes present in different aerosol phases (Table 5 and Table 6). Fifteen analytes in THS2.2 FR1 (benzo[*a*]pyrene, 3- and 4-aminobiphenyl, quinoline, NAT, pyrene, nitrobenzene, vinyl chloride, dibenz[*a,h*]anthracene, and all the elements except mercury) and 11 analytes in THS2.2 D2 (2-aminonaphthalene, 3- and 4-aminobiphenyl, vinyl chloride, dibenz[*a,h*]anthracene, and all the elements except mercury) were below the LOQ. In THS2.2 FR1 M, the yields of 11 analytes (2-aminonaphthalene, 4-aminobiphenyl, quinoline, NAT, all the elements except mercury and selenium, vinyl chloride, and dibenz[*a,h*]anthracene and 8 analytes in the THS2.2 D1 M (2-aminonaphthalene, 3-aminobiphenyl, arsenic, cadmium, lead, selenium, vinyl chloride, and dibenz[*a,h*]anthracene) were below the LOQ. The 54 HPHCs which were targets for reduction (Section 1) were lower in the THS2.2 aerosol than in the 3R4F smoke. The yields of analytes expressed on a per-mg nicotine basis are presented in Table A and in Table B of the supplementary material.

The pie charts in Fig. 3 illustrates the differences in TPM composition between the 3R4F and the THS2.2 FR1. While the THS2.2 FR1 delivered about the same TPM yield as the 3R4F, the THS2.2 aerosol composition was qualitatively and quantitatively different from that of 3R4F. The quantities of water and glycerol relative to total TPM were considerably higher for the THS2.2, whereas the amount of nicotine was approximately 30% lower for the THS2.2. Therefore, the relative yields of the 'other' aerosol constituents were noticeably lower in the THS2.2 TPM. This is also evident when comparing the color of the Cambridge glass-fiber filter pads after collection of the same amount of aerosol mass and TPM from THS2.2 tobacco sticks and 3R4F cigarettes, respectively (Fig. 3). Previous *heat-not-burn* products such as Premier (deBethizy et al., 1990) and Eclipse (Borgerding et al., 1998) also produce TPM compositions that contained mainly water and glycerin. Visually, the collected aerosol from THS2.2 on a Cambridge glass-fiber filter pad appears similar to that seen for both Premier and Eclipse.

3.2. Chemistry of the THS2.2 aerosol generated under different climatic conditions

The three climatic conditions, "Mediterranean", "desert", and "tropical", were selected according to ICH (International Council

Table 5
Analyte yields from THS2.2 FR1, THS2.2 FR1 M, and 3R4F obtained under HCI machine-smoking conditions and expressed on a per-cigarette/tobacco stick basis.

Parameter	Unit	THS2.2 FR1		THS2.2 FR1 M		3R4F	
		Mean ± $CI_{95\%}$	N	Mean ± $CI_{95\%}$	N	Mean ± $CI_{95\%}$	N
TPM	mg/stick	48.2 ± 2.4	4	43.5 ± 1.5	4	49.0 ± 4.8	4
Water	mg/stick	36.5 ± 3.1	4	29.7 ± 3.6	4	15.8 ± 2.9	4
Nicotine	mg/stick	1.32 ± 0.16	4	1.21 ± 0.09	4	1.89 ± 0.16	4
NFDPM	mg/stick	10.3 ± 0.9	4	12.6 ± 2.2	4	31.2 ± 1.8	4
Carbon monoxide	mg/stick	0.531 ± 0.068	4	0.594 ± 0.110	4	32.8 ± 2.4	4
Benzo[*a*]pyrene	ng/stick	<1.00	4	1.29 ± 0.10	4	14.2 ± 0.3	4
Puff Count	/stick	12 ± 0	4	12 ± 0	4	10.6 ± 0.4	4
Menthol	mg/stick	n.a.		2.62 ± 0.1	4	n.a.	
Glycerin	mg/stick	4.63 ± 0.83	4	3.94 ± 0.87	4	2.42 ± 0.14	4
1-aminonaphthalene	ng/stick	0.077	4	0.086	4	20.8 ± 1.3	4
2-aminonaphthalene	ng/stick	0.046 ± 0.008	4	<0.035	4	11.0 ± 0.6	4
3-aminobiphenyl	ng/stick	<0.032	4	0.032	4	3.77 ± 0.47	4
4-aminobiphenyl	ng/stick	<0.051	4	<0.051	4	3.26 ± 0.12	4
Acetaldehyde	µg/stick	219 ± 31	4	205 ± 12	4	1555 ± 184	4
Acetone	µg/stick	40.7 ± 6.2	4	39.4 ± 2.3	4	736 ± 129	4
Acrolein	µg/stick	11.30 ± 2.36	4	9.15 ± 0.43	4	154 ± 20	4
Butyraldehyde	µg/stick	26.1 ± 2.3	4	26.7 ± 2	4	88.4 ± 10.7	4
Crotonaldehyde	µg/stick	4.14 ± 0.23	4	3.24 ± 0.21	4	68.8 ± 14.4	4
Formaldehyde	µg/stick	5.53 ± 0.69	4	4.55 ± 0.25	4	56.5 ± 12.1	4
Methyl ethyl ketone	µg/stick	7.18 ± 1.19	4	6.93 ± 0.64	4	187 ± 30	4
Propionaldehyde	µg/stick	14.5 ± 2.4	4	13.9 ± 0.7	4	125 ± 16	4
Acrylonitrile	µg/stick	0.258 ± 0.041	4	0.220 ± 0.014	4	31.9 ± 1.8	4
1,3-butadiene	µg/stick	0.294 ± 0.042	4	0.265 ± 0.024	4	63.8 ± 3.5	4
Benzene	µg/stick	0.649 ± 0.074	4	0.640 ± 0.040	4	97.6 ± 4.7	4
Isoprene	µg/stick	2.35 ± 0.39	4	2.11 ± 0.18	4	798 ± 49	4
Pyridine	µg/stick	7.54 ± 0.26	4	7.21 ± 0.25	4	36.1 ± 2.2	4
Quinoline	µg/stick	<0.012	4	<0.012	4	0.513 ± 0.023	4
Styrene	µg/stick	0.608 ± 0.058	4	0.561 ± 0.033	4	24.5 ± 1.2	4
Toluene	µg/stick	2.59 ± 0.43	4	2.39 ± 0.16	4	188 ± 11	4
Catechol	µg/stick	16.3 ± 1.5	4	17.1 ± 1.1	4	91.4 ± 5.6	4
o-cresol	µg/stick	0.069 ± 0.008	4	0.095 ± 0.025	4	4.47 ± 0.16	4
m-cresol	µg/stick	0.029 ± 0.004	4	0.033 ± 0.006	4	3.03 ± 0.08	4
p-cresol	µg/stick	0.072 ± 0.008	4	0.083 ± 0.010	4	9.17 ± 0.44	4
Hydroquinone	µg/stick	8.10 ± 0.48	4	8.98 ± 1.02	4	83.1 ± 5.5	4
Phenol	µg/stick	1.16 ± 0.12	4	1.60 ± 0.4	4	13.6 ± 0.9	4
Resorcinol	µg/stick	0.041 ± 0.003	4	0.048 ± 0.004	4	1.85 ± 0.08	4
NAB	ng/stick	<3.15	4	<3.15	4	33.7 ± 8.5	4
NAT	ng/stick	20.5 ± 0.5	4	19.7 ± 3.6	4	318 ± 74	4
NNK	ng/stick	6.7 ± 0.6	4	5.9 ± 0.4	4	266 ± 15	4
NNN	ng/stick	17.2 ± 1.25	4	13.7 ± 1.21	4	309 ± 41	4
Ammonia	µg/stick	14.2 ± 1.1	4	13.8 ± 0.7	4	39.3 ± 3.2	4
Hydrogen cyanide	µg/stick	4.81 ± 0.35	4	5.14 ± 0.70	4	493 ± 78	4
Nitric oxide	µg/stick	16.8 ± 2.3	4	12.3 ± 1.7	4	491 ± 38	4
Nitrogen oxides	µg/stick	17.3 ± 2.6	4	12.6 ± 1.7	4	537 ± 43	4
Arsenic	ng/stick	<1.13	4	<1.13	4	8.51 ± 0.34	4
Cadmium	ng/stick	<0.350	4	<0.350	4	161 ± 4	4
Chromium	ng/stick	<0.55	4	<0.55	4	<0.55	4
Lead	ng/stick	<3.35	4	<3.35	4	37.0 ± 0.7	4
Mercury	ng/stick	1.17 ± 0.05	4	1.34 ± 0.18	4	4.80 ± 0.13	4
Nickel	ng/stick	<0.55	4	<0.55	4	<0.55	4
Selenium	ng/stick	<0.550	2	0.780	4	1.62 ± 0.32	4
Pyrene	ng/stick	<5.00	4	9.06 ± 0.68	4	87.3 ± 2.5	4
o-toluidine	ng/stick	1.260 ± 0.187	4	0.777 ± 0.287	4	85.5 ± 2.7	4
Acetamide	µg/stick	4.02 ± 0.18	4	4.30 ± 0.24	4	13.9 ± 0.5	4
Acrylamide	µg/stick	1.73 ± 0.12	4	1.91 ± 0.16	4	4.8 ± 0.3	4
Ethylene oxide	µg/stick	0.201 ± 0.014	4	0.202 ± 0.013	4	29.4 ± 2.0	4
Nitrobenzene	ng/stick	<0.188	4	0.335 ± 0.164	4	8.62 ± 1.10	4
Propylene oxide	µg/stick	0.148 ± 0.018	4	0.149 ± 0.017	4	1.32 ± 0.12	4
Vinyl chloride	ng/stick	<3.54	4	<3.54	4	96.7 ± 2.0	4
Benz[*a*]anthracene	ng/stick	1.45 ± 0.14	4	2.49 ± 0.17	4	28.0 ± 0.6	4
Dibenz[*a,h*]anthracene	ng/stick	<0.100	4	<0.100	4	1.70 ± 0.11	4

N is the number of determinations, CI is the confidence interval of the mean, n.a.: not analyzed.
<: median lower than the limit of quantitation, in this case LOQ is given.
If at least one value is below the LOQ, the median is given and the CI is not mentioned.
TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosonornicotine.

Table 6
Analytes yields from THS2.2 D2, THS2.2 D1 M, and 3R4F obtained under HCI machine-smoking conditions and expressed on a per-cigarette/tobacco stick basis.

Parameter	Unit	THS2.2 D2		THS2.2 D1 M		3R4F	
		mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N
TPM	mg/stick	54.1 ± 2.4	4	53.8 ± 3.6	4	46.3 ± 2.9	4
Water	mg/stick	39.4 ± 4.6	4	39.1 ± 3.6	4	13.3 ± 1.6	4
Nicotine	mg/stick	1.26 ± 0.24	4	1.32 ± 0.11	4	2.09 ± 0.14	4
NFDPM	mg/stick	13.4 ± 2.8	4	13.4 ± 0.6	4	30.9 ± 1.9	4
Carbon monoxide	mg/stick	0.598 ± 0.072	4	0.620 ± 0	4	30.7 ± 3.0	4
Benzo[a]pyrene	ng/stick	1.19 ± 0.08	4	1.08 ± 0.09	3	13.7 ± 0.8	4
Puff Count	/stick	12 ± 0	4	12 ± 0	4	10.7 ± 0.7	4
Menthol	mg/stick	n.a.		2.98 ± 0.21	4	n.a.	
Glycerin	mg/stick	4.1 ± 1.07	4	4.59 ± 0.47	4	2.39 ± 0.15	4
1-aminonaphthalene	ng/stick	0.063 ± 0.006	4	<0.061	4	19.7 ± 1.6	4
2-aminonaphthalene	ng/stick	<0.035	4	<0.035	4	14.8 ± 1.9	4
3-aminobiphenyl	ng/stick	<0.013	4	<0.013	4	3.90 ± 0.42	4
4-aminobiphenyl	ng/stick	<0.021	4	n.a.		3.13 ± 0.60	4
Acetaldehyde	µg/stick	213 ± 19	4	220 ± 22	4	1589 ± 76	4
Acetone	µg/stick	33.8 ± 6.4	4	42.6 ± 8.1	4	729 ± 36	4
Acrolein	µg/stick	9.44 ± 0.87	4	10.91 ± 2.98	4	193 ± 21	4
Butyraldehyde	µg/stick	25.3 ± 2.7	4	26.4 ± 0.9	4	103.9 ± 8.3	4
Crotonaldehyde	µg/stick	3.75 ± 0.34	4	4.15 ± 0.64	4	92.1 ± 13.2	4
Formaldehyde	µg/stick	5.22 ± 0.24	4	6.19 ± 2.00	4	68.7 ± 7.8	4
Methyl ethyl ketone	µg/stick	7.94 ± 0.75	4	10.19 ± 2.23	4	241 ± 16	4
Propionaldehyde	µg/stick	13.6 ± 1.5	4	15.9 ± 2.2	4	147 ± 8	4
Acrylonitrile	µg/stick	0.186 ± 0.028	4	0.196 ± 0.016	4	31.6 ± 2.3	4
1,3-butadiene	µg/stick	0.319 ± 0.073	4	0.411 ± 0.093	4	91.8 ± 11.0	4
Benzene	µg/stick	0.575 ± 0.072	4	0.628 ± 0.073	4	100.4 ± 2.8	4
Isoprene	µg/stick	2.44 ± 0.50	4	2.63 ± 0.60	4	869 ± 50	4
Pyridine	µg/stick	9.38 ± 0.95	4	10.08 ± 0.46	4	51.8 ± 7.5	4
Quinoline	µg/stick	0.014 ± 0.002	4	0.010 ± 0.003	4	0.390 ± 0.101	4
Styrene	µg/stick	0.672 ± 0.063	4	0.632 ± 0.079	4	28.9 ± 2.2	4
Toluene	µg/stick	1.61 ± 0.17	4	1.67 ± 0.37	4	198.8 ± 10.9	4
Catechol	µg/stick	16.4 ± 0.6	4	12.8 ± 1.3	4	88.7 ± 2.6	4
o-cresol	µg/stick	0.105 ± 0.017	4	0.059 ± 0.007	4	4.86 ± 0.50	4
m-cresol	µg/stick	0.042 ± 0.006	4	0.032 ± 0.005	4	3.71 ± 0.34	4
p-cresol	µg/stick	0.073 ± 0.009	4	0.042 ± 0.007	4	8.50 ± 0.78	4
Hydroquinone	µg/stick	7.86 ± 0.63	4	6.21 ± 0.86	4	84.1 ± 3.3	4
Phenol	µg/stick	1.51 ± 0.23	4	1.00 ± 0.17	4	13.2 ± 0.9	4
Resorcinol	µg/stick	0.055 ± 0.013	4	0.036 ± 0.005	4	1.95 ± 0.55	4
NAB	ng/stick	3.52 ± 0.48	4	3.27 ± 0.15	4	34.1 ± 3.0	4
NAT	ng/stick	22.3 ± 1.6	4	18.6 ± 2.9	4	300 ± 53	4
NNK	ng/stick	10.1 ± 0.4	4	7.9 ± 1.1	4	257 ± 39	4
NNN	ng/stick	10.3 ± 0.4	4	7.7 ± 1.0	4	268 ± 50	4
Ammonia	µg/stick	15.6 ± 1.1	4	13.9 ± 1.1	4	39.2 ± 4.1	4
Hydrogen cyanide	µg/stick	3.78 ± 0.44	4	5.57 ± 0.35	4	451 ± 47	4
Nitric oxide	µg/stick	21.0 ± 8.1	3	18.4 ± 3.6	4	501 ± 33	3
Nitrogen oxides	µg/stick	22.6 ± 8.8	3	19.4 ± 4.0	4	541 ± 74	3
Arsenic	ng/stick	<1.13	4	<1.13	4	6.56 ± 0.46	4
Cadmium	ng/stick	<0.350	4	<0.350	4	122 ± 12	4
Chromium	ng/stick	<0.17	4	0.44	4	2.70[a]	2
Lead	ng/stick	<3.35	4	<3.35	4	25.1 ± 2.1	4
Mercury	ng/stick	1.02 ± 0.05	4	1.12 ± 0.19	4	4.17 ± 0.74	4
Nickel	ng/stick	<0.55	4	0.88	4	1.30[a]	2
Selenium	ng/stick	<0.550	4	<0.550	4	1.43 ± 0.15	4
Pyrene	ng/stick	7.93 ± 0.78	4	7.71 ± 0.63	3	87.3 ± 4.1	4
o-toluidine	ng/stick	1.204 ± 0.149	4	0.868 ± 0.087	4	90.5 ± 3.1	4
Acetamide	µg/stick	4.13 ± 0.21	4	3.43 ± 0.17	4	13.7 ± 0.7	4
Acrylamide	µg/stick	2.27 ± 0.28	4	1.90 ± 0.12	4	5.3 ± 0.4	4
Ethylene oxide	µg/stick	0.314 ± 0.011	4	0.273 ± 0.036	4	34.2 ± 3.6	4
Nitrobenzene	ng/stick	0.092 ± 0.008	4	0.155 ± 0.004	8	0.55 ± 0.04	4
Propylene oxide	µg/stick	0.175 ± 0.03	4	0.14 ± 0.019	4	1.72 ± 0.16	4
Vinyl chloride	ng/stick	<3.47	4	<3.47	4	95.3 ± 12.3	4
Benz[*a*]anthracene	ng/stick	2.58 ± 0.17	4	2.50 ± 0.06	3	26.6 ± 1.7	4
Dibenz[*a,h*]anthracene	ng/stick	<0.100	4	<0.100	4	1.79 ± 0.14	4

N is the number of determinations, CI is the confidence interval of the mean, n.a.: not analyzed.
<: median lower than the limit of quantitation, in this case LOQ is given.
If at least one value is below the LOQ, the median is given and the CI is not mentioned.
TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosonornicotine.
[a] CI not calculated.

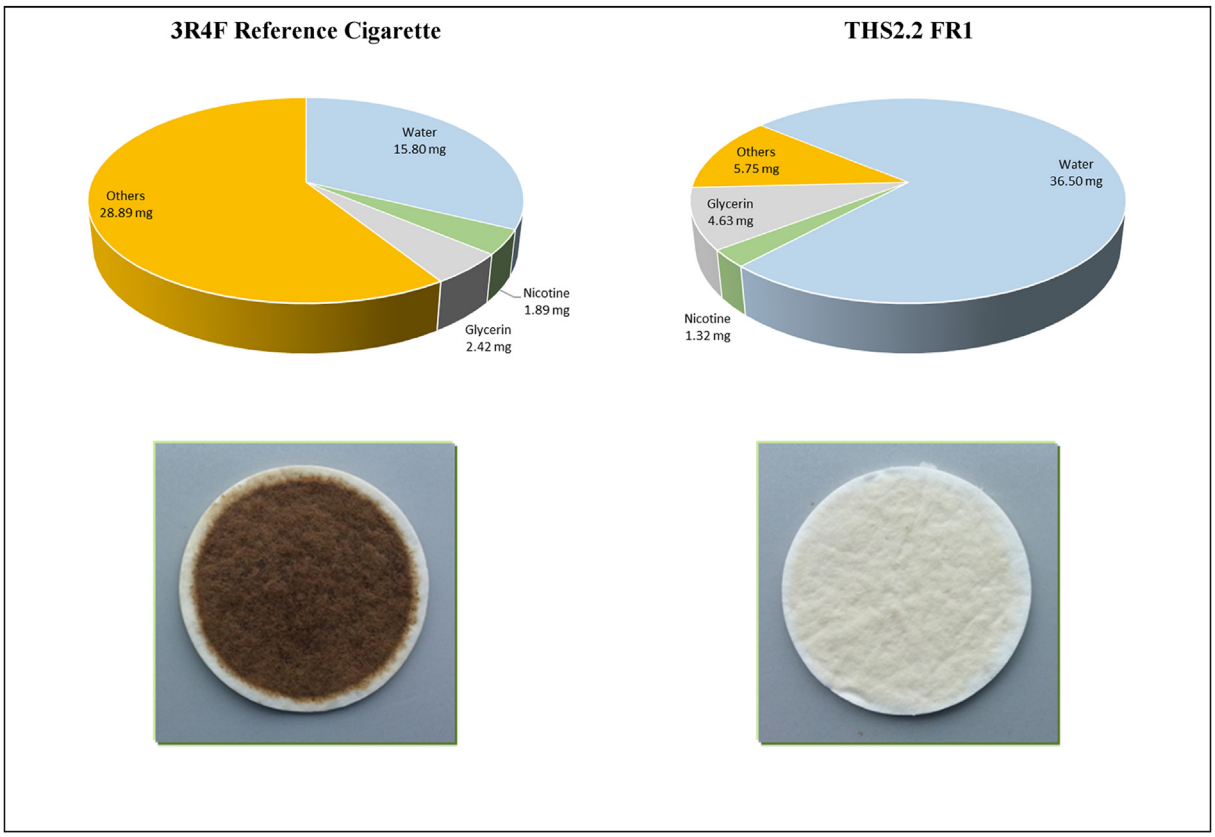

Fig. 3. Cigarette total particulate matter (TPM) compared with THS2.2 aerosol composition generated according to the HCI machine-smoking conditions for measuring emissions. The photographs of the Cambridge glass-fiber filter pads after the collection of cigarette smoke (left) and THS2.2 aerosol (right) are also shown.

for Harmonisation, 2003) and WHO (World Health Organisation Technical Report, 2015) guidelines for stability testing. Since the generation of HPHCs either through pyrosynthesis or distillation should be enhanced when increasing the temperature, lower temperatures were not considered for this comparison. The impact of temperature and relative humidity (RH) on the deliveries of the different analytes in the THS2.2 FR1 aerosol is presented in Table 7. The data expressed on a per-mg nicotine basis are presented in Table C of the supplementary material.

3.3. Aerosol chemistry of the THS2.2 FR1 generated with different machine-smoking regimens

The range of machine-smoking regimens used during this test was quite broad, and ranged from a total puff volume of 210 ml for the ISO conditions to 1120 ml for the LR-3 regimen (see Table 2). Despite these substantial differences in puff volumes, benzo[*a*]pyrene, 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, dibenz[*a,h*]anthracene, and vinyl chloride remained below the LOQ for all machine-smoking regimens. For the other analytes, ISO and SR-1 delivered the lowest yields of HPHCs, while machine-smoking regimens SR-4, SR-6, and LR-3 delivered the highest HPHC yields. A summary of the obtained results is presented in Table 8 together with the yields obtained for the 3R4F reference cigarette smoked in the HCI conditions. All the individual results are presented in Tables D and E of the supplementary material.

The LR-3 smoking regimen could not be performed with the Borgwaldt linear smoking machine type used to quantify NO and NO_x. Therefore, this value was not reported. For TPM and carbonyls, the breakthrough was specifically tested with the most intense smoking regimens to ensure that losses were negligible and did not affect the accuracy of the measurements.

3.4. Physical measurement of the aerosol

Ten series of measurement were performed on the 3R4F reference cigarette and on the THS2.2 FR1. All the calculated MMAD, GSD, and boundary values are presented in Table 9. The upper boundaries (UB) were below 2.5 μm. Consequently, the smoke generated from the 3R4F and the aerosol generated from the THS2.2 were respirable for all replicates with a margin of error of 5% and, according to Hinds (Hinds, 1999), more than 85% of the aerosol droplets could reach the alveoli in the lung.

3.5. Neutral red uptake (NRU) assay

Two versions of the THS2.2 Regular (THS2,2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M) were tested and compared with the 3R4F under HCI machine-smoking conditions in the NRU assay in independent studies. The cytotoxic activity of both the TPM and GVP fractions from the THS2.2 and the 3R4F reference cigarette was determined. A clear concentration-dependent decrease in the number of viable cells was observed for the aerosol fractions generated from the THS2.2 Regular (FR1 and D2) and THS2.2. Menthol (FR1 and D1) and the smoke fractions generated from the 3R4F reference cigarette. The cytotoxicity levels induced by both products covered a range spanning from no or low to high cytotoxicity. The $1/EC_{50}$ values (expressed on a per-mg TPM basis and on a per-mg nicotine

Table 7
Yields on tobacco stick basis from THS2.2 FR1 obtained under three climatic conditions.

Parameter	Unit	Mediterranean 22 °C, 60% RH		Desert 30 °C, 35% RH		Tropical 30 °C, 75% RH		Range[a]
		mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N	mean ± $CI_{95\%}$	N	
TPM	mg/stick	47.1 ± 2.0	5	27.9 ± 1.4	5	65.1 ± 4.5	5	37.2
Water	mg/stick	33.5 ± 1.5	5	15.1 ± 0.4	5	50.6 ± 3.7	5	35.5
Nicotine	mg/stick	1.42 ± 0.05	5	1.46 ± 0.05	5	1.21 ± 0.02	5	0.25[b]
NFDPM	mg/stick	12.2 ± 1.1	5	11.3 ± 1.4	5	12.6 ± 1.5	5	1.3
Carbon monoxide	mg/stick	0.612 ± 0.020	5	0.454 ± 0.047	5	0.468 ± 0.024	5	0.158
Benzo[*a*]pyrene	ng/stick	<1.00	5	1.03	5	<1.00	5	
Glycerin	mg/stick	4.68 ± 0.36	5	4.25 ± 0.25	5	4.23 ± 0.09	5	0.45[b]
1-aminonaphthalene	ng/stick	<0.069	5	<0.069	5	<0.069	5	
2-aminonaphthalene	ng/stick	<0.035	5	<0.035	5	0.088 ± 0.173	5	
3-aminobiphenyl	ng/stick	<0.032	5	<0.032	5	<0.032	5	
4-aminobiphenyl	ng/stick	<0.051	5	<0.051	5	0.073 ± 0.022	5	
Acetaldehyde	µg/stick	193 ± 2	5	179 ± 11	5	229 ± 5	5	50
Acetone	µg/stick	37.7 ± 1.7	5	37.0 ± 5.5	5	43.0 ± 2.9	5	6.0
Acrolein	µg/stick	9.76 ± 0.91	5	8.87 ± 1.81	5	11.54 ± 0.81	5	2.67
Butyraldehyde	µg/stick	27.3 ± 0.7	5	26.0 ± 0.5	5	29.9 ± 2	5	3.9
Crotonaldehyde	µg/stick	4.13 ± 0.55	5	3.69 ± 0.51	5	4.64 ± 0.41	5	0.95
Formaldehyde	µg/stick	3.52 ± 0.3	5	3.65 ± 0.36	5	3.57 ± 0.3	5	0.13[b]
Methyl ethyl ketone	µg/stick	7.58 ± 0.71	5	6.75 ± 0.84	5	8.68 ± 0.57	5	1.93[b]
Propionaldehyde	µg/stick	14.4 ± 0.6	5	13.5 ± 2.1	5	18.1 ± 0.7	5	4.6
Acrylonitrile	µg/stick	0.167 ± 0.036	5	0.178 ± 0.021	5	0.189 ± 0.035	5	0.022[b]
1,3-butadiene	µg/stick	0.277 ± 0.035	5	0.248 ± 0.016	5	0.318 ± 0.027	5	0.070[b]
Benzene	µg/stick	0.603 ± 0.042	5	0.591 ± 0.031	5	0.613 ± 0.030	5	0.022[b]
Isoprene	µg/stick	2.19 ± 0.21	5	1.83 ± 0.17	5	2.60 ± 0.25	5	0.77
Pyridine	µg/stick	7.47 ± 0.31	5	5.76 ± 0.42	5	7.26 ± 0.29	5	1.71
Quinoline	µg/stick	<0.012	5	<0.012	5	<0.012	5	
Styrene	µg/stick	0.640 ± 0.035	5	0.619 ± 0.041	5	0.695 ± 0.038	5	0.076[b]
Toluene	µg/stick	2.11 ± 0.2	5	1.85 ± 0.15	5	2.25 ± 0.19	5	0.4[b]
Catechol	µg/stick	16.9 ± 1.2	5	15.0 ± 2.0	5	15.2 ± 1.4	5	1.9
o-cresol	µg/stick	0.109 ± 0.006	4	0.123 ± 0.021	5	0.135 ± 0.017	5	0.026[b]
m-cresol	µg/stick	0.031 ± 0.003	4	0.061 ± 0.01	5	0.071 ± 0.029	5	0.040
p-cresol	µg/stick	0.070 ± 0.006	4	0.099 ± 0.019	5	0.099 ± 0.012	5	0.029[b]
Hydroquinone	µg/stick	8.51 ± 0.63	5	7.11 ± 1.17	5	8.21 ± 0.25	5	1.40
Phenol	µg/stick	1.66 ± 0.36	5	2.49 ± 0.45	5	2.23 ± 0.26	5	0.83
Resorcinol	µg/stick	0.054 ± 0.004	5	0.047 ± 0.007	5	0.044 ± 0.005	5	0.01
NAB	ng/stick	3.37 ± 0.21	4	<3.15	5	<3.15	5	
NAT	ng/stick	21.7 ± 1.4	4	20.1 ± 1.7	5	17.0 ± 0.6	5	4.7
NNK	ng/stick	9.2 ± 0.3	4	8.3 ± 1	5	7.7 ± 1.2	5	1.5
NNN	ng/stick	16.4 ± 1.3	4	14.6 ± 1.6	5	13.9 ± 0.8	5	2.5[b]
Ammonia	µg/stick	14.6 ± 0.4	5	15.4 ± 0.2	5	15.3 ± 0.9	5	0.8[b]
Hydrogen cyanide	µg/stick	4.84 ± 0.29	4	4.14 ± 0.32	5	4.39 ± 0.68	5	0.70[b]
Nitric oxide	µg/stick	18.3 ± 1.2	5	16.6 ± 0.3	4	18.2 ± 0.2	4	1.7
Nitrogen oxides	µg/stick	19.5 ± 1.5	5	17.6 ± 0.2	4	19.0 ± 0.6	4	1.9
Pyrene	ng/stick	5.66 ± 0.61	5	7.27 ± 1.63	5	5.85 ± 1.27	5	1.61
o-toluidine	ng/stick	1.144 ± 0.113	5	0.649 ± 0.318	5	0.62 ± 0.59	5	0.524
Acetamide	µg/stick	4.24 ± 0.13	4	4.28 ± 0.55	5	4.19 ± 0.16	5	0.09[b]
Acrylamide	µg/stick	2.31 ± 0.12	5	2.30 ± 0.31	5	1.94 ± 0.05	5	0.37
Ethylene oxide	µg/stick	0.267 ± 0.017	5	0.269 ± 0.012	5	0.355 ± 0.032	5	0.088
Nitrobenzene	ng/stick	0.138 ± 0.003	4	0.087 ± 0.005	5	0.087 ± 0.009	5	0.051
Propylene oxide	µg/stick	0.144 ± 0.01	5	0.101 ± 0.004	5	0.113 ± 0.009	5	0.043
Vinyl chloride	ng/stick	<3.54	5	<3.54	5	<3.54	5	
Benz[*a*]anthracene	ng/stick	1.39 ± 0.1	5	1.51 ± 0.35	5	1.39 ± 0.2	5	0.12
Dibenz[*a,h*]anthracene	ng/stick	<0.100	5	<0.100	5	<0.100	5	

N is the number of determinations, CI is the confidence interval of the mean.
<: median lower than the limit of quantitation, in this case LOQ is given.
If at least 1 value is below LOQ, the median is given and CI is not mentioned.
TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosonornicotine.
[a] Range: largest mean obtained among the three climatic conditions minus the smallest mean obtained among the three climatic conditions.
[b] Range smaller than the Intermediate Precision (IP) (International Council for Harmonisation, 1996) of the analytical method.

basis) were used to compare the relative cytotoxicity of the THS2.2 and the 3R4F reference cigarette. The results showed that the relative *in vitro* cytotoxicity of the THS2.2 Regular (FR1 and D2) and Menthol (FR1 and D1) aerosol fractions was reduced by approximately 95% when expressed on a per-mg TPM basis, compared with the 3R4F reference cigarette (Tables F and G of the supplementary material). The *in vitro* cytotoxicity of THS2.2 Regular (FR1 and D2) and THS2.2 Menthol (FR1 and D1) aerosol fractions, expressed on a per-mg nicotine basis, was reduced by 85–90% compared with the 3R4F reference cigarette (Table 10 and Table 11).

Table 8
Summary of the THS2.2 FR1 yields from extreme puffing regimens; comparison with the Health Canada machine-smoking regimen and the 3R4F reference cigarette.

Parameter	Unit	THS2.2 FR1		THS2.2 FR1		THS2.2 FR1		3R4F
		Extreme Regimen Maximum Yields		Extreme Regimen Minimum Yields		HCI Regimen	Ratio[b]	HCI regimen
		mean	puf. reg.	mean	puf. reg.	mean	Max/HCI	mean
TPM	mg/stick	59.0	SR-4	26.9	ISO	56.8	1.0	49.0
Water	mg/stick	45.1	SR-4	21.4	ISO	44.6	1.0	15.8
Nicotine	mg/stick	2.19	LR-3	0.49	ISO	1.36	1.6	1.89
NFDPM	mg/stick	17.4	LR-3	5.1	ISO	10.8	1.6	31.2
Carbon monoxide	mg/stick	0.660	SR-6	0.238	ISO	0.532	1.2	32.8
Benzo[*a*]pyrene	ng/stick	<1.00		<1.00		<1.00		14.2
Glycerin	mg/stick	5.66	LR-3	1.91	ISO	4.59	1.2	2.42
1-aminonaphthalene	ng/stick	<0.069		<0.069		<0.069		20.8
2-aminonaphthalene	ng/stick	<0.035		<0.035		<0.035		11.0
3-aminobiphenyl	ng/stick	<0.032		<0.032		<0.032		3.77
4-aminobiphenyl	ng/stick	<0.051		<0.051		<0.051		3.26
Acetaldehyde	µg/stick	205	SR-4	145	SR-1	196	1.0	1555
Acetone	µg/stick	40.7	SR-4	22.0	SR-1	37.9	1.1	736
Acrolein	µg/stick	12.9	LR-3	4.89	ISO	8.83	1.5	154
Butyraldehyde	µg/stick	26.7	SR-6	16.7	ISO	22.0	1.2	88.4
Crotonaldehyde	µg/stick	4.90	LR-3	1.88	ISO	3.04	1.6	68.8
Formaldehyde	µg/stick	7.73	LR-3	1.85	ISO	3.77	2.1	56.5
Methyl ethyl ketone	µg/stick	7.39	SR-4	3.78	SR-1	7.28	1.0	187
Propionaldehyde	µg/stick	14.4	SR-4	8.5	SR-1	13.5	1.1	125
Acrylonitrile	µg/stick	0.228	LR-3	<0.111	SR-1[a]	0.163	1.4	31.9
1,3-butadiene	µg/stick	0.357	SR-4	<0.236	ISO[a]	0.295	1.2	63.8
Benzene	µg/stick	0.708	SR-4	0.298	SR-1	0.597	1.2	97.6
Isoprene	µg/stick	2.82	SR-5	1.37	SR-1	2.56	1.1	798
Pyridine	µg/stick	8.00	SR-4	4.21	ISO	7.36	1.1	36.1
Quinoline	µg/stick	0.051	LR-3	<0.011	ISO[a]	0.016	3.2	0.513
Styrene	µg/stick	0.686	SR-6	0.314	SR-1	0.619	1.1	24.5
Toluene	µg/stick	2.15	LR-3	0.97	ISO	2.02	1.1	188
Catechol	µg/stick	17.9	SR-4	5.9	ISO	16.8	1.1	91.4
o-cresol	µg/stick	0.438	LR-3	0.021	ISO	0.108	4.1	4.47
m-cresol	µg/stick	0.212	LR-3	<0.010	ISO[a]	0.046	4.6	3.03
p-cresol	µg/stick	0.399	LR-3	<0.010	SR-1	0.100	4.0	9.17
Hydroquinone	µg/stick	9.99	SR-6	3.66	SR-1	8.76	1.1	83.1
Phenol	µg/stick	10.87	LR-3	0.06	ISO	1.93	5.6	13.6
Resorcinol	µg/stick	0.056	SR-6	0.020	ISO	0.047	1.2	1.85
NAB	ng/stick	4.31	LR-3	<3.15	ISO[a]	3.46	1.2	33.7
NAT	ng/stick	26.8	SR-6	8.5	ISO	22.4	1.2	318
NNK	ng/stick	10.2	SR-6	4.1	ISO	8.7	1.2	266
NNN	ng/stick	19.1	LR-3	6.5	ISO	16.1	1.2	309
Ammonia	µg/stick	31	LR-3	4.1	ISO	15	2.1	39.3
Nitric oxide	µg/stick	19.4	SR-4	11	ISO	18	1.1	491
Nitrogen oxides	µg/stick	20.3	SR-4	11.2	ISO	19	1.1	537
Pyrene	ng/stick	6.50	SR-4	<5.00	ISO[a]	<5.00		87.3
o-toluidine	ng/stick	2.146	SR-6	0.489	SR-1	1.195	1.8	85.5
Acetamide	µg/stick	6.62	LR-3	1.32	ISO	4.18	1.6	13.9
Acrylamide	µg/stick	4.23	LR-3	0.69	ISO	2.33	1.8	4.8
Ethylene oxide	µg/stick	0.323	LR-3	0.157	SR-1	0.242	1.3	29.4
Vinyl chloride	ng/stick	<3.54		<3.54		<3.54		96.7
Benz[*a*]anthracene	ng/stick	1.61	SR-4	<1.00	ISO[a]	<1.00		28.0
Dibenz[*a*,*h*]anthracene	ng/stick	<0.100		<0.100		<0.100		1.7

<: median lower than the limit of quantitation, in this case LOQ is given.
TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosonornicotine.
[a] At least one other smoking regimen was also below LOQ; see Tables D a in the supplementary material.
[b] When the, minimum value was inferior to the LOQ, the LOQ value was used to calculate the ratios.

3.6. Ames assay

The TPM from the THS2.2 D2, THS2.2 D1 M, and the 3R4F was tested with the *S. typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in both the presence and absence of S9 (Table 12).

A positive response in the Ames test for the TPM from the 3R4F reference cigarette was detected in 3 of the 5 *S. typhimurium* tester strains in the presence of the S9 fraction, namely TA98, TA100, and TA1537 (see Table 12). Nevertheless, despite testing up to 10 mg of TPM for THS2.2 D2 and 5 mg/per plate for THS2.2 D1 M, no mutagenicity in any of the tester strains was detected under the conditions of this assay.

3.7. Mouse lymphoma assay (MLA)

The *in vitro* MLA was used to assess the mutagenicity of both

Table 9
MMAD and GSD results from 3R4F and THS2.2 FR1.

Repetition	3R4F				THS2.2 FR1			
	MMAD	GSD^2	LB	UB	MMAD	GSD^2	LB	UB
	[μm]		[μm]	[μm]	[μm]		[μm]	[μm]
1	0.9	2.1	0.4	1.8	0.8	2.6	0.3	2.1
2	0.8	1.9	0.4	1.5	0.7	2.3	0.3	1.7
3	0.8	1.9	0.4	1.5	0.7	2.1	0.3	1.4
4	0.9	1.7	0.5	1.5	0.7	1.9	0.4	1.3
5	0.9	1.6	0.5	1.4	0.7	2.0	0.3	1.3
6	0.8	1.9	0.4	1.5	0.7	2.2	0.3	1.6
7	0.9	1.9	0.5	1.6	0.8	2.5	0.3	1.9
8	0.6	1.4	0.5	0.9	0.6	2.3	0.3	1.3
9	0.8	1.8	0.5	1.5	0.6	2.3	0.3	1.5
10	0.7	1.8	0.4	1.3	0.7	3.2	0.2	2.3
Mean	0.8	1.8			0.7	2.3		
Mean GSD		1.3				1.5		

MMAD: Mass median aerodynamic diameter; GSD: Geometric standard deviation.
LB: Lower boundaries with a 95% confidence interval; UB: Upper boundaries with a 95% confidence interval.

TPM and GVP derived from THS2.2 D2, THS2.2 D1 M, and 3R4F. In both tests under the three treatment conditions, TPM and GVP derived from THS2.2 D2 and 3R4F induced concentration-dependent increases in cytotoxicity and mutagenicity. In the presence of S9, the mutagenic responses reproducibly surpassed the GEF threshold for mutagenicity at or just above the cytotoxicity limit of the assay, i.e. RTG 10%–20%. Increases in both large and small colonies were observed for TPM and GVP derived from both test articles. For THS2.2 D2, LOGELs for the TPM fraction were markedly higher (on average 17-fold) than for 3R4F-derived TPM (Fig. 4A and Table 13). A similar mutagenicity profile was observed for the GVP (Fig. 4B). In both treatment conditions (4 h and 24 h) conducted in the absence of S9, mutagenicity which exceeded the GEF threshold was also observed, however, this finding was not always reproducible between the tests. When it was the case, LOGELs for THS2.2 D2 -derived TPM were again markedly higher (on average, at least 14-fold) than for 3R4F TPM (exemplar responses illustrated in Fig. 5A and Table 13). Moreover, in these treatment conditions, the LOGELs for TPM always occurred at the RTG 10%–20% cytotoxicity level. Similar mutagenicity profiles were also observed for GVP under the same treatment conditions. Similar results were obtained for the mentholated version of THS2.2 (exemplar responses illustrated in Figs. 5B and 6, and Table 13). Mutagenicity data expressed on a per-mg TPM basis are presented as part of the supplementary material (Supplementary Figures A–C and Supplementary Table H).

4. Discussion

The objective of this study was to assess the potential for reduced exposure to HPHCs from THS2.2 compared with 3R4F based on chemical analysis of HPHCs, *in vitro* genotoxicity, and cytotoxicity assessments. To evaluate the robustness of the data for the products under different conditions, HPHC yields were also measured when using the product under simulated real-life smoking conditions, additional tests were performed under different climatic conditions and with different puffing regimens.

Table 11
Cytotoxicity of TPM and GVP, expressed as 1/EC50 (ml/mg nicotine).

	THS2.2 FR1		THS2.2 FR1 M		3R4F	
	TPM	GVP	TPM	GVP	TPM	GVP
Mean	21.33	30.86	27.43	28.61	186.78	242.01
SEM	1.57	2.87	1.6	2.53	7.58	14.06
N	3	3	3	3	3	3
Relative cytotoxicity (%)	11.4	12.8	14.7	11.8	100	100

SEM: Standard error of the mean.
TPM: Total particulate matter, GVP: Gas-vapor phase.
Relative cytotoxicity (%) = (cytotoxicity of THS2.2 / 3R4F) × 100.

4.1. Aerosol physics and aerosol chemistry; comparison WITH the 3R4F reference cigarette

During this assessment of the THS2.2, following the feedback from taste panels and to ensure sustainability of tobacco sources, the blend FR1 was replaced by blend D2 for the regular product and by blend D1 for the menthol product (Smith et al., 2016). Therefore, it was considered important to present the chemical characterization of all the four products (THS2.2 FR1, THS2.2 FR1 M, THS2.2 D2 and THS2.2 D1 M) in this publication. When comparing the four products, it can be observed that they delivered HPHC yields that were in the same range. The influence of the tobacco blend composition on the HPHC yields is presented in the 3rd publication of this issue (Schaller et al., 2016-b). When comparing menthol and regular THS2.2 products, no substantial influence of menthol on the HPHC yields was detected. Schmeltz and Schlotzhauer have reported that menthol pyrolysis in a closed system produced benzo[*a*]pyrene leading them to suggest that menthol pyrolysis could act as a potential precursor to benzo[*a*]pyrene to the smoke of mentholated cigarette products (Schmeltz and Schlotzhauer, 1968). A significant contribution of the menthol to the yield of benzo[*a*]pyrene was not observed in the THS2.2. The FR1 Menthol product deliverd a slightly higher yield of benzo[a]pyrene (1.29 ± 0.10 ng/stick) than the FR1 Regular product (<1.00 ng/stick), but the yield of menthol in the D1 Menthol product (1.08 ± 0.09 ng/stick) was on the low side compared to the D2 Regular product (1.19 ± 0.08 ng/stick). In addition, in the pyrolytic conditions used by Schmeltz and Scholtzhauer, benzo[*a*]pyrene was detected only on pyrolysis of menthol at 860 °C, but not during pyrolysis at 600 °C. Other studies have reported that when isotopically labeled menthol was added to tobacco of cigarettes, most of the menthol was transferred to the smoke unchanged and the production of labeled benzo[*a*]pyrene

Table 10
Cytotoxicity of TPM and GVP, expressed as 1/EC50 (ml/mg nicotine).

	THS2.2 D2		3R4F analyzed during the THS2.2 D2 study		THS2.2 D1 M		3R4F analyzed during the THS2.2 D1 M study	
	TPM	GVP	TPM	GVP	TPM	GVP	TPM	GVP
Mean	17.34	28.40	208.55	289.06	19.73	26.07	239.51	276.21
SEM	0.52	1.20	6.92	22.38	0.87	1.78	6.07	22.51
N	3	3	3	3	3	3	3	3
Relative cytotoxicity (%)	8.3	9.8	100	100	8.2	9.4	100	100

SEM: Standard error of the mean.
TPM: Total particulate matter, GVP: Gas-vapor phase.
Relative cytotoxicity (%) = (cytotoxicity of THS2.2/3R4F) × 100.

Table 12
Revertant colonies obtained following exposure to the TPM (1 mg per plate) from THS2.2 D2, THS2.2 D1 M, or 3R4F.

Salmonella typhimurium Strain		THS2.2 D2		3R4F[a]		Solvent Control		Positive[b] Control		THS2.2 D1 M		3R4F[a]		Solvent Control		Positive[b] Control	
		Mean[d]	SD	Mean[d]	SD	Mean[d]	SD	Mean[d]	SD	Mean[d]	SD	Mean[d]	SD	Mean[d]	SD	Mean[d]	SD
+S9	TA98	22	4	658	89	21	1	109	7	21	2	636	24	25	2	97	17
	TA100	94	21	428	25	87	2	481	22	93	12	440	20	90	6	471	67
	TA102	358	12	409	15	272	22	1005	29	290	15	399	16	265	23	968	25
	TA1535	9	3	17	6	6	1	70	8	15	6	15	6	10	3	113	11
	TA1537	8	5	98	9	6	2	50	5	15	3	94	9	7	2	35	5
−S9	TA98	16	4	17	5	23	6	81	6.2	22	3	10[c]	6	26	3	93	10
	TA100	61	8	87	13	66	3	195	25	81	11	96	21	62	8	187	17
	TA102	291	15	282	12	267	21	709	5	230	56	264	4	258	26	620	8
	TA1535	12	3	7	3	6	2	37	8	9	4	16	6	12	2	51	6
	TA1537	6	5	3	2	6	3	84	5	15	5	17	5	7	2	83	6

[a] These samples were generated and tested concurrently with the respective THS variant.
[b] Details of dose and substance are provided in the Ames methods section.
[c] Toxicity was detected at this dose.
[d] Each mean and SD value was derived from 3 plates and the values were rounded.

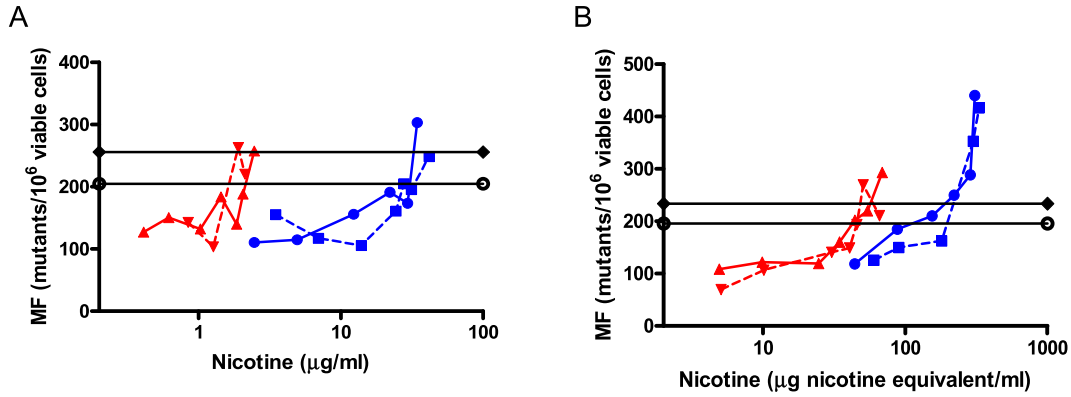

Fig. 4. The mutagenic responses induced by aerosol fractions derived from THS2.2 D2 and 3R4F in the 4 h + S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM. MFs for the DMSO-treated controls in tests #1 and #2 were 129.77 ± 22.57 and 78.73 ± 1.55 mutants/10^6 viable cells, respectively. ●THS2.2 D2 #1; ■THS2.2 D2 #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + DMSO MF #1; ○ GEF + DMSO MF #2. B. GVP. MFs for the PBS-treated controls in tests #1 and #2 were 107.73 ± 10.40 and 69.44 ± 3.10 mutants/10^6 viable cells, respectively. ●THS2.2 D2 #1; ■THS2.2 D2 #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + PBS MF #1; ○GEF + PBS MF #2.

was not detected (Jenkins et al., 1970; Baker and Bishop, 2004). Since (i) the heater blade temperature in the THS2.2 only reaches a maximum temperature of 350 °C, and (ii) available literature on menthol pyrolysis to yield benzo[*a*]pyrene is limited, it was concluded that menthol is unlikely to be a significant source of benzo[*a*]pyrene in the THS2.2 aerosol.

The mainstream aerosols produced by all the analyzed THS2.2 products were similar regarding analyte yields including HPHC yields, but substantially different from the yields in mainstream smoke of the 3R4F reference cigarette. To quantify the exposure reduction, the yields of each HPHC for THS2.2 relative to those in 3R4F were calculated, and are presented on a per Tobacco Stick/cigarette (Fig. 7). The graph presenting the results on a mg-nicotine basis is included in the supplementary material (Figure D).

Fig. 7 presents a general view of the ratios between THS2.2 and 3R4F. The 100% line represents the yields of the 3R4F on a per-cigarette basis. When considering the HPHC yields on a Tobacco Stick/cigarette basis, it can be observed that acrylamide, ammonia, butyraldehyde, butyraldehyde, acetamide, and mercury presented ratios between 25 and 50%. Even if this denotes a substantial reduction compared to 3R4F, it was not surprising to see ammonia, acrylamide, acetamide and mercury at this level, since they could be formed or could distill out of tobacco at relatively low temperatures (McDaniel et al., 2001; Stadler et al., 2002; Becalski et al., 2003; Blank et al., 2005; Moldoveanu, 2010; Becalski et al., 2011). The other analytes presented smaller ratios. Therefore, when compared with 3R4F mainstream smoke, all HPHCs are considerably reduced in the THS2.2 aerosol of the four products. This data supports the hypothesis that the controlled heating of tobacco in THS2.2 resulted in a significant reduction in the pyrosynthesis of HPHCs. When compared with 3R4F, the formation of polycyclic aromatic hydrocarbons, aromatic amines, phenols, and aldehydes was reduced by more than 75%, and for the majority of HPHCs, by more than 90% under the HCI machine-smoking conditions. The analyzed HPHCs covered a broad range of chemical compounds, and several of these HPHCs have been described as markers for the pyrolysis of tobacco (Moldoveanu, 2010). Since the reduction of individual HPHCs was consistent across the different HPHC groups,

Table 13
The LOGELs (expressed on a nicotine basis) achieved following treatment with TPM (μg/ml) and GVP (μg nicotine equivalent/ml) derived from THS2.2 variants and 3R4F values are shown when GEF threshold was exceeded.

		4 h −S9		4 h +S9		24 h −S9	
THS2.2 D2	TPM	18.66		34.48	41.80	17.31	
	GVP	71.90	83.17	249.87	300.99	43.14	
3R4F	TPM	1.31		2.47	1.91	1.14	1.05
	GVP	[a]		69.07	50.79	8.64	12.61
THS2.2 D1 M	TPM	12.55		27.82	21.71	[a]	
	GVP	83.30	74.53	240.34	267.92	[a]	
3R4F	TPM	0.96	1.11	3.05	1.99	[a]	
	GVP	16.27	10.311	53.74		6.14	

[a] Concentration-dependent increase in MF observed but below the GEF threshold.

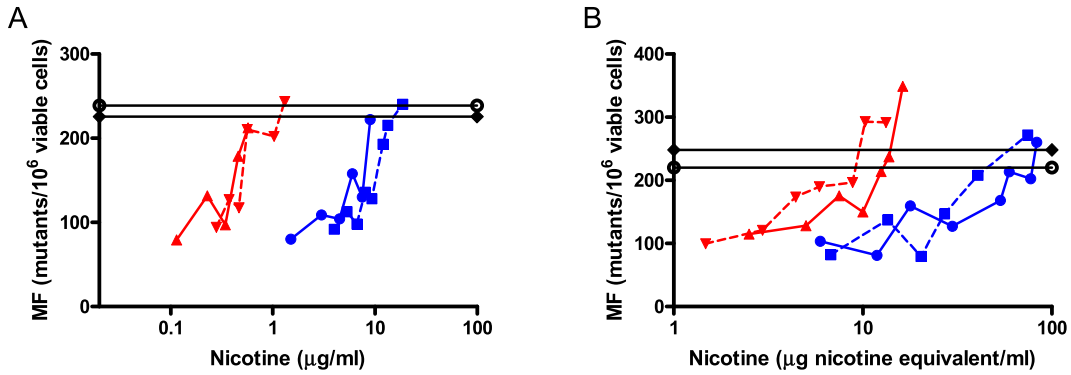

Fig. 5. The mutagenic responses induced by aerosol fractions derived from THS2.2 variants and 3R4F in the 4 h−S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM from THS2.2 D2 and 3R4F. MFs for the DMSO-treated controls in tests #1 and #2 were 99.52 ± 19.21 and 112.81 ± 23.41 mutants/10^6 viable cells, respectively. ●THS2.2 D2 #1; ■THS2.2 D2 #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + DMSO MF #1; ⊙GEF + DMSO MF #2. B. GVP from THS2.2 D1 M and 3R4F. MFs for the PBS-treated control(s) in tests #1 and #2 were 122.33 and 94.10 ± 26.37 mutants/10^6 viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + PBS MF #1; ⊙GEF + PBS MF #2.

it may be assumed that other HPHCs, although not measured, were similarly reduced. In addition, it can also be observed that some HPHCs that could distill out of tobacco in 3R4F were also reduced in the THS2.2 aerosol. The transfer of cadmium to the aerosols of the four THS2.2 products could not be quantified (results below LOQ), and the yield of TSNAs was minimal (Tables 5 and 6). Since the nicotine yield was lower in the analyzed THS2.2 products than in 3R4F, the ratios calculated on a per-mg nicotine basis were somewhat higher. However, the trend remained the same, and the reductions expressed on a per-mg nicotine basis were also substantial (Figure D in the supplementary material).

The mainstream smoke of 3R4F and the THS2.2 aerosol generated under the HCI machine-smoking conditions were both shown to be respirable aerosols (Table 9). The MMAD values were similar: 0.8 μm for 3R4F and 0.7 μm for THS2.2. The GSD was somewhat higher for THS2.2 (Section 3.4). Therefore, THS2.2 presents respirable properties that are similar to those of 3R4F, while reducing substantially the levels of the measured HPHCs.

4.2. Chemical composition of the THS2.2 FR1 aerosol collected under different climatic conditions and extreme PUFFING regimens

Since the THS2.2 may be used by consumers using puffing regimens under climatic conditions that deviate significantly from those considered in the HCI machine-smoking standard (55 ml puff every 30 s, 22 °C, 60% RH), the aerosol of the THS2.2 FR1 was collected under different atmospheric and puffing conditions described in sections 2.5 and 2.6.

The climatic conditions may have a significant impact on the deliveries to the mainstream smoke of CC (Dymond and Hirji, 1972; Boder and Senehi, 1984). In previous studies, the filtration efficiency of the tobacco rod and of the filter and the puff count were affected when CC were conditioned and smoked under different climatic conditions. The effect could be observed on both gas phase and particulate phase components. For instance, a temperature increase of 10 °C resulted in an increased delivery of nitric oxide by 18% for an American blend CC, while the phenol delivery decreased correspondingly (Dymond and Hirji, 1972). It was anticipated that the careful control of the heater blade temperature up to a maximum of 350 °C (Smith et al., 2016) and use of a low efficiency filter would make THS2.2 less sensitive to variations in ambient conditions and alterations in the yields of the different analytes. The yield of water in the THS2.2 aerosol was considered the only exception, because of the high humectant content of the tobacco plug which contains glycerin in about 20% of the tobacco plug weight. When the *tobacco sticks* were conditioned for at least 48 h and used under “desert” conditions, the delivery of water was considerably reduced when compared with the “tropical” conditions (Table 7). The differences in the yield of water under both “tropical” and “desert” conditions explained nearly all the

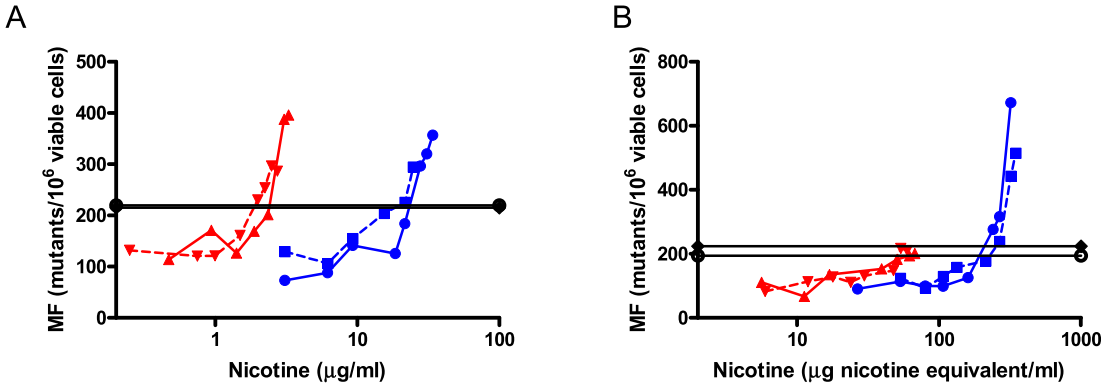

Fig. 6. The mutagenic responses induced by aerosol fractions derived from THS2.2 D1 M and 3R4F in the 4 h + S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM. MFs for the DMSO-treated controls in tests #1 and #2 were 89.01 ± 6.76 and 93.90 ± 13.64 mutants/10^6 viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + DMSO MF #1; ⊙GEF + DMSO MF #2. B. GVP. MFs for the PBS-treated control in tests #1 and #2 were 97.43 and 68.20 mutants/10^6 viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + PBS MF #1; ⊙GEF + PBS MF #2.

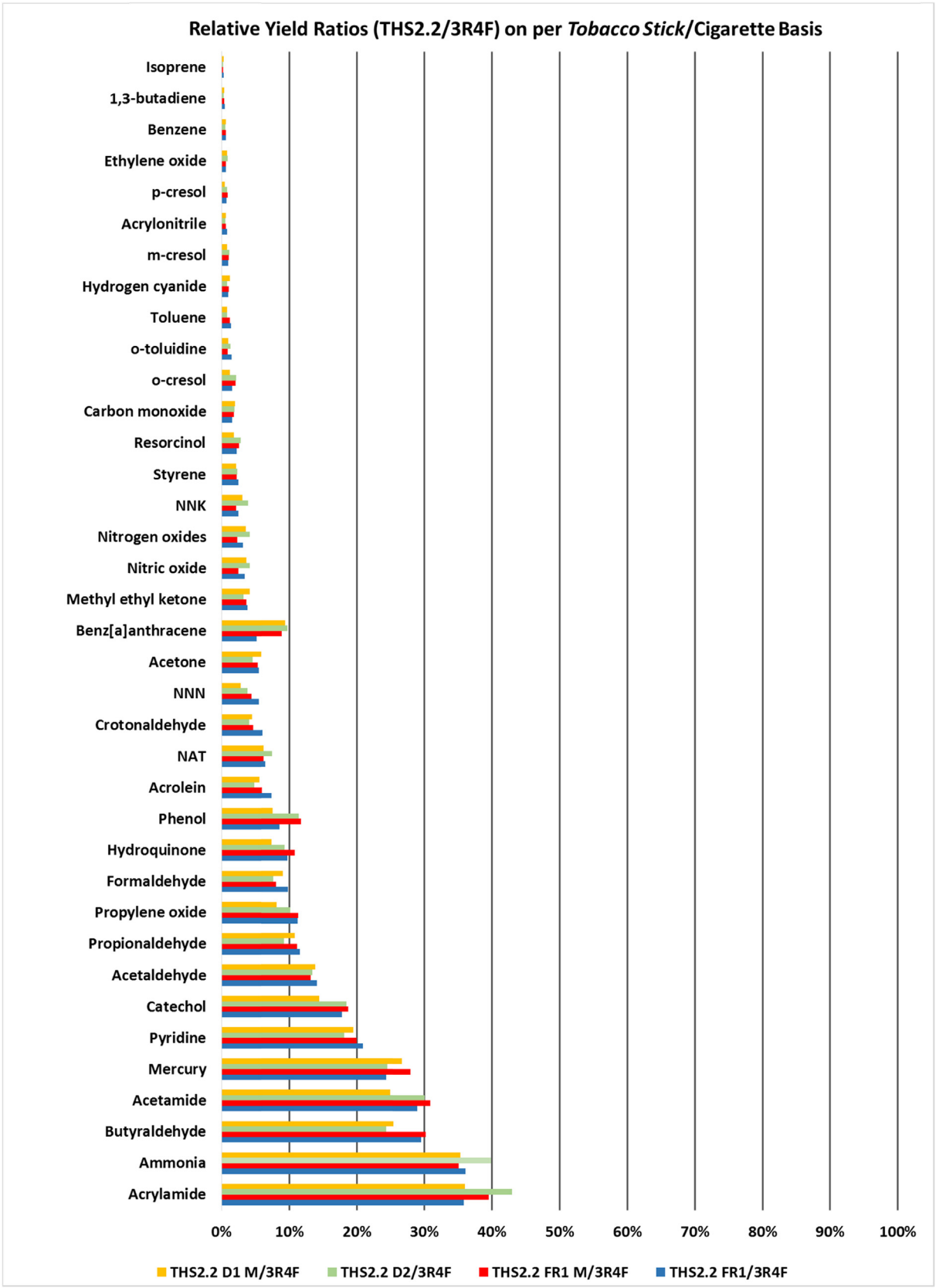

Fig. 7. Mainstream aerosol HPHCs from THS2.2 compared to the mainstream smoke HPHCs from the 3R4F reference cigarette (constituent levels set at 100%) on a per-unit basis under the Health Canada Intense (HCI) machine-smoking regimen. When one value or more was below the LOQ, the results were not presented in the graphs (NAT: N-nitrosoanatabine, NNK: 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: N-nitrosonornicotine).

variability in the TPM yields, since the NFDPM yields obtained from the *tobacco sticks* in the three conditions ("Mediterranean", "tropical" and "desert") were similar. The ranges for nicotine, formaldehyde, ethyl methyl ketone, acrylonitrile, 1,3-butadiene, benzene, styrene, toluene, *o*-cresol, *p*-cresol, NNN, ammonia, and acetamide were lower than the Intermediate Precision (IP) (International Council for Harmonisation, 1996; Walfish, 2006) of the respective analytical methods, and the climatic conditions were considered not to have a significant impact on the yields of these HPHCs. The ranges for other HPHCs were low, except for *m*-cresol, phenol, *o*-toluidine, propylene oxide, and nitrobenzene, which had ranges in excess of 35%. For *o*-toluidine, propylene oxide, and nitrobenzene, the highest yield was obtained when conditioning and machine-smoking the *tobacco sticks* at 22 °C and 60% RH. Therefore, collecting the aerosol under "tropical" or "desert" conditions did not increase the yields of these HPHCs. The yield of *m*-cresol was increased from 0.031 to 0.071 μg/stick under the "tropical" condition, while phenol was increased from 1.66 to 2.49 μg/stick under the "desert" condition. However, the values remained low when compared with the yields obtained from 3R4F: *m*-cresol (3.03 μg/stick) and phenol (13.6 μg/stick) (Table 5). Therefore, the variation of the climatic conditions had only a minor influence on the HPHC yields.

The different machine-smoking puffing regimens (Table 2) were selected to cover the puffing behavior reported for users of THS2.2 (Campelos et al., 2016). They induced significant modifications of the air flow and of the quantity of air used to extract aerosol from the tobacco plug in THS2.2. The minimum and maximum yields obtained from the different machine-smoking regimens are presented in Table 8. In general, the yields of polar HPHCs (e.g. phenol and cresol isomers) were more sensitive than apolar HPHCs to the variation of the machine-smoking puffing conditions (Table 8). For each HPHC, the comparison between the maximum yield and the yield obtained with the HCI smoking regimen should enable the identification of the HPHC for which the standard protocol may underestimate the exposure when using more extreme puffing conditions. The ratios of maximum yield to HCI yield are presented in Table 8. These ratios were less than 2 for 42 of 49 analyzed compounds. Again, phenol and cresol isomers were the HPHCs presenting the largest ratios. However, the HCI machine-smoking protocol gave a relevant estimate of the exposure for the majority of the tested HPHCs. Interestingly, the ratio obtained for nitrogen oxides was only 1.1, and the ratio for CO was only 1.2. Since NO_x and CO can be considered potential markers of combustion (Norman et al., 1983; Reed, 2002; Glarborg et al., 2003; Im et al., 2003; Baker, 2006; Senneca et al., 2007; Cozzani et al., 2016), no evidence of tobacco combustion was found even under extreme machine-smoking puffing conditions. Under extreme machine-smoking puffing conditions, the yields of all toxicologically relevant compounds in the THS2.2 aerosol were lower than those obtained when smoking the 3R4F reference cigarette under HCI machine-smoking conditions (Table 5).

4.3. *In vitro* toxicology

The *in vitro* toxicology results reflect the chemistry data; THS2.2 aerosol fraction-induced effects are distinctly different in terms of potency from those induced by counterpart fractions from 3R4F. The THS2.2 aerosol demonstrates a substantial reduction in toxicological activity compared with 3R4F smoke. In the NRU assay, both the particulate phase and GVP *in vitro* cytotoxicity of THS2.2 Regular (FR1 and D2) and THS2.2 Menthol (FR1 and D1) were reduced by 85%–95% compared with the 3R4F, independent of the basis used to express the activity (per-mg TPM or per-mg nicotine) (Table 10, Table 11 and Tables F and G of the supplementary material).

The Ames assay did not reveal significant mutagenicity of the TPM fraction for either THS2.2 regular or THS2.2 menthol under the conditions of this test. In contrast, the TPM fraction from 3R4F was mutagenic in tester strains TA98, TA100, and TA1537 in the presence of the S9 metabolizing fraction from Aroclor-treated rat liver. The MLA data show that both the TPM and GVP aerosol fractions derived from THS2.2 D2 and THS2.2 D1 M were mutagenic in this assay. However, the LOGELs demonstrate a lower *in vitro* mutagenic potency of the THS2.2 aerosol fractions compared with 3R4F. While a conclusion underlying the mechanism(s) of this phenomenon cannot be definitively made on the basis of these data, it is reasonable to suggest that the overall reduction in the burden of toxicants present in the THS2.2 aerosols may play a role in the manifestation of reduced cytotoxic and mutagenic potency *in vitro*.

5. Conclusion

The low operating temperature of THS2.2 results in significantly lower concentrations of HPHCs in the mainstream aerosol compared with the mainstream smoke of the 3R4F reference cigarette when expressed on either a per-Tobacco Stick/cigarette or a per-mg nicotine basis, while the MMAD of both aerosols remains similar. The reductions in the concentrations of most HPHCs in the THS2.2 aerosol were greater than 90% when compared with 3R4F, and were not affected by machine-smoking of THS2.2 under extreme climatic conditions. No evidence of tobacco combustion was found when using the THS2.2 device with puffing regimens that were significantly more intense than the HCI conditions. The mutagenic and cytotoxic potencies of the mainstream aerosol fractions from THS2.2, when evaluated by the Ames, mouse lymphoma, and NRU assays were reduced by at least 85%–95% compared with the mainstream smoke aerosol of 3R4F.

Conflict of interest statement

The work reported in this publication involved a candidate Modified Risk Tobacco Product developed by Philip Morris International (PMI) and was solely funded by PMI. All authors are (or were) employees of PMI R&D or worked for PMI R&D under contractual agreements.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2016.10.001.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.10.001.

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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 System 2.2 aerosol

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ABSTRACT

The Tobacco Heating System (THS2.2), 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 THS2.2 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 THS2.2 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 THS2.2 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.

1. Introduction

The U.S. Family Smoking Prevention and Tobacco Control Act (FSPTCA) defines a Modified Risk Tobacco Product (MRTP) as "any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products" (Family Smoking Prevention and Tobacco Control Act). This publication is part of a series of nine publications describing the nonclinical and part of the clinical assessment of a candidate MRTP, THS2.2 regular and a mentholated version (THS2.2M). The series of publications provides part of the overall scientific program to assess the potential for THS2.2 to be a reduced risk product. The first publication in this series describes THS2.2 and the assessment program for MRTPs (Smith et al., submitted (this issue)). This is followed by six publications, including this one, that describe the nonclinical assessment of THS2.2 regular and THS2.2M (Kogel et al., submitted (this issue); Oviedo et al., submitted (this issue); Schaller et al., submitted (this issue)-a; Schaller et al., submitted (this issue)-b; Sewer et al., submitted (this issue); Wong et al., submitted (this issue)). The eighth publication in the series describes a clinical study to assess whether the reduced formation of Harmful and Potentially Harmful Constituents (HPHCs) for THS2.2 regular also leads to reduced exposure to HPHCs when the product is used in a clinical setting (Haziza et al., submitted (this issue)). A final publication utilizes data gathered from the reduced exposure clinical study on THS2.2 regular to determine if a systems pharmacology approach can identify exposure response markers in peripheral blood of smokers switching to THS2.2 (Martin et al., submitted (this issue)). This publication is the third of the series and presents the aerosol

Abbreviations: NNK, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; HPHC, harmful and potentially harmful constituent; HCI, Health Canada Intense; IARC, International Agency for Research on Cancer; LOQ, limit of quantification; NFDPM, nicotine-free dry particulate matter; NAB, *N*-nitrosoanabasine; NAT, *N*-nitrosoanatabine; NNN, *N*-nitrosonornicotine; NO_x, nitrogen oxides; PCA, principal component analysis; UL, upper limit; PAH, polycyclic aromatic hydrocarbon; THS2.2, Tobacco Heating System 2.2; TPM, total particulate matter; TSNA, tobacco specific nitrosamine.

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chemistry of 43 tobacco blends to assess the influence of the blending on HPHC generation in the THS2.2 aerosol.

The three major classes of tobacco used to manufacture classical lit-end cigarettes (CCs) are flue-cured (bright), air-cured, and Oriental tobaccos. The classic "American blend" cigarette usually contains all three types of tobacco, whereas "Virginia" cigarettes mainly contain flue-cured tobacco. The type and the quality of tobacco used for cigarette manufacturing not only impacts the sensory perception of the resulting smoke, but also affects the delivery of HPHCs in the smoke. According to Piadé et al. (Piadé et al., 2013), Virginia blend cigarettes have higher yields of formaldehyde, cadmium and, to a lesser extent, unsaturated aldehydes, hydrogen cyanide and aromatic compounds. In contrast, American blend cigarettes have higher yields of nitrogen-containing compounds (except hydrogen cyanide). These blend effects are essentially unchanged by differences in cigarette construction. With minor exceptions, both pyrogenesis and pyrosynthesis of HPHCs in cigarettes result from the thermal decomposition of organic tobacco compounds at elevated temperatures (Torikaiu et al., 2005; Baker and Dixon, 2006).

In the heat-not-burn THS2.2, tobacco is heated with a blade reaching a maximum temperature of 350 °C (Smith et al., submitted (this issue)) resulting in an aerosol containing lower concentrations of HPHCs compared to the mainstream cigarette smoke (Schaller et al., submitted (this issue)-a). Since the temperature at which tobacco is heated in the THS2.2 is considerably lower than the temperatures of up to 900 °C found in a cigarette during puffing (Baker, 1974, 1975b), both the formation and delivery of HPHCs from the tobacco blend to the mainstream aerosol may be different and not reflected by knowledge gained from the study of HPHC formation and chemistry prevailing in a cigarette. Consequently, the influence of the tobacco plug blend composition on the deliveries in the THS2.2 aerosol was studied using the analyte list, including HPHCs, defined in the in the previous paper of the series (Schaller et al., submitted (this issue)-a).

A detailed chemical characterization of 59 analytes in the mainstream aerosol produced by 43 different experimental tobacco plug blends when tested for use in the THS2.2 using the Health Canada Intense (HCI) machine-smoking protocol (Health Canada, 2000) is reported. Principal Component Analysis (PCA) was used to identify clustering of HPHCs in the aerosols from different tobacco plug blends and to identify which tobacco types/tobacco blend compositions would result in the largest reductions of the yields of HPHCs in the mainstream aerosol.

2. Materials and methods

2.1. Selection of tobaccos and production of the blends

The effects of using plugs containing different tobacco blends on HPHC yields in mainstream THS2.2 aerosol were studied using a blending strategy in which different types of tobacco with distinctive sensorial characteristics were used. The blends were based on four categories of tobaccos selected to provide different flavor directions:

1. Air-cured: burley tobaccos and other air-cured tobaccos, used to provide the blend with dark notes.
2. Bright: flue-cured tobaccos, used to provide the blend with sweet and spicy aromas.
3. Aromatic: Oriental tobaccos and a fire-cured tobacco, used to provide aromatic complexity.
4. Flue-cured stems or strips, with little flavor, used as fillers or to dilute the flavor.

A second level of classification, the country of origin of the tobacco, was used to provide more granularity to the primary tobacco classification and to maximize the coverage of different sensory perceptions. All the studied tobaccos were also used for the production of commercial lit-end cigarettes or roll-your-own tobaccos. The air-cured tobaccos were burley tobaccos and other tobaccos for which the curing induced the transformation of most of the tobacco carbohydrates. The bright, Oriental and fire-cured tobaccos were cured according to their usual respective curing procedures (Davis and Nielsen, 1999). Nineteen different tobaccos covering the four primary tobacco classes mentioned above were selected. The tobaccos were blended and formed into *tobacco sticks* to produce different sensory perceptions when used in the THS2.2. The 19 selected tobaccos are listed in Table 1.

The selected tobaccos (shown in Table 1) were homogenized and ground, then blended as required. Cast leaf was then formed from each blend (Coggins et al., 2013). Tobacco plugs were produced from the different cast leaf materials. The compositions of the monitor blend (FR1) and the 43 different tobacco blends that were used (BL1–BL43) are listed in Table 2.

The HPHCs in THS2.2 aerosol produced by each type of tobacco in the absence of interactions that might occur in a blend were determined by performing tests using samples of each single tobacco, but the air-cured tobaccos AC1, AC2, AC4, and AC5 could not be tested because it was not possible to use these tobaccos to form cast leaf suitable for manufacturing tobacco plugs. The impact of blending beyond the typical usage level of different tobacco types was investigated using blends containing up to 40% of single grades of air-cured tobaccos (e.g., BL6, BL7, BL8, BL9, BL10), up to 40% of fire-cured tobacco (BL1, BL5 and BL9) and up to 20% of filler tobacco (BL25, BL27). The FR1 blend was used as a control to determine the reproducibility of the manufacturing process. This blend was selected as control because it contains the main tobacco categories (Air-cured, Bright, and Aromatic) and it had been particularly well characterized since it was used as reference blend during all the product development process. The FR1 blend was produced on six different occasions using the same tobacco lots and the HPHC yields in mainstream aerosol produced from each FR1 blend production were determined.

2.2. THS2.2 tobacco sticks

The *tobacco stick* was designed to be used only with the THS2.2

Table 1
Single grade tobaccos selected for use in the tests.

Tobacco type	Country of origin	Label
Air-cured	Malawi	AC1
Air-cured	USA	AC2
Air-cured	Indonesia	AC3
Air-cured	Brazil	AC4
Air-cured	Malawi	AC5
Bright	China	FC1
Bright	India	FC2
Bright	USA	FC3
Bright	Brazil	FC4
Bright	Tanzania	FC5
Bright	USA	FC6
Aromatic	Tanzania	AR1[a]
Aromatic	Greece	AR2[b]
Aromatic	Greece	AR3[b]
Aromatic	Turkey	AR4[b]
Aromatic	Turkey	AR5[b]
Aromatic	Greece	AR6[b]
Filler	Brazil	FI1
Filler	Various	FI2

[a] Fire-cured tobacco.
[b] Oriental tobacco.

Table 2
Blends produced for the study.

Blend	Percentage contribution of each tobacco type																		
ID	AC1	AC2	AC3	AC4	AC5	FC1	FC2	FC3	FC4	FC5	FC6	AR1	AR2	AR3	AR4	AR5	AR6	FI1	FI2
FR1			20				20		50				10						
BL1								60				40							
BL2						60				40									
BL3							30		60										10
BL4								60			40								
BL5										40		40						20	
BL6					40					60									
BL7				40		60													
BL8			40						60										
BL9	40						60												
BL10		40					60												
BL11									60				40						
BL12								60									40		
BL13											60			40					
BL14								60							40				
BL15								60								40			
BL16					30	60												10	
BL17				30						60								10	
BL18			30				60											10	
BL19	30								60									10	
BL20		30								60									10
BL21											60		30						10
BL22								60						30					10
BL23											60					30		10	
BL24		40						40										20	
BL25									40								40		20
BL26							40								40			20	
BL27							40									40			20
BL28				40					30			30							
BL29	30										30	40							
BL30			30							30			40						
BL31	40							30					30						
BL32				30			30										40		
BL33			40								30						30		
BL34					30		30							40					
BL35		30							30					40					
BL36	30									30					40				
BL37		30									30				40				
BL38					30				30							40			
BL39[a]	3.0	3.0	3.0	3.0	3.0	10.0	10.0	10.0	10.0	10.0	10.0	3.0	3.0	3.0	3.0	3.0	3.0	3.3	3.3
BL40[a]	2.1	2.1	2.1	2.1	2.1	11.7	11.7	11.7	11.7	11.7	11.7	2.1	2.1	2.1	2.1	2.1	2.1	3.3	3.3
BL41[a]	8.0	8.0	8.0	8.0	8.0	5.4	5.4	5.4	5.4	5.4	5.4	2.4	2.4	2.4	2.4	2.4	2.4	6.7	6.7
BL42[a]	2.8	2.8	2.8	2.8	2.8	5.5	5.5	5.5	5.5	5.5	5.5	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
BL43[a]	5.3	5.3	5.3	5.3	5.3	5.6	5.6	5.6	5.6	5.6	5.6	4.5	4.5	4.5	4.5	4.5	4.5	6.7	6.7

[a] Each contribution is rounded to one decimal place, so the sum of the contributions is not exactly 100%.

holder (Smith et al., submitted (this issue)). The structure of a *tobacco stick* is shown in Fig. 1.

A *tobacco stick* is constructed by sequentially assembling the following components:

1. Tobacco plug wrapped in paper over-wrap.
2. Hollow acetate tube wrapped in paper over-wrap.
3. Polymer-film filter, wrapped in paper over-wrap.
4. Mouthpiece filter wrapped in paper over-wrap.

All of these elements were wrapped in an outer sheet of paper, and tipping paper was added at the mouth end (Fig. 1).

Unlike cigarettes, THS *tobacco sticks* do not burn when used in the THS2.2 holder, and they remain the same length after use. *Tobacco sticks* were made of cast leaf containing the tobaccos and tobacco blends listed in Tables 1 and 2, binders, and humectants. The humectants were added to prevent the cast leaf becoming too brittle. Heating the humectants caused them to evaporate and recondense to form small droplets, generating a visible aerosol. No

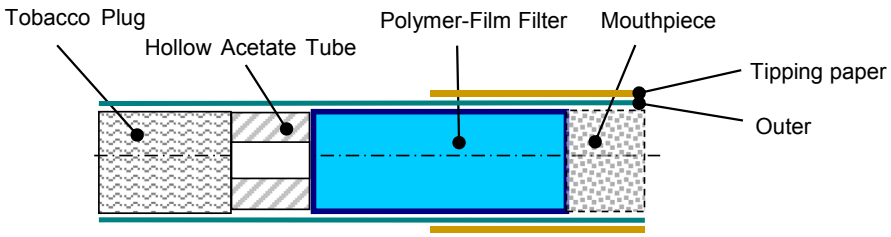

Fig. 1. Schematic of a *tobacco stick* for use in the Tobacco Heating System 2.2.

flavorings were added to the tobacco of the *tobacco sticks* of this study.

2.3. Chemical analyses

Samples of each of the *tobacco sticks* containing the different tobacco plug blends (Table 2) were sent to Labstat International ULC (Kitchener, Ontario, Canada), where the analytes in the mainstream aerosol produced when the *tobacco sticks* were used in a THS2.2 were quantified. Each *tobacco stick* was conditioned following the ISO 3402 protocol (International Organisation for Standardization, 2010), then mainstream aerosol was produced from the stick using the Health Canada intense machine-smoking regimen (Health Canada, 2000). The analytes in the mainstream aerosol produced by each *tobacco stick* were determined using the analytical methods shown in Table 3. All analyses were performed in triplicate. It should be pointed out that the analytical methods used by Labstat were not exactly the same as those used by the Philip Morris Research Product Testing Laboratories for the previous paper of the series (Schaller et al., submitted (this issue)-a). This resulted in different limits of quantification (LOQs) and different analytical results for some analytes. For instance Labstat was not using the in-situ extraction technique (Ghosh and Jeannet, 2014) which resulted in lower water results and higher NFDPM values.

The analyte yields of the mainstream smoke of the University of Kentucky 3R4F reference cigarettes (Lexington, KY, USA; https://ctrp.uky.edu/) were used as quality control samples for the analytical methods. The blend of the 3R4F reference cigarettes was made of 35.41% flue-cured, 21.52% burley, 1.35% Maryland, 12.07% Oriental and 29.55% reconstituted tobaccos. The 3R4F reference cigarettes were conditioned according to ISO 3402 (International Organisation for Standardization, 2010) and machine-smoked using the Health Canada intense protocol (Health Canada, 2000).

For the determination of the tobacco-specific nitrosamines (*N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), and *N*-nitrosonornicotine (NNN)) in cast leaf and tobacco materials, a published method (CORESTA, 2016) was adapted. After addition of labelled internal standards the sample is extracted into an aqueous buffer and filtered. The filtrate is analyzed by Liquid Chromatography - Triple Quadrupole Mass Spectrometry (LC-MS/MS).

The determination of total alkaloids (as nicotine), reducing carbohydrates, nitrates (NO_3^-) and ammonia in tobacco materials was performed by 4 parallel lines of continuous flow analysis after grinding and extraction of the tobacco with a 5% acetic acid solution. The determination of total alkaloids (as nicotine) was performed according to the CORESTA recommended method #35 (CORESTA, 2010a) by reaction of the alkaloids with sulphanilic acid and cyanogen chloride. Cyanogen chloride was generated in situ by the reaction of potassium cyanide and chloramine T. The determination of reducing carbohydrates was performed according to the CORESTA recommended method #38 (CORESTA, 2010b) by reaction of reducing carbohydrates with *p*-hydroxybenzoic acid hydrazide in alkaline medium to produce a yellow osazone. The determination of nitrates (NO_3^-) was performed according to the CORESTA recommended method #36 (CORESTA, 2015) by reduction of nitrate to nitrite with hydrazinium sulphate in the presence of a copper catalyst, followed by reaction with sulphanilamide to form a diazo compound. The determination of ammonia was performed by oxidation of ammonia with sodium hypochlorite to react with salicylate ions in the presence of nitroprusside to form the salicylic acid analog of indophenol blue (Digard et al., 2013). The analytical results for the single grade tobaccos are presented in supplementary material Table III.

In order to detect potential signs of combustion in the studied blends, the presence of relevant quantities of nitrogen oxides in the aerosol, not formed from the decomposition of nitrates present in the original tobacco, was monitored (Cozzani et al., 2016). The quantification of nitrogen oxides in aerosols was performed by Labstat according to the Official Method T-110 from Health Canada ((Health Canada, 2000). Carbon monoxide, a second potential marker of combustion, was quantified in the aerosols produced from the different blends in the THS2.2. The quantification of carbon monoxide in aerosols was performed by Labstat according to the Official Method T-115 from Health Canada (Health Canada, 2000). Potential signs of combustion were identified when both the production of nitrogen oxides in aerosol was not correlated with the nitrate content of the tobacco blend and the carbon monoxide yields increased above the upper limit of the 99% confidence interval of the monitor blend FR1.

2.4. Statistical analyses

The analytes produced by the 43 blends and by the single tobacco grades were analyzed in triplicates. For each analyte the mean is reported. The LOQ is given if the mean was below the LOQ.

Descriptive statistics (the number of values, the arithmetic mean, and the 99% confidence interval) were obtained for all of the

Table 3
Analytical methods used to determine the concentrations of harmful and potentially harmful constituents of mainstream aerosol.

Compound[a]	Labstat International Analytical Method (November 2013)
Ammonia	T-101
Acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde	T-104
Hydrogen cyanide	T-107
Mercury	T-108
Arsenic, cadmium, chromium, lead, nickel, selenium	T-109
Nitric oxide, nitrogen oxides	T-110
Nitrobenzene, pyridine, quinoline, styrene	T-112/TMS-00112
Carbon monoxide, glycerin, nicotine, NFDPM, TPM, water	T-115/TMS-00115a
Benz [*a*]anthracene, benzo [*a*]pyrene, dibenz [*a,h*]anthracene, pyrene	TMS-00120
Acrylonitrile, benzene, 1,3-butadiene, ethylene oxide, isoprene, propylene oxide, toluene, vinyl chloride	TMS-00124
3-Aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, *o*-toluidine	TMS-00128
NAB, NAT, NNK, NNN	TMS-00135
Acetamide, acrylamide	TMS-00137
Catechol, *m*-cresol, *o*-cresol, *p*-cresol, hydroquinone, phenol, resorcinol	TMS-00139

[a] TPM: total particulate matter; NFDPM: nicotine-free dry particulate matter; NAB: *N*-nitrosoanabasine; NAT: *N*-nitrosoanatabine; NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NNN: *N*-nitrosonornicotine.

analytes found in the mainstream aerosol produced by blend FR1 on a *tobacco stick* basis. The monitor blend FR1 was used as reference for comparison of the analyte yields of the 43 tobacco plug blends and the data obtained from the analysis of aerosols from the monitor blend FR1 manufactured on 6 different occasions was used to estimate the variability of the monitor blend for each analyte. A confidence interval of 99% was calculated around the average yield obtained when analyzing each analyte of the monitor blend to identify tobacco plug blends that were significantly different based on the upper confidence limit value. For analytes which were systematically below the LOQ in the aerosol of monitor blend FR1, an upper limit of 4 times the LOQ was defined.

Principal component analysis (PCA) was performed using XLSTAT version 2013.4.03 software (Addinsoft SARL, Paris, France). PCA was performed on the data after the data had been mean-centered and scaled to a variance of one, based on the covariance matrix for the entire dataset.

3. Results

The mean ± $CI_{99\%}$ and the upper limit (UL) of the analyte yields in the aerosol of the monitor blend FR1, the extreme yields (minimum and maximum) of each analyte in the aerosols from the 43 different tobacco plug blends, and the yields of analytes in mainstream smoke of the reference cigarette 3R4F which was smoked as monitor cigarette are presented in Table 4. The individual analyte yields found when blends BL1–BL43 were used in the THS2.2 are presented in supplementary material Table I.

The yields of seven HPHCs (cadmium, chromium, crotonaldehyde, dibenz [*a,h*]anthracene, nickel, nitrobenzene, and selenium) in the aerosols produced by the FR1 blend and the BL1–BL43 blends were lower than the LOQs. The yields of 28 of the other HPHCs that were analyzed in the aerosols produced by blends BL1–BL43 were all lower than the upper limit for the FR1 blend. These 28 HPHCs were 3-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, acetaldehyde, acetone, acrolein, acrylonitrile, arsenic, benzene, 1,3-butadiene, butyraldehyde, carbon monoxide, catechol, *p*-cresol, ethylene oxide, formaldehyde, hydrogen cyanide, lead, mercury, methyl ethyl ketone, phenol, propionaldehyde, pyridine, quinoline, resorcinol, styrene, toluene, and vinyl chloride. The yields of 11 nitrogen-containing HPHCs compounds (acetamide, acrylamide, 4-aminobiphenyl, ammonia, nitric oxide, nitrogen oxides, *o*-toluidine, and tobacco-specific nitrosamines (TSNAs)), three polycyclic aromatic hydrocarbons (PAHs) (benz [*a*] anthracene, benzo [*a*]pyrene, and pyrene), three phenols (*m*-cresol, *o*-cresol, and hydroquinone), isoprene, and propylene oxide were higher than the UL of the monitor blend FR1 in aerosols produced by some of the tobacco plug blends.

The data were subjected to PCA to allow the ranges of chemicals found in the aerosols produced by the different blends to be investigated and to allow correlations between the yields of different analytes to be identified. The glycerin, nicotine-free dry particulate matter (NFDPM), total particulate matter (TPM), and water yields were removed from the data to allow the PCA to be focused on specific chemicals with potential toxicological relevance. HPHCs with yields below their LOQs for more than 15 blends were also removed to eliminate unnecessary “noise” from the data. The PCA results shown in Fig. 2 were obtained from the yields of the 41 remaining analytes, which were acetaldehyde, acetamide, acetone, acrolein, acrylamide, acrylonitrile, 3-aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, ammonia, benz [*a*]anthracene, benzene, 1,3-butadiene, butyraldehyde, carbon monoxide, catechol, *m*-cresol, *o*-cresol, *p*-cresol, ethylene oxide, formaldehyde, hydroquinone, isoprene, methyl ethyl ketone, mercury, nicotine, nitric oxide, nitrogen oxides, NAB, NAT, NNK, NNN, phenol, propionaldehyde, propylene oxide, pyrene, pyridine, styrene, toluene, and *o*-toluidine.

About 26% of the total variance was explained by PC1. Nitrogen-containing HPHCs contributed 80% of PC1. The main contributors to PC1 were, in decreasing order, acetamide, NNN, NAB, *o*-toluidine, NAT, NNK, 4-aminobiphenyl, and ammonia. About 17% of the total variance was explained by PC2. The phenols contributed 33% of PC2, the nitrogen-containing HPHCs contributed 27%, and the aldehydes and ketones contributed 11%. The main contributors to PC2 were, in decreasing order, phenol, methyl ethyl ketone, nitrogen oxides, nitric oxide, *m*-cresol, *p*-cresol, and *o*-cresol. About 11% of the total variance was explained by PC3. The aldehydes and ketones contributed 42% of PC3, the nitrogen-containing HPHCs contributed 15%, and the PAHs contributed 10%. The main contributors to PC3 were, in decreasing order, butyraldehyde, acetone, propionaldehyde, acetaldehyde, benzene, acrylonitrile, styrene, toluene, benz [*a*]anthracene, and pyrene. HPHCs in all of the chemical families contributed to the fourth and fifth components, and it was difficult to identify any trends. In terms of distribution of the components variance to PCs, it should be pointed out that, for none of the HPHCs, the majority of the variance was distributed on a single axe of the PCA.

The scores for the blends containing at least 40% air-cured tobacco were mostly in the positive PC1 zone of the plot of PC1 and PC2, but they overlapped substantially with some bright blends (e.g., BL17, BL19, BL20, and BL21) and Oriental blends (e.g., BL30, BL 35, BL37, and BL38). Two blends containing at least 40% fire-cured tobacco (BL1 and BL29) were at extreme positions, with high scores for both PC1 and PC2. The third fire-cured blend (BL5) was not separated from the other blends. On the PC1/PC3 plot, the scores for the air-cured tobacco blends were mostly positioned in the zone of positive PC1 and PC3 with a significant overlap with Bright and Oriental blends. The fire-cured tobaccos (BL1, BL5 and BL29) were separated in the zone of positive PC1 and negative PC3. PC1, PC2, and PC3 together represented only 55% of the variance (Fig. 2) but the fourth and fifth components (which each contributed 8% of the total variance) did not offer additional clustering information.

4. Discussion

The objective of this study was to assess the variations in HPHC yields caused by using different tobacco blends in THS2.2 tobacco plugs. The FR1 blend was used as a reference blend, and the HPHC yields from the other blends were compared with the HPHC yields from the FR1 blend. The physical properties of monitor blend FR1 aerosol (particle size distribution determined as the mass medium aerodynamic diameter) and aerosol chemistry (determination of 59 analytes) using analytical methods developed by Philip Morris International have also been reported in another paper contained in this issue (Schaller et al., submitted (this issue)-a).

4.1. Markers of combustion

The THS2.2 operates by heating tobacco with a blade operating at a maximum temperature of 350 °C to generate an aerosol, compared to combustion of tobacco at temperatures up to 900 °C in a cigarette (Smith et al., submitted (this issue)), confirmation of the absence of tobacco combustion occurring in all the experimental tobacco plug blends was investigated by analyzing for the presence of nitrogen oxides (Norman et al., 1983; Glarborg et al., 2003; Im et al., 2003) and carbon monoxide (CO) (Reed, 2002; Baker, 2006; Senneca et al., 2007), two chemical markers of potential tobacco combustion (Cozzani et al., 2016), in the mainstream aerosols of THS2.2. The yields of nitrogen oxides detected in the aerosols produced by the different experimental tobacco plug blends varied

Table 4
Summary of the yields of the FR1 blend and blends BL1–BL43 used in the Tobacco Heating System 2.2 (THS2.2) and of 3R4F reference cigarettes.

Parameter*	Unit	FR1 blend used in the THS2.2		Blends BL1–BL43 used in the THS2.2			3R4F cigarette
		Mean ± $CI_{99\%}$[a]	UL[b]	Max value[c]	Min value[c]	No. higher than UL[b]	Mean
TPM	mg/stick	54.7 ± 3.2	57.9	57.8	46.8	0	44.7
Water	mg/stick	32.1 ± 6.5	38.6	40.9	25.6	6	15.8
Nicotine	mg/stick	1.38 ± 0.20	1.58	1.64	0.62	1	1.88
NFDPM	mg/stick	21.2 ± 8.5	29.7	25.5	10.6	0	26.8
Carbon monoxide	mg/stick	0.446 ± 0.246	0.692	0.567	<0.223	0	30.6
Benzo [*a*]pyrene	ng/stick	1.02 ± 0.69	1.71	4.46	<0.35	7	15.0
Glycerin	mg/stick	4.63 ± 1.01	5.64	5.69	3.72	1	2.28
1-Aminonaphthalene	ng/stick	0.069 ± 0.077	0.146	0.091	<0.027	0	21.2
2-Aminonaphthalene	ng/stick	0.045 ± 0.060	0.105	0.056	<0.012	0	16.2
3-Aminobiphenyl	ng/stick	0.012 ± 0.012	0.024	0.014	<0.004	0	4.09
4-Aminobiphenyl	ng/stick	0.012 ± 0.012	0.024	0.028	<0.005	2	2.77
Acetaldehyde	µg/stick	211 ± 60	271	267	181	0	1694
Acetone	µg/stick	35.0 ± 11.3	46.3	41.9	28.7	0	685
Acrolein	µg/stick	10.96 ± 5.16	16.12	14.17	5.83	0	161
Butyraldehyde	µg/stick	24.0 ± 8.1	32.1	25.6	15.3	0	83.5
Crotonaldehyde	µg/stick	<3.29	13.16	<3.29	<3.29	0	51.7
Formaldehyde	µg/stick	10.16 ± 10.08	20.24	13.42	4.58	0	88.9
Methyl ethyl ketone	µg/stick	7.95 ± 6.65	14.60	10.15	6.42	0	183
Propionaldehyde	µg/stick	14.6 ± 10.5	25.1	15.2	12.3	0	122
Acrylonitrile	µg/stick	0.177 ± 0.173	0.350	0.335	<0.107	0	24.0
1,3-Butadiene	µg/stick	0.272 ± 0.101	0.373	0.347	<0.095	0	97.0
Benzene	µg/stick	0.700 ± 0.540	1.240	1.010	0.442	0	81.1
Isoprene	µg/stick	2.14 ± 0.44	2.58	4.34	1.01	13	885
Pyridine	µg/stick	8.27 ± 3.06	11.33	11.18	5.53	0	31.5
Quinoline	µg/stick	<0.011	0.044	0.011	<0.011	0	0.440
Styrene	µg/stick	1.067 ± 2.528	3.595	1.128	0.468	0	16.5
Toluene	µg/stick	2.49 ± 1.69	4.18	3.05	1.77	0	137
Catechol	µg/stick	13.2 ± 5.6	18.8	16.3	10.6	0	89.6
o-Cresol	µg/stick	0.052 ± 0.036	0.088	0.113	0.041	2	4.11
m-Cresol	µg/stick	0.031 ± 0.036	0.067	0.116	<0.019	2	3.61
p-Cresol	µg/stick	0.068 ± 0.097	0.165	0.122	<0.034	0	8.86
Hydroquinone	µg/stick	6.23 ± 2.46	8.69	9.39	4.77	2	88.3
Phenol	µg/stick	1.12 ± 0.52	1.64	1.59	0.72	0	13.90
Resorcinol	µg/stick	<0.055	0.220	0.080	<0.055	0	1.75
NAB	ng/stick	3.01 ± 1.13	4.14	8.89	0.77	13	30.3
NAT	ng/stick	17.5 ± 9.3	26.8	63.9	4.9	13	269
NNK	ng/stick	7.1 ± 2.8	9.9	29.3	2.0	24	261
NNN	ng/stick	14.2 ± 5.9	20.1	57.1	3.0	16	284
Ammonia	µg/stick	12.0 ± 5.2	17.2	97.2	5.3	29	31.2
Hydrogen cyanide	µg/stick	<4.37	17.48	10.07	<4.37	0	364
Nitric oxide	µg/stick	13.0 ± 2.4	15.4	51.4	3.7	24	510
Nitrogen oxides	µg/stick	13.8 ± 2.4	16.2	51.4	4.2	24	571
Arsenic	ng/stick	<1.20	4.80	1.43	<1.20	0	7.99
Cadmium	ng/stick	<0.280	1.120	<0.280	<0.280	0	94
Chromium	ng/stick	<11	44	<11	<11	0	<11
Lead	ng/stick	<1.62	6.48	3.80	<1.62	0	31.9
Mercury	ng/stick	1.25 ± 0.48	1.73	1.60	<0.70	0	4.67
Nickel	ng/stick	<53	212	<53	<53	0	<53
Selenium	ng/stick	<0.83	3.32	<0.83	<0.83	0	1.49
Pyrene	ng/stick	8.01 ± 4.80	12.81	74.09	1.97	9	79.3
o-Toluidine	ng/stick	1.616 ± 0.883	2.499	3.094	0.542	3	103.9
Acetamide	µg/stick	3.31 ± 1.69	5.00	6.28	2.24	5	13.0
Acrylamide	µg/stick	1.85 ± 1.33	3.18	3.56	0.78	2	4.5
Ethylene oxide	µg/stick	0.199 ± 0.141	0.340	0.324	<0.119	0	24.10
Nitrobenzene	ng/stick	<37.84	151.36	<37.84	<37.84	0	<37.84
Propylene oxide	µg/stick	0.078 ± 0.021	0.099	0.109	0.065	5	1.11
Vinyl chloride	ng/stick	<2.19	8.76	3.92	<2.19	0	100.8
Benz [*a*]anthracene	ng/stick	2.64 ± 2.46	5.10	20.52	0.36	8	27.2
Dibenz [*a*,*h*]anthracene	ng/stick	<0.413	1.652	<0.413	<0.413	0	0.79

* TPM: total particulate matter; NFDPM: nicotine-free dry particulate matter; NAB: *N*-nitrosoanabasine; NAT: *N*-nitrosoanatabine; NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NNN: *N*-nitrosonornicotine.
[a] Mean: mean of six determinations, each performed in triplicate (6 × 3); CI: confidence interval, determined from one set of triplicate analyses.
[b] UL: upper limit of the confidence interval ($CI_{99\%}$) or four times the limit of quantitation.
[c] <: the average yield was lower than the limit of quantitation, the value given is the limit of quantitation.

from less than 4.2–51.4 µg/stick (Table 4). As expected, a correlation (r = 0.849) was found between the presence of nitrogen oxides in the aerosol and the concentration of nitrates in the different experimental tobacco plug blends (Fig. 3). Blend BL33 which resulted in a yield of 51.4 µg/stick nitrogen oxides in the mainstream aerosol, one order of magnitude lower than 571 µg nitrogen oxides found in the mainstream smoke from the reference cigarette 3R4F (Table 4), deviated considerably from the regression line for all experimental tobacco plug blends. Removal of blend BL33 from the data set improved the correlation (r = 0.911). Such a

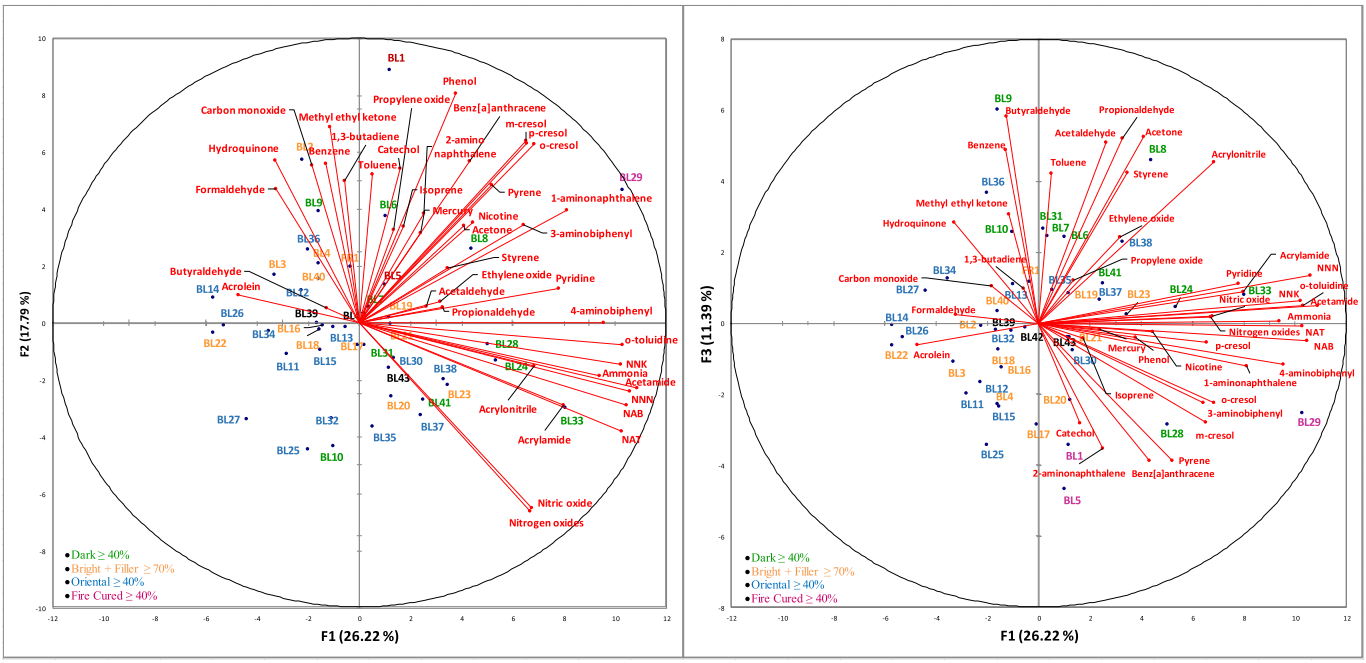

Fig. 2. Principal component analysis scores and loadings for the 41 aerosol constituents (acetaldehyde, acetamide, acetone, acrolein, acrylamide, acrylonitrile, 3-aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, ammonia, benz [*a*]anthracene, benzene, 1,3-butadiene, butyraldehyde, carbon monoxide, catechol, *m*-cresol, *o*-cresol, *p*-cresol, ethylene oxide, formaldehyde, hydroquinone, isoprene, methyl ethyl ketone, mercury, nicotine, nitric oxide, nitrogen oxides, NAB, NAT, NNK, NNN, phenol, propionaldehyde, propylene oxide, pyrene, pyridine, styrene, toluene, and *o*-toluidine) produced by the 43 test blends, plus the FR1 blend. The first three principal components are shown. Loadings are shown in red, scores for blends containing at least 40% air-cured tobacco are shown in green, scores for blends containing at least 70% bright and filler tobaccos are shown in orange, scores for blends containing at least 40% Oriental tobacco are shown in blue, and scores for blends containing at least 40% fire-cured tobacco are shown in purple.

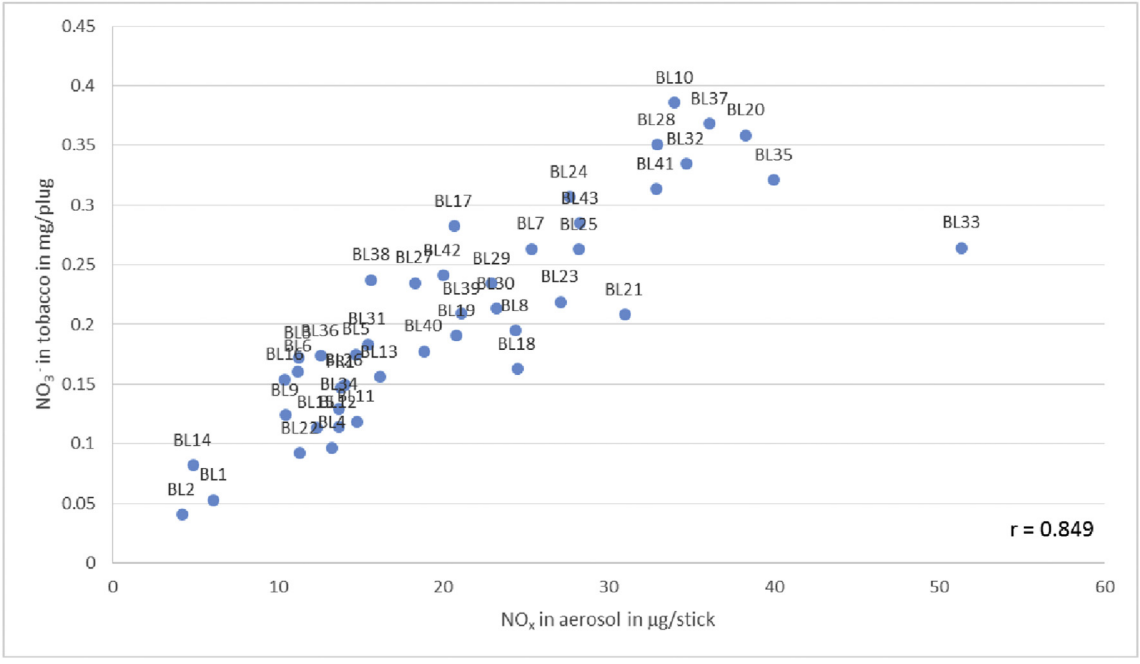

Fig. 3. Correlation between the nitrogen oxides yield in the aerosol produced and the nitrate concentration in the tobacco plug.

strong correlation should be expected since the presence of nitrogen oxides in the mainstream aerosol results primarily from decomposition of inorganic nitrates present in tobacco during heating at temperatures <350 °C, rather than from oxidative decomposition (i.e., combustion) of nitrogen-containing organic material in the tobacco plug blend at higher temperatures (Im et al., 2003; Cozzani et al., 2016). Blend BL33 produced 0.433 mg/stick CO in the mainstream aerosol of THS2.2, lower than the yield of CO produced by monitor blend FR1 (0.446 ± 0.246 mg/stick), while all other experimental tobacco plug blends produced CO concentrations in the mainstream aerosols below the UL for monitor blend FR1 (0.692 mg/stick). CO is not an unambiguous chemical marker

for a combustion process and can be generated from non-combustion processes, e.g., lower temperature thermal process such as torrefaction of biomass (Baker, 1975a; Senneca et al., 2007; Basu, 2010). Therefore, low levels of CO can be observed without tobacco combustion and combustion can only be inferred to have occurred when a significant increase in the yield of CO is observed. Further confirmation of the absence of combustion phenomena in the blend FR1 was obtained by collecting the THS2.2 aerosol in absence of oxygen. Under these inert conditions, nitrogen oxides and CO yields were similar as those obtained when collecting the aerosol in air (Cozzani et al., 2016). Therefore, no sign of combustion could be detected and the low concentrations of CO and nitrogen oxides in the THS2.2 aerosol confirm that the tobacco plugs of the analyzed blends were heated rather than burnt in the THS2.2.

4.2. Nitrogen-containing HPHCs

Ammonia was the only nitrogen-containing HPHC detected in the mainstream THS2.2 aerosols produced by 29 of 43 experimental tobacco plug blends which exceeded the UL for monitor blend FR1. Six experimental tobacco plug blends (BL23, BL24, BL28, BL29, BL33, and BL38) produced more ammonia than was found in 3R4F CC smoke (Table 4). Ammonia could be produced by either the distillation of endogenous ammonia already present in the tobacco plug or by the decomposition of organic amines and amides present in the tobacco plug blend to yield ammonia in the mainstream aerosol. Ammonia can be formed through the pyrolysis of amino acids in tobacco at temperatures as low as 200 °C (Moldoveanu, 2010), so the pyrosynthesis of ammonia from amino acids, peptides, and proteins may also contribute to the generation of ammonia into the THS2.2 aerosols. The conditions used when producing cast leaf and the conditions during the heating process when a THS2.2 is used are appropriate to initiate reactions between amines or ammonia and sugars (Maillard reaction (Ames, 1990)). Blending tobaccos that are rich in ammonia or amino acids with tobaccos with high sugar contents could therefore modify the availability of ammonia to be distilled or pyrosynthesized. As a result, ammonia produced by using single tobacco grades in the tobacco plug of THS2.2 could not be used to calculate the yield of ammonia generated in the mainstream aerosol of a tobacco blend containing the corresponding single grade tobaccos. The single grade air-cured tobacco AC3, fire-cured tobacco AR1, and Oriental tobacco AR5 gave higher ammonia yields than did the other single grade tobaccos (supplementary material Table II). Pure bright tobacco blends (e.g., BL2, BL3 and BL5) produced aerosols with a low ammonia content, while limiting the level of nitrogen-rich air-cured and aromatic tobaccos in the tobacco plug blend resulted in the lowest ammonia levels.

Several blends gave TSNA yields higher than the yield given by the FR1 blend but significantly lower than the 3R4F CC yield (Table 4). Both NNK and NNN have been classed as Group 1 carcinogens (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2015). Green tobacco leaves do not contain NNK and NNN, and these chemicals are formed during the curing and storage of tobacco (Fischer et al., 1990). The individual TSNA concentrations in the tobacco blends and yields in the mainstream THS2.2 aerosols correlated well, the correlation coefficients (r) for NNN, NNK and NAT being 0.885, 0.930, and 0.833, respectively, as is shown in Fig. 4. The NAB concentrations in the tobacco blends and yields in the aerosols correlated less well ($r = 0.763$) because of the limitations of the analytical method used and because the NAB concentrations in the tobacco blends and yields in the aerosols were lower than the concentrations and yields of the other TSNAs. Thus, selecting tobaccos with low concentrations of TSNAs should reduce exposure to these HPHCs when used in the THS2.2.

A small number of tobacco blends produced aerosols with higher acetamide and acrylamide yields than the upper limits of the FR1 blend yields. The BL23 and BL33 acetamide and acrylamide yields were both higher than the upper limits of the FR1 blend yields, and the BL24, BL29, and BL38 acetamide yields were higher than the upper limit of the FR1 blend yield. None of the blends produced aerosols with higher acetamide and acrylamide yields than the 3R4F CC. Acetamide is classed as a Group 2B carcinogen (possibly carcinogenic to humans) by the IARC (International Agency for Research on Cancer, 2015) and can be formed through the pyrolysis of Amadori products and the decomposition of ammonium acetate at around 250 °C (Moldoveanu, 2010). Acrylamide is classed as a Group 2A carcinogen (probably carcinogenic to humans) by the IARC (International Agency for Research on Cancer, 2015). Acrylamide can be formed from asparagine through a Maillard-type reaction at between 120 and 200 °C in food and other biological material, such as tobacco (Stadler et al., 2002; Blank et al., 2005; Becalski et al., 2011). Both acetamide and acrylamide could therefore be produced at the temperatures used in the THS2.2. The air-cured tobaccos (AC1 to AC5) and some of the aromatic tobaccos (the fire-cured AR1 and the Oriental tobacco AR5) were the main contributors of acetamide and acrylamide in the THS2.2 aerosols, and the bright blends gave lower acetamide and acrylamide yields. Adding together the acetamide and acrylamide yields given by each individual tobacco type in a blend in the proportions the tobaccos were used in the blend gave a good approximation of the acetamide and acrylamide yields in the aerosol produced by the blend (supplementary material Table II).

The yields of the other nitrogen-containing HPHCs in the aerosols produced by most of the blends were similar. Three blends (BL28, BL29, and BL38) gave higher *o*-toluidine yields than the upper limit of the FR1 blend yield, but the BL28, BL29, and BL38 *o*-toluidine yields were still more than 95% lower than the 3R4F CC *o*-toluidine yield. The BL33 and BL38 4-aminobiphenyl yields were also higher than the upper limit of the FR1 blend yield but were more than 99% lower than the 3R4F CC yield. None of the blends gave yields of the other nitrogen-containing compounds (acrylonitrile, 3-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, hydrogen cyanide, nitrobenzene, pyridine, and quinoline) higher than the upper limits of the FR1 blend yields.

4.3. Other HPHCs

Ten blends (BL1, BL2, BL5, BL6, B17, BL20, BL28, BL29, BL42 and BL43) gave higher PAH yields than the FR1 blend yield (Table 4). Good approximations of the benz [*a*]anthracene, benzo [*a*]pyrene, and pyrene yields given by the different blends were obtained by adding together the yields of the single tobacco types in the proportions they were used in the blends (supplementary material Table II). The blending process therefore had a minimal influence on the benz [*a*]anthracene, benzo [*a*]pyrene, and pyrene yields. None of the single tobaccos or blends gave higher PAH yields than did the 3R4F CCs, but some blends (e.g., BL1, BL5, BL28, and BL29) gave higher PAH yields than did other blends (supplementary material Table I). Benzo [*a*]pyrene is classed as a Group 1 carcinogen, benz [*a*]anthracene as a Group 2B carcinogen, and pyrene as a Group 3 carcinogen by the IARC (International Agency for Research on Cancer, 2015). PAHs are formed through the incomplete combustion of organic materials, such as tobacco (Rodgman, 2001; Baker, 2006; McAdam et al., 2013). PAHs do not usually occur naturally in plant materials, and their presence is caused by contamination or decomposition during pyrolytic processes. For tobacco in particular, PAHs can be introduced into tobacco leaves during curing if the tobacco is exposed to the exhaust gases from

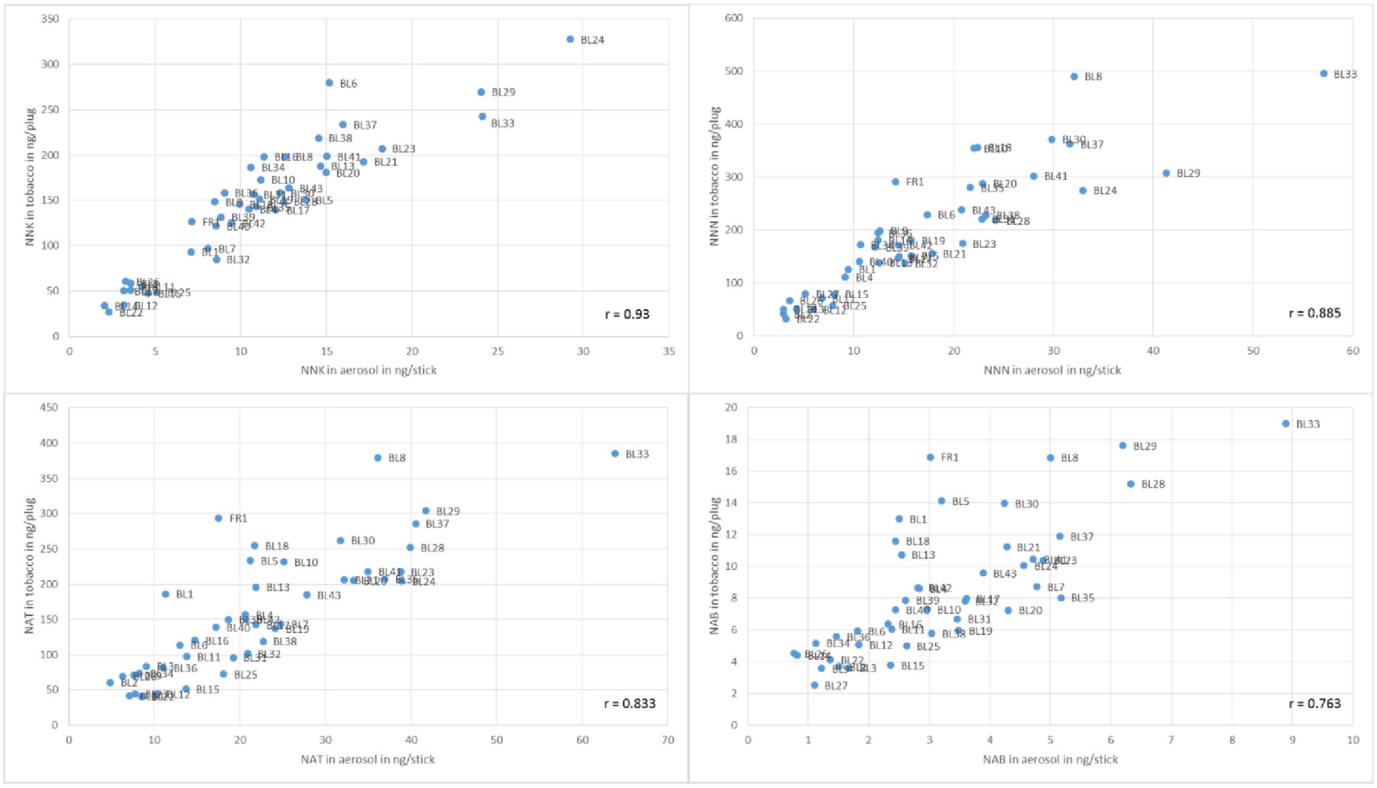

Fig. 4. Correlations between the *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), and *N*-nitrosonornicotine (NNN) concentrations in the tobacco plug blends and yields in the THS2.2 mainstream aerosols produced using the blends.

wood, or other organic fuel, heat sources (Bentley and Burgan, 1960). This type of contamination during curing can be minimized by using curing barns equipped with heat exchangers that prevent flue gases from coming into contact with the tobacco. Fire-curing, in which the tobacco leaves are in direct contact with wood smoke, gives cured tobaccos that contain particularly high PAH concentrations (McAdam et al., 2013). PAHs may form when tobacco pyrolysis occurs at between 550 and 600 °C, whereas PAHs have not been found to form below 350 °C when heating tobacco (McGrath et al., 2007). The pyrosynthetic formation of PAHs should therefore not occur at the temperatures the THS2.2 operates (Smith et al., submitted (this issue)). As suspected, tobacco blends containing large proportions of fire-cured tobaccos (e.g., BL1, BL5, BL28, and BL29) gave the highest PAH yields (supplementary material Table I). The bright tobacco F5 and the flue-cured stems FL1 gave a higher PAH yield than did the other tobacco materials (supplementary material Table II). Consequently, PAH yields in the THS2.2 aerosol could be minimized by avoiding the use of fire-cured or bright tobaccos which have been cured in tobacco barns not fitted with a heat-exchange system.

Only minor differences were found in the phenol yields given by the different tobacco blends. Two blends (BL1 and BL29) gave higher *m*-cresol and *o*-cresol yields than the upper limits of the FR1 blend yields (Table 4). These two experimental tobacco plug blends contained large proportions of fire-cured tobacco (40%). High concentrations of phenols in fire-cured tobaccos may be caused by the transfer of these phenols from wood smoke to tobacco during the curing process (Naghski et al., 1944). Two blends (BL9 and BL36) gave higher hydroquinone yields than the upper limit of the FR1 blend yield, but the yields were about 90% lower than the 3R4F CC yield.

Thirteen experimental tobacco plug blends (BL1, BL4, BL6, BL7, BL8, BL12, BL13, BL14, BL21, BL23, BL24, and BL33and BL40) gave higher isoprene yields than the upper limit of the FR1 blend yield (Table 4). The confidence interval for determination of isoprene was proportionally smaller than for most of the other analytes, but the reductions of isoprene in the THS2.2 aerosols using different experimental tobacco plug blends were still larger than 99% when compared to the mainstream smoke of reference cigarette 3R4F. Similar results were found for propylene oxide. A major source of propylene oxide in mainstream CC smoke is the dehydration of propylene glycol, which is used as a humectant or for flavor application (Diekmann et al., 2006; Laino et al., 2012). The *tobacco sticks* we used were not flavored, and propylene glycol was not used as a humectant, so we expected to find low propylene oxide yields. The propylene oxide yields given by the single tobaccos and tobacco blends were one order of magnitude lower than the 3R4F CC propylene oxide yield (Table 4).

4.4. Effects of the blend used on the generation of HPHCs

In chemometrics, PCA is widely used to extract relevant information from spectroscopic or chromatographic datasets, and other types of dataset. This un-supervised process without prior class identification (Jackson, 1991) has been used to differentiate tobacco types and cigarette brands by analyzing profiles of gases produced by tobacco pyrolysis (Adam et al., 2005, 2007, 2009). Piadé et al (Piadé et al., 2013). analyzed the concentrations of HPHCs in mainstream smoke from market cigarettes using PCA and found that “Virginia brands are higher in formaldehyde, cadmium and, to a lesser extent, unsaturated aldehydes, hydrogen cyanide and aromatic compounds. In contrast, American blend brands are higher in nitrogen-containing compounds (except hydrogen cyanide).” In the current study, PCA of the concentrations of HPHCs in the aerosol of THS2.2 produced by different experimental tobacco plug blends with a high proportion of bright tobacco did not result in

significantly more aldehydes, aromatic compounds and hydrogen cyanide than the other tobacco plug blends. In a classical American blend cigarette the percentage of air-cured (burley) tobacco is usually about 20% ranging up to a maximum of 35%. In the experimental tobacco plug blends investigated in this study, increasing the percentage of air-cured tobaccos in the blend to 40% only had a small effect on the concentrations of nitrogen-containing HPHCs in the mainstream aerosols. With the exception of the blends containing up to 40% fire-cured tobacco, the PCA did not efficiently cluster the different blend types. For the majority of HPHCs, the blend compositions did not result in significant differences in HPHC yields, which were lower than found in the reference cigarette 3R4F.

For the few HPHCs presenting significant variability across blends, the mechanisms governing their transfer to the aerosol was mainly based on distillation processes (e.g., TSNAs and PAHs), simple thermal reactions (e.g., nitrogen oxides and acetamide) or early steps in the Maillard reaction (e.g., acrylamide). The Maillard reaction could modify the level of available ammonia and ammonia precursors in nitrogen-rich tobaccos (e.g., air-cured tobaccos); however, this reaction is not quantitative (Ledl and Schleicher, 1990; Cerny, 2008) and the aerosols with the lowest concentrations of ammonia were obtained when blending bright tobaccos with a low nitrogen content. Consequently, both the tobacco blend and the blending process had only a limited impact on the presence of HPHCs in mainstream aerosol of THS2.2.

5. Conclusions

The 43 experimental tobacco plug blends investigated in this study were generated from a large range of different tobacco types and included extreme blend compositions, compared to normal tobacco blending practices used for cigarettes. The aerosols produced by 43 different experimental tobacco plug blends in the THS2.2 contained significantly lower concentrations of HPHCs than found in the mainstream smoke of reference cigarette 3R4F. For most of the analyzed HPHCs, the tobacco blend composition had only a minimal impact on the yields of HPHCs in the resulting aerosols. Only ammonia, TSNAs, nitrogen oxides, PAHs, acrylamide and acetamide concentrations in the THS2.2 mainstream aerosols showed significant variability across the 43 experimental tobacco plug blends. Pyrosynthesis did not appear to play a role in the generation of PAHs and TSNAs in the mainstream aerosol of THS2.2 and these HPHCs most likely resulted from the distillation of endogenous preformed compounds already present in the tobacco blends. Tobacco plug blends containing high proportions of nitrogen-rich tobaccos such as air-cured tobaccos and some Oriental tobaccos produced higher yields of ammonia, nitrogen oxides, acrylamide and acetamide in the THS2.2 aerosol. Finally, the low concentrations of chemical markers of tobacco combustion such as CO and nitrogen oxides in the THS2.2 aerosol confirm that the tobacco plug was heated rather than burnt in the THS2.2.

Conflict of interest statement

The work reported in this publication involved a candidate Modified Risk Tobacco Product developed by Philip Morris International (PMI) and was solely funded by PMI. All authors are (or were) employees of PMI R&D or worked for PMI R&D under contractual agreements.

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Appendix A. Supplementary data

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.10.016

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