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Key Challenges and Recommendations for *In Vitro* Testing of Tobacco Products for Regulatory Applications: Consideration of Test Materials and Exposure Parameters

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Abstract

The Institute for In Vitro Sciences (IIVS) is sponsoring a series of workshops to identify, discuss and develop recommendations for optimal scientific and technical approaches for conducting *in vitro* assays, to assess potential toxicity within and across tobacco and various next generation nicotine and tobacco products (NGPs), including heated tobacco products (HTPs) and electronic nicotine delivery systems (ENDS). The third workshop (24–26 February 2020) summarised the key challenges and made recommendations concerning appropriate methods of test article generation and cell exposure from combustible cigarettes, HTPs and ENDS. Expert speakers provided their research, perspectives and recommendations for the three basic types of tobacco-related test articles: i) pad-collected material (PCM); ii) gas vapour phase (GVP); and iii) whole smoke/aerosol. These three types of samples can be tested individually, or the PCM and GVP can be combined. Whole smoke/aerosol can be bubbled through media or applied directly to cells at the air–liquid interface. Summaries of the speaker presentations and the recommendations developed by the workgroup are presented. Following discussion, the

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workshop concluded the following: that there needs to be greater standardisation in aerosol generation and collection processes; that methods for testing the NGPs need to be developed and/or optimised, since simply mirroring cigarette smoke testing approaches may be insufficient; that understanding and quantitating the applied dose is fundamental to the interpretation of data and conclusions from each study; and that whole smoke/aerosol approaches must be contextualised with regard to key information, including appropriate experimental controls, environmental conditioning, analytical monitoring, verification and performance criteria.

Keywords
Ames test, chromosome aberrations, cigarettes, e-cigarettes, electronic nicotine delivery systems, ENDS, gene mutation, genetic toxicology, micronucleus, mouse lymphoma assay, smokeless tobacco, tobacco product toxicity evaluation

Introduction

The evolving regulatory landscape for tobacco product assessment has shifted in light of new and emerging categories such as e-cigarettes and heated tobacco products. As such, there has been a drive to establish best practices for the assessment of these products, given the new regulatory oversight. Historically, tobacco products have not been covered by the same regulatory frameworks as other consumer products; however, the implementation of new regulatory oversight for tobacco products in a number of countries has created a need to develop appropriate approaches, particularly for toxicology evaluations. In 2005, Canadian Tobacco Reporting Regulations initiated a requirement to annually conduct the reverse bacterial mutation assay (Ames), the *in vitro* micronucleus assay (IVMN) and the Neutral Red Uptake cytotoxicity test (NRU) on cigarette emissions from mainstream tobacco smoke. Recent updates to these guidelines were posted in 2017 by Health Canada.[1–3] To comply with the *European Union Tobacco Product Directive*, tobacco companies have submitted genotoxicity test data, particularly as a part of the assessment for priority additives (*2014/40/EU*).[4,5] In 2009, with the passage of the *Family Smoking Prevention and Tobacco Control Act* (FSPTCA), an amendment to the *Federal Food, Drug and Cosmetic Act*,[6] the US Food and Drug Administration (FDA) gained regulatory authority over tobacco products. In 2016, FDA's regulatory authority was extended to include all tobacco products, including electronic nicotine delivery systems (ENDS), which were not previously covered by the FSPTCA. FDA tobacco product guidance documents recommend toxicological testing, including genetic toxicology evaluations. The FDA Center for Tobacco Products (CTP) website includes links to the latest versions of all their guidance documents (https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/guidance).

For a number of years prior to the promulgation of these regulatory requirements and frameworks, tobacco companies and other organisations have used genetic toxicology tests. Given that combusted cigarettes are and have been recognised as genotoxic (and carcinogenic), tobacco product stewardship has not focused on simple hazard identification. Rather, researchers have compared relative genotoxic response for different types of combusted cigarettes based on the types of tobacco, cigarette components, additives and designs.[7–12] Comparisons have also been made concerning the genotoxic potency of different categories of tobacco products — for example, combusted cigarettes *versus* heated tobacco products (HTPs, also termed heat-not-burn or tobacco heating systems).[13–15] More recently, new generation vapour products such as electronic nicotine delivery systems (ENDS), including e-cigarette devices/systems and e-liquids, have been evaluated to understand their potential genotoxicity. The genotoxicity of these next generation nicotine and tobacco products (NGPs) has also been compared to the genotoxicity of traditional combusted products.[16–19] While there is a need to evaluate the genotoxicity of single chemicals that are tobacco product constituents or additives, generally the test material used for tobacco product evaluation is a complex mixture. For all of these product evaluations, samples are prepared and cells are exposed *in vitro*. There are various types of samples that can be prepared and evaluated from aerosolised tobacco products, e.g. pad-collected material (PCM), gas vapour phase (GVP) and whole smoke/aerosols. Historically, many evaluations for combustible cigarettes were conducted using PCM, i.e. condensates or total particulate matter (TPM). More recently, it has been recognised that, for many tobacco products, optimal, and perhaps more clinically relevant, hazard assessment requires an understanding of the genotoxic potential of smoke/aerosols, rather than (or in addition to) the more commonly studied solid or liquid fractions. This adds complexity to the choice of appropriate test samples, the evaluation of exposure and the interpretation of the biological responses. Thus, the use of *in vitro* genetic toxicology tests to evaluate tobacco products involves numerous technical challenges that are not encountered when evaluating single chemicals.

As already indicated, tobacco companies and other organisations have conducted a substantial amount of *in vitro* (and some *in vivo*) genetic toxicology research. Literature

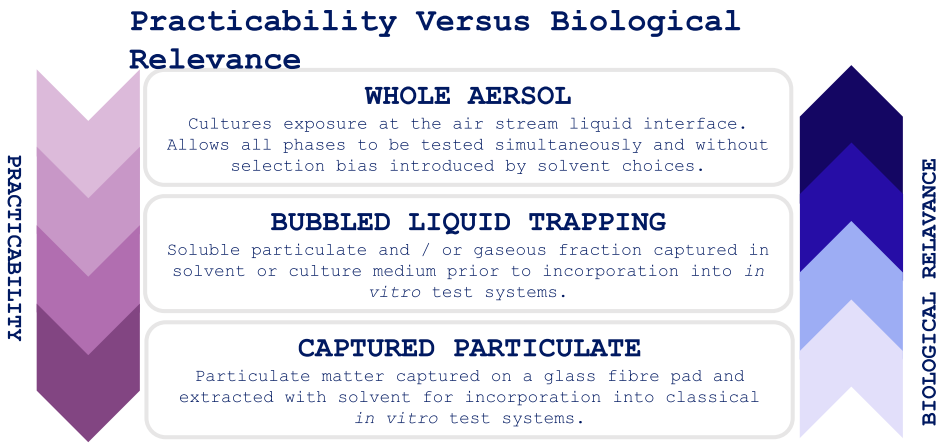

Figure 1. The relationship between practicability *versus* the biological relevance of utilising the various types of samples that can be generated from tobacco products. Modification of a figure from Thorne and colleagues,[27] with permission from the copyright holder, Elsevier.

summaries/reviews provide a survey of the many published studies.[20–25] While there is considerable technical information in the published literature, much of the research data and technical expertise required to evaluate tobacco products resides within the individual tobacco companies and/or within a very limited number of contract testing laboratories.

With the evolving regulatory requirements for tobacco products and NGPs, it is important for the various stakeholders to communicate and to share expertise. Recognising the need for such interactions, the Institute for In Vitro Sciences (IIVS) is sponsoring a series of workshops where best practices for *in vitro* hazard assessment can be discussed. Participants include scientists from tobacco companies, contract research organisations, US regulatory agencies, academia and other *in vitro* assay experts with tobacco product experience. To optimise the presentations and discussion during face-to-face meetings, subgroups meet virtually between workshops to hold detailed discussions, and to bring background information and recommendations to the whole workgroup.

A summary of the first two workshops (27–28 November 2018 and 4–5 June 2019) is published in Moore et al.[12] Prior to the first workshop, the invited experts identified issues that are important to the use of genetic toxicology (and other *in vitro*) assays for evaluating tobacco products. During the first workshop, these issues were discussed and triaged based on the amount of available information, the ease of developing recommendations for the particular issue and whether the workgroup wanted to tackle the issue as a part of the workshop series. A subgroup was established to conduct a literature-based summary of methods that have been used to generate ENDS aerosol samples, and this review has been published.[26] The second workshop focused on a preliminary discussion of sample types that can be generated for tobacco products, and initiated a subgroup to develop schematic graphics for these sample types for presentation and the development of consensus terminology, which were discussed during the third workshop held on 24–26 February 2020.

The third workshop is summarised in this current publication (see Table 1). Keynote presentations provided essential background information. Following the keynote presentations, experts provided summaries of their perspectives for critical factors, pitfalls and recommendations for sample types (PCM, GVP, and whole smoke/aerosol samples that can be bubbled through culture medium to expose cells or applied directly to cells). The recommendations provided by the individual speakers were discussed by the workgroup members. This discussion generated a series of workgroup-endorsed conclusions/recommendations which are provided below.

Keynote presentation summaries

Key challenges when testing tobacco products using the standard regulatory in vitro *genetic toxicology assays*

(*Presented by Julie Clements, Labcorp Early Development Laboratories Limited*) *In vitro* genetic toxicology assays have traditionally been used to test additives and tobacco product condensates in a routine way by following standard protocols, e.g. the Ames test[1,27] and the *in vitro* micronucleus assay.[2,28] The study design may need careful consideration and adaptation depending on the question being asked — i.e. does this product induce an effect (Yes/No), or is this product more or less toxic than other materials to

which it is being compared, and potentially by how much? Adaptations to both the assay design and data analysis methods are required to perform product comparisons with any degree of confidence.

The advent of a vast array of new vapour products (such as ENDS), combined with a desire for achieving the most relevant exposure of the test system, has prompted significant research in many laboratories. There are various approaches, ranging from testing the GVP and PCM either separately or in combination, bubbling whole smoke/aerosol into cell culture medium, or direct exposure of cells at the air–liquid interface (ALI) or air–agar interface (AAI) to whole smoke or aerosol. The direct exposure approach may be the gold standard, but comes with an array of technical and practical challenges, including the absence of standardised procedures. The schematic diagram in Figure 1 shows the association between practicability and biological relevance.

Aerosol generation itself is technically challenging, and exposure of cells at the ALI or AAI requires a multidisciplinary team who must address practical considerations, such as:

— generating and handling aerosol;
— exposure and recovery of cells;
— appropriate cell survival and dose range;
— dosimetry; and
— compliance with regulatory guidelines, e.g. Organisation for Economic Co-operation and Development (OECD), quality standards, etc.

Other considerations for aerosol testing are: i) having a sufficient number of cells exposed and recovered to adequately assess the endpoint in question; ii) the choice of cell type (cells in monolayers are more amenable to the testing conditions than cells grown in suspension culture); and iii) determining the doses, so that an appropriate toxicity range is covered. A number of parameters also need to be defined, such as what determines the top dose (in the absence of toxicity) and whether the standard exposure times are acceptable? Additionally, there are a multitude of dosimetry measures to choose from — for example, µg/ml, puff number, cigarette number, nicotine quantification, quantification of Hoffmann analytes, dilution factor, quartz crystal microbalance (QCM) weight, photometer and soft photoionisation mass spectrometry. Furthermore, from a regulatory compliance perspective, thought should be given as to how the test material should be characterised, as well as what considerations are needed with regard to validation of all the software used for conducting the experiment, including the data capture.

Thus, there are many challenges to be considered throughout this series of workshops, but the plan for this particular workshop was to focus on the various test materials and exposure methods that can be used for *in vitro* testing. Broadly speaking, the topics covered fall into the following areas: sample type and relative merits; optimal sample preparation; and sample characterisation. In terms of sample type, PCM, combined samples (e.g. PCM and GVP), bubbling methods and conditioned medium and aerosol exposures were all considered with a view to defining optimal exposure methodologies, dosimetry, and availability and use of reference products.

Importance of, and approaches to, assessing dosimetry when exposing cells to both traditional and aerosol samples

(*Presented by Gene Gillman, Enthalpy Analytical*) Analytical aerosol dosimetry is key to understanding the biological response due to cell exposure to chemical constituents in the *in vitro* studies. The challenges of aerosol dosimetry not only depend on the aerosol being studied but also on how the aerosol is delivered to the cell surface. Aerosol constituents can be delivered in a variety of forms, from condensates trapped in media to whole aerosol delivered at the ALI/AAI.

Because of the diversity and complexity of aerosol delivery systems, investigators must characterise their individual systems for the aerosol constituents delivered to the cell surface, in order to set dosimetry standards by which aerosol from test materials may be compared/measured when using the same equipment. Trapping of aerosol in liquid impingers has been widely used to prepare condensate samples from cigarette smoke and e-cigarette aerosols. Condensate collection methods vary significantly and often are not fully characterised for trapping efficiency or compound stability in the media. Volatile organic compounds are known to be poorly trapped in aqueous media, while reactive compounds (such as free radicals or epoxides) can react with the trapping media. Inefficient trapping or lack of stability in the trapping media will lead to condensates that do not accurately represent the composition of the parent aerosol.

For ALI systems, dosimetry endpoints can range from determining the mass of aerosol delivered to the cell surface, to determining the cellular surface dosing rate over the time course of the study. The ALI exposure system's characterisation should also include the specific cells used and any measured endpoints. Most of the cells used in ALI exposure systems are maintained at 37°C during exposure and the generated aerosol is diluted with humidified air. Heating and dilution of the aerosol with humidified air may change the phase (particle to gas phase) of semi-volatile chemical constituents of an aerosol, complicating accurate cellular surface dose determination.

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Results of a literature search: Summary and recommendations for methods used to generate whole aerosol samples

(*Presented by Daniel Smart, PMI, and Gary Phillips, Life Science Technologies Ltd*) For the testing of ENDS-derived aerosol in submerged cell culture-based *in vitro* assays, the aerosol first must be collected. While, to date, there is no standardised collection method for this purpose, the growing number of publications in this area indicates that such *in vitro* research is being conducted. In order to map the types of methods hitherto utilised by the scientific community, a literature search of the MEDLINE health and medical sciences bibliographic database was performed by using PubMed, according to the following search terms: ((“electronic cigarette”[All Fields] OR “electronic cigarettes”[All Fields]) OR “e-cigarette”[All Fields]) OR (((“electronic nicotine delivery systems”[MeSH Terms] OR (((“electronic”[All Fields] AND “nicotine”[All Fields]) AND “delivery”[All Fields]) AND “systems”[All Fields])) OR “electronic nicotine delivery systems”[All Fields]) OR “e cigarettes”[All Fields]).

The search retrieved 47 relevant publications, among which seven were distinct aerosol sample collection methods for ENDS products. The most frequently cited methods were bubbled liquid trapping in impingers (57%) and collection of particulate matter on a Cambridge filter pad (CFP), followed by extraction with polar solvents (18%). The five other methods (aerosol collected material (ACM) plus bubbled liquid trapping; condensation; cotton filters; settle-upon; settle-upon plus dry) were cited less often (2–10%). Further insights from this review indicated

Table 2. Terminology, synonyms and abbreviations.

Terminology[a]	Synonym(s)[b]	Abbreviation[a]	Description[c]
Aerosol	N/A	N/A	Umbrella-term for cigarette smoke, HTP and ENDS aerosols.
Aerosol collected material	eTPM (ENDS TPM)	ACM	Refers to the particulate material captured on a filter pad specific to HTP and ENDS and is traditionally eluted from the pad using solvent extraction.
Air–agar interface	N/A	AAI	Refers to the method by which cells are maintained and exposed to whole smoke/aerosol. Specifically, the cells are maintained on an agar bed and exposed to freshly generated aerosols.
Air–liquid interface	N/A	ALI	Refers to the method by which cells are maintained and exposed to whole smoke/aerosol. The cells can be maintained on various surfaces, and their apical side exposed directly to freshly generated aerosols.
Bubbled liquid trapping	AqE (aqueous extracts), conditioned media, aqueous trapping, aqueous bubbling, bubble through, whole smoke conditioned media (WSCM), whole aerosol conditioned media (WACM)	N/A	Umbrella-term for the technique used to trap aerosol, irrespective of inhalable product type. Aerosol (usually the soluble fraction) is captured by using a glass impinger. The aerosol is normally directly bubbled through the impinger. Capture efficiency will differ between products and techniques used (impinger design, fritted, glass beads, cold trap, solvent extraction, etc.).
Collected aerosol fractions	N/A	N/A	Umbrella-term for all methods used to collect, sample and fractionate cigarette, HTP and ENDS aerosols.
Electronic nicotine delivery system	Electronic cigarette, e-cig, e-cigarette	ENDS	An electronic device that heats and aerosolises a liquid consisting of a mixture of propylene glycol, vegetable glycerol, nicotine and potentially various flavours.
Electronic nicotine delivery system bubbled liquid trapping	Bubbled liquid trapping	N/A	Refers specifically to the trapping of ENDS aerosol via an impinger approach.
Gas vapour phase	Vapour phase	GVP	Umbrella-term for the portion of the aerosol that does not contain any particulates, consisting of volatile and semi-volatile compounds. The particulate is often removed from the aerosol via filter pad extraction. This fraction differs in chemical composition for the three product categories, cigarette smoke, HTP and ENDS. A filter can be used to extract the particulates as part of the TPM generation process or before bubbled liquid trapping.
Heated tobacco product	Heat not burn (HnB), tobacco heating product (THP), tobacco heating system (THS)	HTP	A tobacco product that heats the tobacco rather than using traditional combustion process.
Heating tobacco product bubbled liquid trapping	Bubbled liquid trapping	N/A	Refers specifically to the trapping of HTP aerosol via an impinger approach.

(continued)

Table 2. (continued)

Terminology[a]	Synonym(s)[b]	Abbreviation[a]	Description[c]
Heating tobacco product total particulate matter	N/A	HTP-TPM	Refers to the particulate material captured on a filter pad specific to HTP and is traditionally eluted from the pad by using solvent extraction.
Next generation nicotine and tobacco products	N/A	NGPs	Umbrella-term for HTP, ENDS categories and other oral products such as tobacco-free nicotine products.
Pad-collected material	TPM, ACM, HTP-TPM	PCM	Umbrella-term for particulate material captured on a filter pad irrespective of product category (cigarette, HTP and ENDS).
Total particulate matter	Particulate matter (PM), particulate collected material (PCM), total particulate material, smoke condensates	TPM	Refers to the particulate material captured on a filter pad specifically from a cigarette and is traditionally eluted from the pad by using solvent extraction.
Whole aerosol	N/A	WA	Umbrella-term for freshly machine generated aerosols from HTP (whole HTP aerosol) and ENDS (whole ENDS aerosol).
Whole ENDS aerosol	N/A	N/A	Freshly machine generated ENDS aerosol which consists of droplets suspended in a gas cloud.
Whole smoke	Cigarette smoke, mainstream cigarette smoke, whole cigarette smoke	WS	Freshly machine generated mainstream cigarette smoke consisting of both a particulate and vapour phase suspended as an aerosol.
Whole HTP aerosol	N/A	N/A	Freshly machine generated HTP aerosol consisting of both a particulate and vapour phase suspended as an aerosol.

[a]Terminology and abbreviation used in this manuscript.
[b]Not all synonyms are used in this manuscript, but they are used within the wider research environment.
[c]Description based on workshop outcomes and common usage.

that the collected aerosol fractions were only minimally characterised chemically and, in addition, there was large heterogeneity in other experimental parameters (e.g. vaping regimen). More comprehensive research on the composition of these collected aerosol fractions is necessary to facilitate the identification of the method(s) that produces the fraction(s) most representative of the native aerosol. Greater standardisation of the aerosol generation process should also be considered. These are potential opportunities for increasing the value of *in vitro* assessments in relation to ENDS-derived aerosols. Complete details for this review can be found in the Smart and Phillips publication from 2021.[26]

Schematics and terminology for tobacco product samples

The subgroup charged with developing draft graphics and recommended terminology for clearly describing the types of samples that can be generated from tobacco products and used to expose cells for *in vitro* toxicological evaluations, including genetic toxicology, presented their suggestions to the full workgroup. Based on subsequent discussions, several figures and terminology were agreed upon. Table 2 provides a summary of the terminology that the workgroup will be using, as well as previously used synonyms and abbreviations.

Three common types of test samples that can be generated from combusted cigarettes, HTPs and ENDS, are shown in Figure 2. When cigarettes are combusted or HTPs/ENDS activated, either whole smoke or aerosol is produced. This whole smoke or aerosol is passed through a CFP and the material collected on the pad (PCM) can be extracted for testing. The material that passes through and is not collected on the CFP, is the GVP. Common terminology was developed by the workgroup for all of these various sample types:

— For combusted cigarettes, these test samples are i) whole smoke, ii) TPM and iii) GVP.
— For HTPs, the test samples are i) whole HTP aerosol, ii) HTP total particulate matter (HTP-TPM) and aerosol collected material (ACM) and iii) GVP.

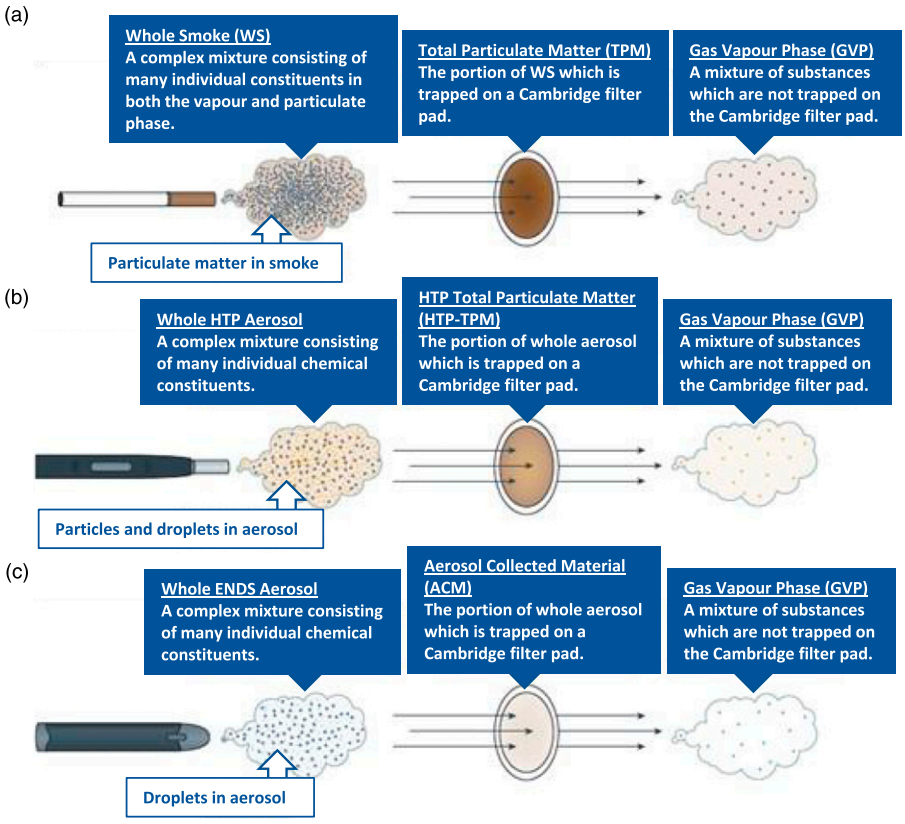

Figure 2. Shown are: a) common cigarette smoke fractions; b) common HTP aerosol fractions; and c) common ENDS aerosol fractions. A schematic representation of the various fractions of cigarette smoke, HTP aerosol and ENDS aerosol, and their respective generation. Irrespective of product type, the whole smoke or aerosol can be fractionated into the particulate phase and the GVP via the selective filtration of the particulate material by using a filter pad. The chemicals and ratios of particulate matter to vapour phase will differ depending on the category of product. Products and aerosols are representative of the category. For ENDS, a variety of open and closed-type systems exist; a closed modular-type system is depicted here. Products are not to scale.

— For ENDS products, the samples are i) whole ENDS aerosol, ii) aerosol collected material (ACM) and iii) GVP.

To differentiate between HTP and ENDS collected mass (which can be both referred to as ACM), here we have used HTP-TPM for heated tobacco products and ACM for ENDS products.

An alternative to passing whole smoke or aerosol through a CFP is a method to trap chemicals within a liquid by using an impinger (as shown in Figure 3). In this sample generation method, the whole smoke or aerosol is bubbled into phosphate buffered saline (PBS), culture medium or other solvent. Depending upon the tobacco product, the workgroup defined this method as cigarette smoke bubbled liquid trapping, HTP aerosol bubbled liquid trapping or ENDS aerosol bubbled liquid trapping.

Samples may also be generated by fractionating the whole smoke/aerosol. This is accomplished by first passing the whole smoke or aerosol through a CFP, and then trapping the resulting vapour within a liquid (PBS, culture medium or other solvent) by using an impinger (Figure 4). Depending upon the tobacco product, the workshop defined this method as cigarette smoke TPM and GVP bubbled liquid trapping, HTP aerosol HTP-TPM and GVP bubbled liquid trapping, or ENDS aerosol ACM and GVP bubbled liquid trapping. This method generates a fractionated sample — that is, the whole smoke aerosol is separated into particulates and vapour that can both be captured.

Figure 5 provides a schematic overview of the various types of samples that can be generated from combustible cigarettes, HTPs and ENDS, and the types of cell exposures that can be used. Exposure to whole smoke/aerosol can be accomplished by exposing cells immediately and directly to the sample generated from the tobacco product(s). Samples can also be prepared from whole smoke or aerosol by capturing particles and vapour substances in an impinger that is filled with PBS, cell medium or other solvent. If culture medium is used, the cells can be contained and exposed in the impinger. The prepared liquid sample can also be stored for future cellular exposure. Whole smoke/

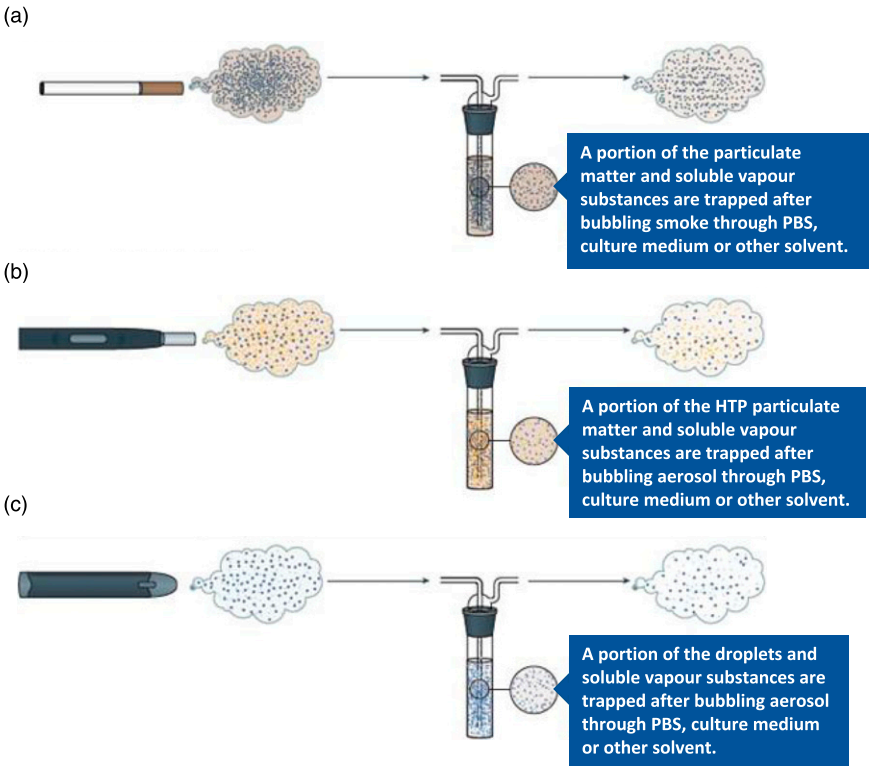

Figure 3. Shown are: a) cigarette smoke bubbled liquid trapping; b) HTP aerosol bubbled liquid trapping; and c) ENDS aerosol bubbled liquid trapping. A schematic representation of the bubbled liquid trapping method for the capture of aqueous aerosol fractions. The chemicals and ratios of particulate matter to vapour phase will differ depending on the category of product. Products and aerosols are representative of the category. For ENDS, a variety of open and closed-type systems exist; a closed modular-type system is depicted here. Products are not to scale. PBS = phosphate-buffered saline.

aerosol fractionation (shown on the right side of Figure 5), yields PCM and GVP which can either be evaluated separately or combined. For the NRU assay, Health Canada recommends a combination of TPM and GVP exposure for combusted cigarettes (Health Canada HC test method T-502, Appendix).[3] This combined particulate + gaseous phase exposure method has also been used with other tobacco products and in genetic toxicology assays.

Perspectives on critical factors, pitfalls and recommendations for test sample types

Several experts presented their research and their perspectives concerning the general test sample types, namely: i) fractionated samples (PCM, GVP or the combination thereof); ii) bubbled liquid trapping of whole smoke/aerosol/vapour samples; and iii) direct or 'fresh' exposure of whole smoke/aerosol/vapour samples.

Fractionated tobacco product test samples derived from smoke or aerosol have been used for a number of years. Although the majority of studies have been conducted with PCM from combusted cigarettes, these general methods utilised for combusted cigarettes have been implemented for aerosol sample generations from NGPs. However, as noted in the abstracts below, there are differences. The methods used for generating test samples from the ENDS and HTP products do involve some adaptations, as compared to combustible product samples. Because the evaluation of tobacco products by using smoke and aerosol test samples is still an emerging research field, there are important lessons to be learned from *in vitro* methods previously developed for assessing single chemical aerosols both within and beyond genetic toxicology. Thus, speakers with broader aerosol testing expertise were included in the workshop presentations for this sample type. The following sections feature the summaries of these presentations.

Pad-collected material (TPM/HTP-TPM/ACM)

Critical factors, pitfalls and recommendations for using pad-collected material to evaluate cigarettes, HTPs and ENDS

(*Presented by Mark Ballantyne, Labcorp Early Development Laboratories Limited*) It is known and accepted that

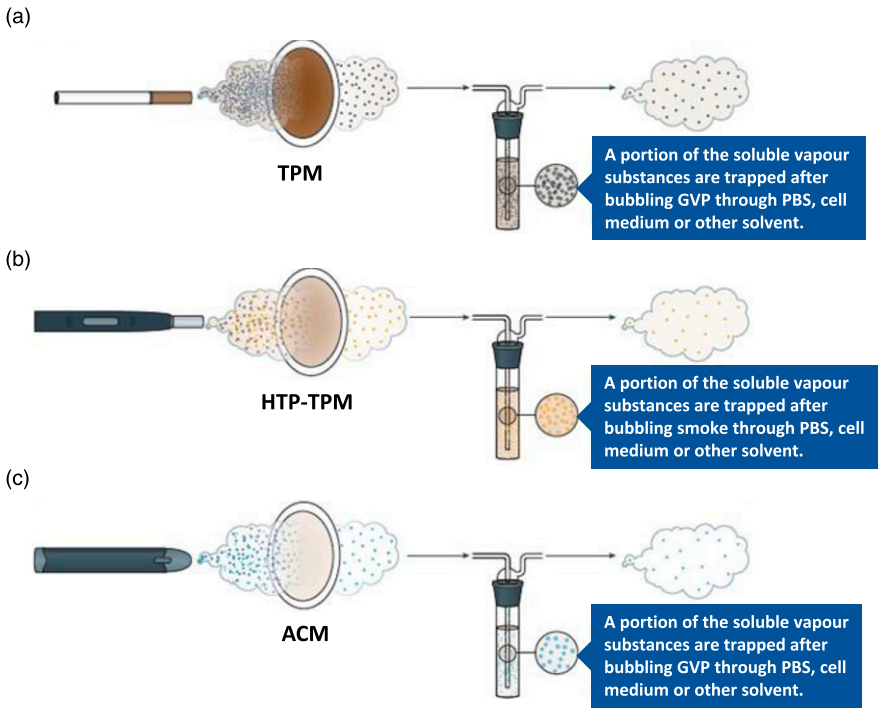

Figure 4. Shown are: a) cigarette smoke TPM and GVP bubbled liquid trapping; b) HTP aerosol HTP-TPM and GVP bubbled liquid trapping; and c) ENDS aerosol ACM and GVP bubbled liquid trapping. A schematic representation showing the generation of TPM, HTP-TPM, ACM and bubbled GVP, for cigarettes, HTP and ENDS respectively. Products and aerosols are representative of the category. For ENDS, a variety of open and closed-type systems exist; a closed modular-type system is depicted here. Products are not to scale. ACM = aerosol collected mass; GVP = gas vapour phase; PBS = phosphate-buffered saline; TPM = total particulate matter.

there are limitations associated with testing PCM from whole cigarette smoke, HTP aerosols or ENDS aerosols. In general, PCM accounts for only those chemicals found within the particulate phase, and therefore it is not fully representative of the entire aerosol. However, generating PCM is a convenient way of obtaining test material that can readily be used in a variety of *in vitro* assays, which can then be conducted in compliance with current regulatory test guidelines.

There are clear differences in the PCM from cigarette smoke, HTP aerosols and ENDS aerosols, including for example, much higher liquid content from whole ENDS aerosols, and very different proportions of chemicals, including humectants, tobacco-specific nitrosamines (TSNAs) and aldehydes. Other factors that can affect the nature of the PCM include the puffing regimes (which are frequently different between cigarettes, HTPs and ENDS, due to differing regulatory guidance on appropriate regimes, as well as issues such as the smouldering of cigarettes between puffs and the on/off nature of heating coils with ENDS and HTPs), variable pad loading levels between the product types, and, for ENDS in particular, the angle of vaping and differences between puff blocks. Pad material elution methodologies may also vary between product types, as can the elution vehicle used — dimethyl sulphoxide (DMSO) or ethanol (EtOH) are the most commonly used — result in further sources of variation.

PCM can be characterised by chemical analysis, and it is common to conduct a more detailed analysis on the material collected directly from the filter pad than on any resulting pad material eluate that may be used for test system exposure in the *in vitro* assays. However, the PCM from each of the product types should be considered as complex mixtures, and therefore it is technically challenging to fully characterise PCM analytically. If using PCM for GLP compliant *in vitro* studies, generation should be conducted to a recognised quality standard (ideally OECD or US FDA GLP compliant), and characterisation of the PCM should be performed with consideration given to the combination of starting product, puffing regime, pad collection and elution methodologies, along with at least limited chemical analysis, such as nicotine, water and humectant levels.

The *in vitro* assays in which the PCM is tested are often designed to provide a qualitative rather than quantitative assessment of the materials tested. Therefore, the assay design may need to be optimised to provide meaningful comparative assessment between different PCM from different tobacco products or product types. Comparative assessments also need to consider day-to-day variability, and should be meaningful. For example, consideration

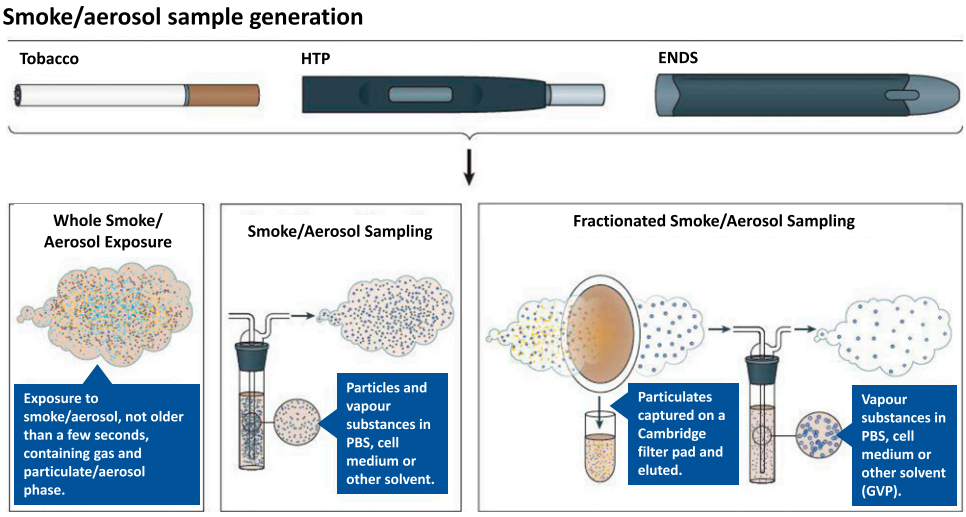

Figure 5. Overview of the three main sampling types for cigarettes, HTP and ENDS. Whole smoke/aerosol can be generated by using an *in vitro* exposure platform and aerosol delivered to the exposure point. The aerosol can also be fractionated to capture the aqueous soluble fractions in an impinger for cell dosing, and finally, the particulate material can be captured on a filter pad and eluted with a solvent to create a particulate test article. Alternatively, the particulate material can be filtered to create an aerosol GVP fraction, or an impinger-captured GVP fraction. Products and aerosols are representative of the category. For ENDS, a variety of open and closed-type systems exist; a closed modular-type system is depicted here. Products are not to scale. ENDS = electronic nicotine delivery system; GVP = gas vapour phase; HTP = heated tobacco product; PBS = phosphate-buffered saline.

should be given to qualitative results and biological relevance before performing statistical analysis.

The recommendations for comparing different cigarettes, HTPs or ENDS by using PCM and *in vitro* assays are:

- Be aware that there are limitations to PCM testing, and inherent differences between the material eluate from different product types. Such testing should be used in conjunction with other sampling methods, such as, GVP and/or whole smoke or whole aerosol testing.
- Consider appropriate puffing regimes, e.g. PCM concentrations, relevance to actual use behaviour, etc., particularly when comparing across different tobacco product types.
- Characterise PCM (generation and analysis) to a recognised quality standard.
- If possible, treat PCM eluates in parallel when conducting the *in vitro* assays (to minimise day-to-day variation), particularly when performing comparative assessments.
- Consider (and justify) appropriate *in vitro* assay design, including numbers of replicates, treatment concentrations, number of experiments, in order to permit meaningful and biologically relevant comparisons.
- Use a stepwise approach to comparative assessments. Initial qualitative assessment should be made before any quantitative assessment, or comparison, is made. It is not appropriate to conduct quantitative evaluations for responses that would not be considered to be positive.

Perspectives and practical considerations on using pad-collected material to evaluate cigarettes, HTPs and ENDS

(*Presented by Leon Stankowski, Charles River Labs*) Until fairly recently, direct exposure of bacterial or mammalian cells to cigarette smoke or aerosols from ENDS and HTP devices has been relatively uncommon and fraught with technical issues. Instead, it has been much more common to evaluate fractions of these substances, such as PCM (TPM, ACM or HTP-TPM) collected on CFPs, as well as GVP — trapped in impingers — as a surrogate for whole smoke or whole aerosol. While the collected materials represent only a fraction of all constituents, these samples are fairly easy to generate, are similar to the more familiar 'white powders' that are routinely tested for genotoxicity and cytotoxicity (for instance in the pharmaceutical industry), and testing throughput is relatively high.

Sample collection must be consistent, using defined and qualified methods and parameters recommended by recognised regulatory or industry governance bodies. Unlike the fractions collected from combustible tobacco products, those produced from ENDS and HTP devices are generally much less cytotoxic or genotoxic, and often show no

measurable activity. A combustible TPM sample prepared at 30 mg/ml is generally more than sufficient to evaluate that sample up to the required cytotoxicity or solubility limits. In contrast, samples generated from ENDS and HTP products are generally limited to ~60 mg/ml owing to physical/technical constraints. The general lack of cytotoxicity or solubility limitations for the latter type of products necessitates the collection of larger sample volumes and the pooling of material collected on multiple pads at concentrations as high as feasible. Thus, for routine testing in a typical *in vitro* battery, producing a large (~50 ml), pooled batch of PCM (± GVP) is recommended.

While the biological activity of combustible TPM has been shown to be stable for at least two years under appropriate storage conditions,[29] the long-term stability of samples collected from the newer products is largely unknown. Unless long-term stability is confirmed, testing should be completed within as short a time as is feasible.

Since samples generated from the newer tobacco products are generally non-cytotoxic and freely soluble, the primary question heard in relation to the dose levels used to test these newer products is 'How much, or how high, is enough?' The OECD and FDA have established limit doses for single chemicals or simple solutions in the various *in vitro* toxicology assays, but the tobacco product test samples are complex mixtures often comprised, substantially, of humectants and carriers. Therefore, common practice in many laboratories is to test up to the maximum feasible dose (MFD), based upon solvent or vehicle cytotoxicity limitations, in the various test systems. This has the benefit of allowing one to forego standard concentration verification of the dose formulations, provided initial and end of use characterisation is performed. Assuming that there is no substantial degradation of the samples or key constituents within the period of use, and the highest concentration evaluated is tested as received, there would be no adverse impact associated with a lack of additional dose formulation analysis (i.e. there is no need to test the actual aliquot used for a study, and no need to analyse lower dose formulations).

One final consideration is logistical, as related to comparing products for potential adverse results. Almost invariably, comparisons are desired for one or more novel ENDS or HTP product relative to an existing combustible product or a same-in-class market comparator, or evaluating various additives or constituents (e.g. nicotine levels, flavours, etc.) against a base formulation of humectants and carriers. The regulatory *in vitro* test battery is comprised of three assays (Ames, *in vitro* MN and NRU), and the samples should be tested as soon as possible after generation; concurrent testing of even two products would thus involve six concurrent assays. Adding additional variables, one can see how quickly the concurrent evaluation of multiple sample types and products can explode in number. Thus, even the largest and most experienced laboratories are likely limited, in practical terms, to testing three or four standard test batteries per day for true concurrent comparisons.

Considerations for in vitro *genetic toxicological tests: Vehicle control and dosing considerations for cigarette and ENDS samples*

(*Presented by Sara Hurtado, Charles River Labs*) While the OECD and ICH have established limit doses for single chemicals and simple mixtures when tested in the various *in vitro* genetic toxicological assays, samples generated from combustible and ENDS/HTP products are complex mixtures, and the ENDS/HTP products are generally much less cytotoxic. Therefore, as stated above, common practice in many laboratories is to test up to the MFD based upon solvent or vehicle limitations in the various test systems.

Our laboratory has generated extensive data on vehicle control limitations for the usual *in vitro* test battery comprised of three assays (Ames, *in vitro* MN and NRU). The two most commonly used vehicle controls for pad-collected samples generated from ENDS and HTP are DMSO and EtOH (absolute ethanol). Within the Ames assay, a range of dose volumes (µl/plate) for both vehicles were evaluated, to determine the limitation for the two commonly used methods of exposure — i.e. plate incorporation and preincubation. Various trials were conducted by altering the dose volume of these vehicles over a wide range. To evaluate cytotoxicity caused by the vehicles, several measures were used. These include: i) a decrease in the background lawn of bacteria resulting from cell growth which occurs before the small amount of residual histidine is consumed; and ii) a decrease in the number of spontaneous revertants and/or the positive control responses. The results from various amounts of vehicle were compared to the historical control data. Our results indicate that up to 100 µl/plate of EtOH or DMSO was not inherently cytotoxic (i.e. inducing a reduction in the background lawn or ≥ 50% reduction in negative control spontaneous revertant frequency), and the vehicle and positive control responses were comparable to the 95% confidence intervals of the historical controls. At higher dose volumes (≥ 200 µl/plate), cytotoxicity and positive control responses were altered, especially for EtOH.

In the *in vitro* MN assay using TK6 cells, a wide range of dose volumes was evaluated. The pH and osmolality of the vehicle in media was also examined. At concentrations ≥ 2% (v/v) DMSO and ≥ 1.4% (v/v) EtOH, the osmolality was increased to > 120% of the media alone (our criterion for an excessive change). However, the pH remained within acceptable ranges (7 ± 1) at concentrations up to 10% (v/v) for both vehicles. Based on the cytotoxicity observed (decreases in relative population doubling), the MFD of DMSO is 2.0% (v/v), and the MFD of EtOH is 1.3% (v/v). At these

Table 3. Summary and recommendations for testing pad-collected material (PCM).

- The limitation is known and accepted that PCM from cigarette smoke or HTP/ENDS aerosols only accounts for those chemicals found within the particulate phase, and therefore is not representative of the entire aerosol.
- However, it is a commonly used way of obtaining test material from aerosols that can readily be assessed in various regulatory *in vitro* testing assays and for which preparation guidelines are available, e.g. for combustibles. There is a need to develop guidelines describing the preparation of PCM from new NGPs.
- Consider appropriate puffing regimes; there is a need to develop appropriate puffing regimes based on topography of consumer use for NGPs.
- TPM from the various types of products have differing degrees of cytotoxicity. Combustible cigarette and HTP will most likely produce cytotoxicity at different levels. ACM, however, may show limited cytotoxicity, and therefore it may be appropriate to consider different solvents or different concentrations when testing the material.
- Maximise pad loading for NGPs without compromising the pad integrity. Elute with a minimal amount of solvent to obtain as high a concentration of material as possible.
- NGP PCM batch considerations should be taken into account: for NGPs, amounts of HTP-TPM/ACM at the concentration required may be higher than those that can be generated in one collection attempt; therefore, possible sample to sample variability needs to be considered; a single pooled sample can be obtained by mixing extracts from multiple filters.
- Need to consider TPM/ACM/HTP-TPM concentrations and constituents, particularly when comparing across product types (e.g. when comparing conventional cigarettes with NGPs).
- Characterise PCM (generation and analysis) to a recognised quality standard, e.g. GLP, GMP, ISO, etc.
- If performing a comparative analysis of different products, treat the PCMs to be compared in parallel in the *in vitro* assays whenever possible. Otherwise, compare with a common standard reference sample.
- Use a stepwise approach to comparative analysis: initial comparison should be a qualitative assessment; only compare quantitatively if both products are positive.

concentrations and below, DMSO and EtOH were negative for cytotoxicity and MN induction in TK6 cells, when following the standard procedure of a 4-hour treatment ± S9 and a 27-hour treatment –S9. The osmolality of common carriers — propylene glycol (PG), glycerin (G), and a 50:50 mix (50:50 PG:G) — for ENDS products were also evaluated and were not different from neat DMSO at the same % (v/v) concentrations. Evaluating these carriers in the MultiFlow assay (a mechanism of action screen that detects molecular events leading to the formation of MN) indicated that PG (alone or as 50:50 PG:G) has a potential aneugenic effect at ≥ 2% (v/v).

The OECD Test Guideline (TG) 129 for the NRU specifies a 2.5 mg/ml limit dose (if solubility permits) or a maximum 0.5% (v/v) dose volume using DMSO, thus requiring a 200× stock at 500 mg/ml to comply (in the absence of cytotoxicity or insolubility). This is not an issue for the TPM samples from combustibles, which have generally shown cytotoxicity at approximately or > 50 µg/ml. However, as stated previously, PCM from ENDS and HTP products are typically prepared at approximately 60–70 mg/ml due to collection limitations. If dosed neat at 0.5% (v/v) this equates to a maximum concentration of only 300 µg/ml (much lower than recommended), thus meeting the guideline for dose volume, but not the concentration limit. Given the restrictions in OECD TG 129, testing beyond the specified volumes is not typically done. However, in our laboratory, we have evaluated DMSO and EtOH up to 8% (v/v) in BALB/c 3T3 cells. Our results indicate that the volume of DMSO could be increased to as high as 1% (v/v) without cytotoxic effects, as viability was > 80% as compared to the concurrent standard negative control (0.5% (v/v) DMSO). However, increases in EtOH are not recommended as concentrations ≥ 1% were considered cytotoxic. Therefore, at present we have adhered to the guideline dose volumes, but further investigation in this area clearly is needed.

The dose volumes and vehicles established above are relevant for evaluation and comparison to TPM of a combustible reference cigarette, as a 30 mg/ml sample can easily be generated in the vehicle of choice. TPM will likely produce cytotoxicity and/or a positive response, therefore vehicles are less limiting for combustible products. Typical assay design when working with combustible TPM can use the anticipation of a positive response or cytotoxicity (or insolubility) to aid in dose selection. HTP-TPM is similar to TPM, with cytotoxicity generally observed (albeit at lower levels than TPM). Products generated from ENDS, however, generally exhibit limited cytotoxicity, precipitation or response. Therefore, the maximum dose for ACM from ENDS products should be the MFD for the vehicle used.

Summary and recommendations for testing pad-collected material

Following all of the presentations related to TPM, HTP-TPM and ACM, the recommendations made by the individual speakers were discussed. The workgroup discussed the specific wording for each of the recommendations and created the summary provided in Table 3.

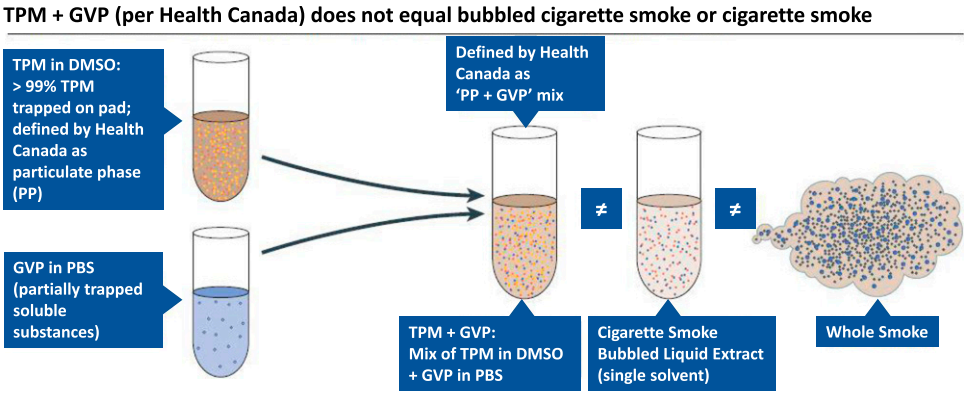

Figure 6. A schematic representation of the Health Canada TPM + GVP testing approach for combustible cigarettes. TPM and aqueous captured GVP is combined to create a TPM + GVP mix. Such approaches are designed to capture the various fractions of aerosols, such as the particulate and vapour phase together, and are a proxy for whole smoke/aerosol approaches, when they cannot be used. However, such combinations of test articles are not an exact representation of either bubbled extracts or the whole aerosol/smoke. DMSO = dimethyl sulphoxide; GVP = gas vapour phase; PBS = phosphate-buffered saline; PP = particulate phase; TPM = total particulate matter.

Fractionated samples (TPM/HTP-TPM/ACM and GVP)

Perspectives on using fractionated smoke and aerosol samples to evaluate tobacco products

(*Presented by Robert Leverette, RAI Services Company*) With the expanding innovative development of NGPs focused on reducing health risks associated with traditional tobacco products, there is a growing need to test these novel products by using *in vitro* genotoxicity models for inclusion in pre-marketing and modified risk applications to the relevant government regulators. Testing methods that are currently used for combustible cigarettes are being adapted and modified in preparation for the testing of samples generated from NGPs.

Historically, the *in vitro* genetic toxicological assessment of combustible tobacco products has predominately used fractionated smoke samples. The two most commonly used cigarette smoke fractions are the TPM and the GVP, with many studies utilising only the TPM, which is that portion of smoke captured on a CFP. The fraction passing through the filter pad is referred to as the GVP (see Figure 2). Interestingly, the particulate fraction is estimated to contribute approximately 4% of the total smoke mass derived/generated from a burning cigarette, with most of the smoke's mass consisting of atmospheric gases drawn through the cigarette and the (gas) vapour phase constituents.[30,31]

A typical smoke fraction collection system consists of a CFP holder positioned behind the cigarette to collect the TPM. The GVP passing through the CFP is then trapped by using an impinger, typically chilled in an ice bath containing either an aqueous (e.g. PBS or culture media) or organic (e.g. EtOH) solvent (see Figure 3). The pad-collected matter is extracted from the CFP with a variety of extraction methods (e.g. press, vacuum, centrifugation) and solvents (e.g. DMSO or EtOH). Once prepared, the TPM and GVP samples can be tested *in vitro*, either separately or combined (as per Health Canada Official Method T-502, Appendix 1;[3] see Figure 6).

In fractionated sample preparations (PCM and GVP), there are numerous critical factors that need to be considered in order to achieve consistent and reproducible results. Starting with the smoke or aerosol generator, utilising either a rotary or linear type machine is acceptable, depending on the laboratory's needs and preferences. However, being consistent in the type of machine used for sample preparation will aid in maintaining sample reproducibility. Generators that are programmable, and verified to perform specified puffing regimes within a certain degree of tolerance, are essential for aerosol sample preparations. Other attributes of aerosol generators to be considered include, but are not limited to: i) minimal smoke/aerosol path length (shorter is better) to minimise aerosol ageing; ii) adaptability of the generator to different tobacco and nicotine product types (HTP, ENDS), including button activation and variable angle adjustment (horizontal to vertical) at which the product is to be puffed; and iii) the ability to collect TPM and GVP simultaneously from the same machine.

In addition to the generator, preparing TPM and GVP samples under controlled conditions is also critical in achieving reproducible tobacco product samples. ISO Standard 3308[32] and ISO Standard 3402[33] specify standard definitions and conditions for the use of analytical smoking machines, conditioning of product prior to puffing, and the conditions under which the products should be puffed. Standard puffing regimes have been established for

combustible (ISO 3308,[32] Health Canada Standard Method T-115[34]) and ENDS products (CORESTA Recommended Method 81; CRM81,[35] ISO 20768:2018[36]), with these being adopted for use with other NGPs.

Having knowledge on the quantity of smoke, or aerosol, that the test products produce under these standard atmospheric and puffing conditions is crucial in preventing overloading the CFP, which could result in pad breakthrough, loss of sample and contamination of the GVP with particulate constituents. For cigarettes, it is known that CFPs of 44 mm and 92 mm in diameter can maintain their integrity and retain up to 150 mg or 600 mg TPM, respectively, as specified in ISO Standard 4387.[37] For NGPs, since they do not have as high a solid particulate percentage as cigarette smoke, the pad breakthrough may happen at higher concentrations. Therefore, it is important that each laboratory conduct CFP breakthrough studies for their particular products. Established procedures exist for the handling of the CFP, including appropriate conditioning, weighing before and after TPM collection, and removal from the pad holder (ISO Standard 4387[37]).

With use of these collection methods, it is inherently understood that the entire composition of the smoke or aerosol will not be captured. The solvents used for the extraction and trapping of the smoke or aerosol will influence what is captured due to the different solubilities, partition coefficients, and other chemical characteristics of the constituents in relationship to the collection (solvent) environment.

TPM samples prepared from combustible cigarettes and extracted in DMSO have been shown to maintain their biological activity (cytotoxicity and genotoxicity) for up to two years when stored frozen.[29] In contrast, GVP is relatively unstable when prepared in aqueous solvents, hence the stipulation in Health Canada Method T-502[3] that the GVP be tested within 60 minutes after collection.

There are clear advantages to using TPM and GVP samples for the *in vitro* assessment of aerosol-generating tobacco products. These samples are easy to collect with the proper equipment, and well-established methods have facilitated reproducible preparations. TPM and GVP samples have been used historically for *in vitro* testing, and dosing with these test sample types in cell-based systems tends to be straightforward although there are limits to maximum dosage based on the concentration of the stock sample and the upper solvent limit that can be tolerated by the cell cultures. The incorporation of analytical chemistry methods to quantify constituents (e.g. nicotine, etc.) adds a more sophisticated level of dosimetry beyond the mass of TPM and/or GVP or the number of cigarettes exposed to the cells.

There are caveats to using TPM and GVP samples as well. As stated above, depending on the exact methods and solvents used, the samples may only contain a portion of the chemical constituents of the whole smoke or aerosol. Moreover, with the inherent instability of the GVP fraction, it can be challenging to prepare and expose the cells within a specified time, and it is even more difficult if the GVP is to be combined with the concurrently prepared TPM. Key recommendations for the collection of TPM and GVP include:

- Use appropriate equipment, including smoke/aerosol generators adaptable to the products to be tested, as well as programmable to achieve, reproducibly, the targeted smoking/puffing parameters.
- Use standard or established methods already available; for example, those provided by ISO and CORESTA.
- Document the parameters and methods used to prepare the samples. Knowing the specific equipment, solvents, volumes, conditions, puffing parameters, methods, etc., used for sample generation are critical to allow comparison across different studies.
- Incorporate analytical chemistry methods. There are established methods available from CORESTA for numerous constituents found in combustibles and NGPs, e.g. *No. 75: Determination of Tobacco Specific Nitrosamines in Mainstream Smoke by LC-MS/MS*.[38] Chemistry data can be used to characterise (to some extent) the collected samples, to provide additional basis for dosimetry, and to allow for comparison(s) across different products and studies.
- Understand the limitations of the cell-based test systems being utilised and how those limitations relate to the samples being tested (e.g. non-specific toxicity).

In vitro *and stability testing of an EtOH collection method combining particulate and gas vapour phase components from cigarette smoke*

(*Presented by Mariano J. Scian, Enthalpy Analytical*) Health Canada (HC) guidelines (method T-502)[3] require the collection and testing of the tobacco smoke as the particulate phase (TPM), the gas vapour phase (GVP), and a combination of both (TPM + GVP; see Figure 6). The TPM is extracted in DMSO while the GVP is collected in PBS. This method has limitations, since smoke is artificially collected as two separate fractions in this procedure. Another limitation of the method is that GVP is collected by bubbling it into PBS, which has limited trapping capacity for volatile and non-water soluble compounds. GVP collected in PBS also has limited stability and therefore must be used in testing within 60 minutes of generation.[3] These limitations could be overcome with a method that allows collection of the TPM and GVP together, in a solvent with enhanced trapping and stability of the GVP components. We evaluated the use of EtOH to collect TPM and GVP

components together and compared it against the traditional HC collection method by using the NRU, Ames and MN assays all following HC guidelines. Reference 3R4F cigarettes were used to generate extracts with a concentration of 10 mg/ml of whole-smoke equivalent (TPM + GVP).

In the NRU assay, extraction of TPM in EtOH produced comparable results to DMSO-extracted TPM (IC_{50} 101.7 µg/ml *versus* 87.6 µg/ml); however, GVP in EtOH resulted in higher cytotoxicity (EtOH concentration was within the allowable limits of the assay) when compared to GVP in PBS (IC_{50} 40.2 µg/ml *versus* 159.1 µg/ml) likely due to better trapping efficiency of GVP components. The combination of TPM + GVP in EtOH showed higher toxicity (IC_{50} 58.2 µg/ml) compared to TPM in DMSO or TPM + GVP collected under HC guidelines. When 1-month old TPM + GVP-EtOH extracts were tested in the NRU assay, no significant differences were observed in the induced cytotoxicity compared to freshly collected samples. Results in the Ames assay showed that, in the absence of S9, EtOH condensate collecting TPM + GVP together resulted in increased bacterial lawn cytotoxicity in TA98, TA100, TA1535 and TA1537 compared to DMSO or PBS extracts at the same concentrations. With S9, TPM + GVP in EtOH induced an 18-fold and 11-fold increase in the number of revertants in TA98 and TA1537, respectively. These responses were more pronounced compared to TPM alone or TPM + GVP in PBS. In TA100 and TA1535 in the presence of S9, the EtOH TPM + GVP combination produced a similar response compared to TPM alone or TPM + GVP in PBS in the presence of S9. Dose-dependent increases in MN were observed in all three types of extracts (TPM + GVP-EtOH, TPM-DMSO or TPM-DMSO + GVP-PBS) when tested in the presence or absence of metabolic activation. Although no differences were noted in MN induction between the three types of extracts in the absence of metabolic activation, when tested in the presence of metabolic activation, the TPM + GVP-EtOH resulted in a mean fold induction of 3.7-fold compared to 5-fold for TPM alone or TPM + GVP in PBS.

Aliquots of the DMSO, PBS and EtOH condensates were analysed for selected chemical components. The results were similar for nicotine, NNN, NAT, NNK, NAB and ammonia. Nicotine values were higher for the EtOH method, but it was not detected in the vapour phase (GVP) of either setup, suggesting that EtOH is likely a more efficient solvent for extracting nicotine from the filter pad. Carbonyl results varied depending on the collection method, with the EtOH method being more efficient overall. Total carbonyl concentrations in the EtOH method (TPM + GVP) were ~12% higher than either TPM (in DMSO) or GVP (in PBS) phases of the HC collection. The largest difference between these two collection methods was seen for volatile organic compounds (VOCs). The aqueous PBS solution does not efficiently trap VOCs. Only acrylonitrile was detected in the GVP of the HC method, while isoprene, butadiene, benzene and toluene were not detected at all. The EtOH setup trapped approximately 234 µg more VOCs per ml of condensate than the traditional PBS collection (a 38-fold increase).

The EtOH extract method allows for the trapping of TPM + GVP yielding a single whole-smoke condensate that is more representative of the smoke/aerosol generated, has increased stability, and induces comparable or higher responses than the traditional HC method extracts when used in *in vitro* assays. The EtOH collection method allows more efficient trapping of VOCs than the aqueous PBS method, and appears to be applicable for e-cigarettes, as carbonyl compounds are one of the primary Hazardous and Potentially Hazardous Chemicals (HPHCs) formed during product use. Carbonyl collection is slightly more efficient in the EtOH setup, and combines the carbonyl compounds that are trapped in both the particulate and gas phases. The results show that this extract is suitable for testing in traditional *in vitro* toxicology assays. Furthermore, stability with the EtOH condensate was acceptable for all compounds for at least three days when stored at –80°C.

Summary and recommendations for testing fractionated samples

Following all of the presentations related to TPM, HTP-TPM, ACM and GVP, the recommendations made by the individual speakers were discussed. The workgroup agreed on the specific wording for each of the statements and created the summary provided in Table 4.

Whole smoke, aerosol and vapour samples

As discussed earlier, there is increasing emphasis and interest in evaluating a whole smoke or aerosol that is generated from combusted cigarettes, or aerosol from HTPs and ENDS. Various methods have been used to try and capture the chemicals that are present in both the particulate and vapour phases, and to use these samples for cell exposure. First, the Health Canada method, mentioned in several abstracts above,[3] combines TPM and GVP for testing. Another approach is to bubble whole smoke into a liquid to form a test sample that may be applied/exposed to the cell culture system. The workgroup wanted to emphasise that these methods, however, are not the same as exposing cells directly to whole smoke. Figure 6 was developed to emphasise this important point.

Methods to expose cells directly to whole smoke or aerosols are rapidly being developed because of the desire to provide more ‘relevant’ evaluations. Therefore, the workshop included a number of talks on this topic. Among the

Table 4. Summary and recommendations for testing fractionated samples.

Advantages of using TPM and GVP for *in vitro* studies
— There are established/standard/favoured methods for the collection of TPM and GVP from combustible products; however, there is still the need for the development of detailed methodologies for NGPs.
— Both fractions are fairly simple to collect with proper equipment and established methods.
— Analytical chemistry methods are available for both TPM and GVP preparations.
— Historical biological data exist for TPM from combustible products.
— Combustible TPM can be collected and stored frozen for up to two years (DMSO preparations) with little effect on the biological activity in genetic toxicology tests and NRU.[28]
— Test fractions may be assessed separately or combined.
— Dosing of cells *in vitro* using fractionated samples is fairly straightforward.
Caveats for the use of TPM and GVP in *in vitro* studies
— The use of TPM + GVP (either combined as a single test sample or as two independent test samples) should not be considered the same as exposing cells to whole smoke/aerosol.
— Fractionated test samples may lack any potential synergistic and/or inhibitory effects of the combined components.
— It should be ensured that different solvents (e.g. DMSO, PBS, EtOH) are compatible with the *in vitro* test system. The organic solvents limit the amount of test material that can be used in *in vitro* test systems.

presentations was an introductory talk broadly dealing with the use of *in vitro* studies for evaluating tobacco products (Robert Tarran), as well as a talk providing an approach developed specifically for evaluating a single chemical aerosol (Xuefei Cao). Both talks provide insight that can be applied to evaluating tobacco products for their potential genotoxicity.

Perspectives on generating smoke/aerosol samples for in vitro *test systems*

(*Presented by Robert Tarran, University of North Carolina*) The act of smoking tobacco is a complex process that typically results in the exposure of multiple cell types in the lung to multiple chemical constituents, often over the course of decades. Although *in vitro* studies lack the complexity or time signature of human smoking, they do allow for the isolation of specific components for more detailed mechanistic study. They are also useful for toxicological screening of new and emerging tobacco and nicotine products. Indeed, as will be discussed, many of the changes seen in smokers' airways are replicated by using *in vitro* systems.

We have extensively studied the impact of tobacco smoking on airway epithelia. For example, human bronchial epithelial cultures (HBECs) can be directly isolated either *post mortem*, or from excess surgical samples and then cultured for 1–2 months as described.[39] At this point, these cultures are fully differentiated, and have similar numbers of ciliated cells and mucus-producing goblet cells as seen *in vivo*.[40] HBECs undergo vectorial ion transport and secrete a thin layer of airway surface liquid (ASL) that contains mucins and ~1000 other proteins similar to what is seen *in vivo*, including antimicrobial peptides, proteases and cytokines.[41] Moreover, HBECs also transport mucus in a fashion that is dependent on ion transport and ciliary beating.

We have previously exposed HBECs to tobacco smoke by using a British American Tobacco (BAT)-style smoke exposure chamber linked to a Borgwaldt smoking engine.[42] This chamber is advantageous since it only exposes the apical side of the HBECs to cigarette smoke, while the basolateral side, which is continuously perfused, sees a much lower level of smoke exposure.

When performing tobacco smoke exposure experiments, one must decide what to measure, and then decide a smoke exposure paradigm that delivers an appropriate amount of smoke exposure and is relevant to your endpoint(s). The lung is the first point of contact with inhaled tobacco smoke, and it makes sense that nicotine levels will be higher in the lung than systemically. We previously measured nicotine levels in human sputum immediately after smoking one cigarette and found a value of ~30 μM. In contrast, nicotine levels are < 100 nM in the human blood.[43] We then measured nicotine levels in the HBEC ASL and found that one cigarette puffed under ISO conditions (10 × 2 seconds 35 ml puff every 60 seconds) also gave a similar level of ASL nicotine.[42] At that time, we were interested in the impact of tobacco smoke on the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated anion channel. We chose CFTR because it is a validated biomarker of harm. Indeed, studies with cystic fibrosis patients show that either partial or full loss of CFTR activity leads to lung disease of varying severity.[44] We first measured CFTR activity in humans and found tobacco smoke exposure diminished it by 50–75%.[45] Next, we measured CFTR activity in tobacco smoke-exposed HBECs and found a similar decrease in CFTR activity.[45] Thus, we felt confident that we were using appropriate tobacco smoke

Table 5. Summary and recommendations for testing whole smoke, aerosols and vapours.

— There is a need to promote greater standardisation in aerosol generation and collection processes, in order to improve consistency between testing laboratories.
— Many *in vitro* whole aerosol studies on NGPs employ methods that were optimised for cigarette smoke and so do not take into account the differences in physical and chemical make-up of aerosols from these new categories of product. It is necessary to develop and optimise methods that are appropriate for these new types of aerosol. It may not be sufficient to simply mirror cigarette smoke testing approaches, as the NGPs are chemically and physically different and should be treated as such.
— Dosimetry approaches that can link diverse studies are needed. For example, nicotine or other markers of exposure can be used as comparators.
— For whole aerosol exposure, it is important to: determine and control temperature and relative humidity; determine and control airflow rates for supply and exhaust from exposure system; determine and control exposure concentrations; include sham controls; verify deposition; and include analytical monitoring when possible.
— In order to evaluate system performance, it is important to: minimise and understand any alteration of the aerosol by the exposure system; demonstrate comparable exposure and deposition among target wells; establish the reproducibility of independent exposures; establish a range of delivery doses; and perform fit-for-purpose characterisation of the system.

exposure regimes, and that we were using an appropriate *in vitro* model to study our protein of interest.

In conclusion, despite being reductionist, *in vitro* models are extremely useful for studying tobacco smoke exposure and will likely be important in the future for studying ENDS. However, it is important to use a relevant model, understand the appropriateness of the exposure system, and use validated biomarkers. This work was funded by NIH/NHLBI HL135642.

Recommendations for generating aerosol/vapour samples for direct exposure to cells in culture

(*Presented by Xuefei Cao, National Center for Toxicological Research*) Conducting direct exposures of lung cells to aerosols or vapours is technically challenging, and the methods employed directly impact the quality of the data that are generated. Over the years, our laboratory has evaluated two *in vitro* exposure systems for conducting such exposures. The Cloud System for aerosol exposure and the Spiking System for vapour exposure were both developed by Vitrocell® (Waldkirch, Germany). In general, these two systems are capable of generating aerosols/vapours in a controlled and reproducible manner for exposing cells at the ALI. These and other similar systems, simulate inhalation exposures in an *in vitro* setting. However, thorough validation of the systems must be conducted for each test substance evaluated, in order to ensure the consistent generation of aerosols/vapours. In the following sections, technical modifications and considerations applicable to most types of aerosol/vapour test system validation will be discussed.

The Cloud System is designed for brief (less than 5 minutes) exposure of cell cultures to aerosols generated from liquids or suspensions. The system consists of a piezeoelectrially driven nebuliser for aerosolising test substances, an enclosed exposure module for confining the aerosol 'cloud' and housing cell cultures, and an integrated digital heating device for controlling the temperature of the system during exposure. The exposure module is available in different configurations, and nebulisers with different mesh pore sizes can be used to accommodate test substances having different volume mean diameters. During the exposure, cell medium is supplied to the cultures in a static manner for nourishing the cells and maintaining the pH of the cellular environment. The Cloud System utilises the principles of cloud dynamics to produce rapid aerosol sedimentation at a high droplet output rate.

Fluorometric and chemistry analyses have demonstrated the spatially uniform deposition of test article aerosols within the Cloud System exposure module.[46,47] Lenz et al.[46] characterised the Cloud System by nebulising 200 µl fluorescein solution and quantifying fluorescein concentration in 6-well-sized cell culture inserts. The authors concluded that aerosol delivery was highly efficient and reproducible. Recently, our laboratory conducted a similar system validation by using 24-well-sized inserts. Contrary to the conclusion from the Lenz study, we observed significant variation between positions within the exposure

module. It is possible that the variation was magnified by use of the smaller inserts. This problem was overcome by modifying the aerosol generation process. We found that the mesh surface on the nebuliser was not flat, especially around the edges. The unevenness of the mesh surface may have accounted for the heterogenous aerosol generation across the mesh and, subsequently, the variation in aerosol deposition across positions in the exposure module. To circumvent this problem, we started with a much larger volume of solution than that employed by Lenz et al., to ensure sufficient coverage of the mesh surface throughout the nebulisation process and controlled nebulisation volume by time. These small modifications significantly improved the homogeneity and repeatability of aerosol deposition within the exposure module.[47]

Another important consideration for Cloud System validation is the use of the quartz crystal microbalance (QCM). The QCM provides real-time monitoring of particle deposition. Previous experience indicates that the QCM performs as expected within its detection limits. However, its performance can be affected by the physical properties of the test substances. For instance, viscous chemicals tend to impede vibration of the crystal on the QCM. Therefore, it is essential that the performance of the QCM is evaluated for each test substance and its readings confirmed.

Our laboratory has used the Spiking System for vapour generation from volatile and semi-volatile substances. The system is composed of a controller, for regulating the airflow and temperature of the system, and a digital syringe drive for injecting the test substance at a predefined speed. Chemicals are vaporised at their boiling point by heating, and vapours are delivered to the exposure module. There are several considerations for validating the Spiking System. First of all, it is important that the chemical properties of the test substances do not change and that undesirable side reactions, e.g. degradation or polymerisation, do not occur as a result of test agent generation. One option for avoiding alterations to test substances is modifying the configuration of the system. For instance, the configuration of the Spiking System can be modified to vaporise test substances by relying on the mechanical forces of the airflow. It is also important that the syringe drive speed be optimised manually and carefully when working with aqueous solutions, such as formaldehyde solution. Aqueous solutions tend to accumulate in the lines of the Spiking System, especially when the system temperature is relatively low, leading to unstable vapour generation. In this case, care should be taken to inject chemicals at speeds that do not overwhelm the vaporisation capacity of the system. Lastly, in-line dose monitoring devices are highly recommended. Although the concentrations of chemical substances in the vapours can be determined by collecting the test substances into impingers connected to the Spiking System, such an approach measures the average concentration over the collection period. Brief variations in vapour generation, which may greatly impact cellular responses, are not captured in these data.

The goal of system validation is to demonstrate the consistency and reproducibility of test substance generation in a manner relevant to human inhalation exposure. In this regard, carefully considering the chemical properties of the test substance and developing a set of criteria that best monitor the properties of the test substance, as well as the performance of the exposure system, are critical for the success of validation studies.

Perspectives on generating smoke and aerosol conditioned media samples to evaluate tobacco products using in vitro *test systems*

(*Presented by Robert Leverette, RAI Services Company*) Historically, the *in vitro* genetic toxicological assessment of combustible tobacco products has predominately used TPM and GVP samples. Another approach for smoke/aerosol sample collection includes bubbling whole smoke or aerosol through cell culture media, resulting in what is referred to as 'whole smoke conditioned media' or 'whole aerosol conditioned media' (WSCM or WACM, respectively). A typical WSCM/WACM collection system is nearly identical to the system used for TPM and GVP collection with only the absence of a CFP (used to collect the particulates) in order to bubble unfiltered smoke/aerosol through an impinger containing the appropriate cell culture media (see Figure 3).

Currently, no standardised methods exist for the preparation of WSCM/WACM. There are methods available throughout the published literature; however, the approaches for sample preparation vary quite extensively. A few examples are listed below:

- Makwana et al.[48] prepared WSCM by bubbling smoke (ISO puffing parameters, ISO 3308[32]) from four 3R4F Kentucky reference cigarettes through 20 ml of culture media in a 30 ml impinger. Glass beads were used to aid in the capture of smoke constituents. WSCM was analysed for nicotine and stored frozen (< –50°C) for up to four weeks without loss of activity. Exposures were based on nicotine concentration (μg/ml).
- Oke et al.[49] prepared WSCM by bubbling smoke (ISO puffing parameters) from a single combustible cigarette through 20 ml of cell culture media and using it immediately. The WSCM was analysed for nicotine, but the dose was based on the percentage of WSCM added to the test system.

— Poussin et al.[50] bubbled smoke (HCI puffing parameters, Health Canada Standard Method T-115[34]) from six 3R4F Kentucky reference cigarettes through 36 ml of PBS. Additionally, whole aerosol (HCI puffing parameters) from 10 heated tobacco products (HTPs) was bubbled through 40 ml of PBS. The preparations were used immediately (added to culture media), but were analysed for nicotine and several carbonyl constituents. The dose was based on puffs/ml.
— Omaiye et al.[51] prepared WACM from ENDS products by using a non-standard puffing regime. Aerosol was bubbled through two impingers in tandem, each containing 25 ml of cell culture media. The WACM was stored frozen (–80°C) prior to use. The dose was based on mg/ml, calculated as the difference in pre-puffed and post-puffed weights of the ENDS device.

From these examples of WSCM and WACM preparation approaches, it would be difficult to compare the results from such a variety of studies when vastly different sample preparations were used. In order to achieve consistent WSCM samples, researchers need to consider those factors deemed important for TPM and GVP collections. Briefly, use controllable and verifiable aerosol generating equipment, and standard product conditioning and atmospheric parameters.

As with the TPM and GVP collections, it is understood that the entire composition of the smoke or aerosol will not be captured in the WSCM. The aqueous cell culture media used for the trapping of the smoke or aerosol will influence what is captured in collected samples due to the different solubilities, partition coefficients, and other chemical characteristics of the constituents in relationship to the aqueous environment. Also, when collecting WSCM or WACM, the presence of serum (e.g. fetal bovine serum) or extraneous protein (e.g. bovine serum albumin) in the media will influence what is captured and how the *in vitro* system responds when exposed.

Stability of WSCM and WACM is not well understood. Makwana et al.[48] showed similar activity of WSCM in their test system with samples stored frozen for up to four weeks. Taylor et al.[52] demonstrated the stability of frozen 3R4F WSCM for up to 31 weeks, based on nicotine content, cytotoxicity and oxidative stress endpoints. However, this may not be the case for different *in vitro* test systems and different biological endpoints. Since WSCM and WACM are aqueous based, they should be used immediately as HC recommends for aqueous GVP preparations (Health Canada Official Method T-502).[3]

The advantages to using WSCM and WACM samples for the *in vitro* assessment of aerosol-generating tobacco and nicotine products are similar to those for TPM and GVP preparations, with use of identical equipment, ease of collection, characterisation and dosing of *in vitro* test systems. The maximum dose will be based on the WSCM stock concentration and assay guideline recommendations for the maximum volume of aqueous based samples that can be added to the *in vitro* system — for example, the OECD TG 487 (Mammalian Cell Micronucleus Test)[28] recommends limiting aqueous samples to 10% (v/v) in the final treatment media.

Currently, no established or standardised methods exist for the preparation of WSCM and WACM samples. There is a wide range of methods in the published literature, with variations in the amount of product bubbled through a range of volumes of media. Key recommendations for the preparation of WSCM and WACM are similar to those for TPM and GVP preparations:

— Use proper equipment, including smoke/aerosol generators adaptable to the products to be tested, as well as programmable to achieve, reproducibly, the targeted smoking/puffing parameters.
— Document the parameters and methods employed to prepare the samples used in a study. Knowing the specific equipment, aqueous media (PBS or cell culture media), volumes, conditions, puffing parameters, methods, etc., used for sample generation are critical to allow comparison across different studies.
— Incorporate analytical chemistry methods. There are established methods available (CORESTA) for numerous constituents found in combustibles and NGPs. Chemistry data can be used to characterise, to some extent, the collected samples, provide additional basis for dosimetry, and allow for comparison(s) across different studies.
— Understand the limitations of the cell-based test systems being utilised, and how those limitations relate to the samples being tested (e.g. non-specific toxicity).
— Develop and establish standard methods for WSCM and WACM preparations for the different tobacco product types (combustible, HTP, ENDS). This is also critical to allow comparison across different studies.

In vitro *advances in whole aerosol approaches for electronic nicotine delivery systems (ENDS) testing*

(*Presented by David Thorne, British American Tobacco*) *In vitro* approaches have been used to assess alternative tobacco and nicotine products, such as ENDS and HTP. HTP and ENDS deliver nicotine to consumers by either heating a consumable tobacco rod or aerosolising a solution (e-liquid)

containing nicotine, by using a variety of aerosolisation mechanisms. Recent data suggest that HTPs and ENDS show > 90 and > 96% reductions, respectively, in chemical yields, in terms of the amounts and levels of chemicals present compared to that of cigarette smoke.[53–55] To increase clinical relevance and better mimic human use, whole aerosol *in vitro* testing approaches are considered the go-to choice for researchers if such an option is available. However, to date, many aerosol testing strategies for these new products are conducted by following pre-established protocols for cigarette smoke exposure and have not been optimised for ENDS or HTP testing. This approach assumes that aerosols from these products are similar, physically and chemically, to cigarette smoke and that consumers use the products comparably. As a result, the data obtained from early ENDS studies were generated by using methods that were designed and developed to deliver and test cigarette aerosols, often with little consideration of dose.

Here, we summarise the development and implementation of a focused *in vitro* aerosol programme, through a series of publications that deal with the challenges of adapting these systems to work with the changing ENDS category. For example, we discuss: synchronisation of ENDS button activation with syringe initiation within the aerosol generation system; maximising dose; elimination of complex dilution principles; comparing within category, as well as with cigarette smoke; establishing an aerosolised positive control for *in vitro* exposures; and comparisons between different exposure systems which can demonstrate ENDS device evolution to a significantly cleaner product.

Neilson et al.[56] published a study comparing aerosol from two commercially available ENDS to cigarette smoke over an extended 6-hour exposure period, using a Vitrocell VC10 (Vitrocell® Systems, Waldkirch, Germany) exposure system and 3-D human reconstructed lung tissue (EpiAirway™, MatTek Corp., Ashland, MA, USA). This study successfully demonstrated that over an equivalent exposure timeframe, cigarette smoke was cytotoxic, whereas ENDS aerosol caused no cytotoxicity at all. However, there is a scientific requirement to investigate not only the potential health effects of ENDS against cigarette smoke as a reference, but also the absolute risk of the ENDS category itself. To do this, more sensitive approaches are required that facilitate within-category comparisons.

Thorne et al.[57] demonstrated that the Vitrocell VC10 exposure system could be adapted to deliver an undiluted aerosol stream, which could be considered more appropriate for ENDS aerosol testing because, when using this approach, chemicals of concern are not diluted. Furthermore, this method enables testing to absolute 'extreme' exposure scenarios. For example, Thorne et al.[57] tested up to 900 puffs and demonstrated that the tested ENDS aerosol (ePen) was negative in all five Ames strains (TA98, TA100, TA97, *E. coli* WP2*uvrA*, and TA104), assessed both with and without S9, while achieving toxicity (observed as thinning of the background lawn and reduction in revertant counts). The authors took data analysis one step further and used this approach to demonstrate that, although the dose delivered was 4.5× greater than daily ENDS consumption (at approximately 200 puffs per day),[58] it still resulted in a negative response.

Bishop et al.[59] modified the approach by using a VC10 exposure system for the assessment of ENDS aerosol in a mammalian system. This study investigated a much more powerful ENDS product, an open tank system (eBox), with modifiable power and vent options, designed to be tailored to consumer use. This study demonstrated that, when using undiluted approaches, a full toxicity dose–effect curve could be obtained for an ENDS in 3-D reconstructed human airway tissue (MucilAir™, Epithelix, Plan-les-Ouates, Switzerland). The study also applied functional endpoints, such as transepithelial resistance, ciliary beat frequency and cilia active area. These functional endpoints decreased with increased exposure as a precursor to toxicity, thus confirming the observations. This study also demonstrated that an e-liquid spiked with a known respiratory system irritant, cinnamaldehyde, can act as a positive control. This positive control was used to confirm system functionality, which is especially important if a negative response is observed.[59]

Finally, we compared the biological response of MucilAir exposed to undiluted ENDS aerosol generated by a Vitrocell VC10 and a Borgwaldt LM4E, and demonstrated equivalent responses on a puff-by-puff basis. This is especially important, as the LM4E is specifically designed to work with a variety of ENDS (and HTPs) — it uses an undiluted approach, having device synchronisation built into the system, and does not require additional software or hardware applications[60] compared to the Vitrocell VC10. In our final study we were able to demonstrate the successful assessment and discrimination between ENDS, comparing an open tank system (eBox) with a closed podmod system (ePen3). When using the same formulation and an undiluted approach, the eBox device produced a full toxicity dose–effect curve within 200 puffs in 3-D human reconstructed lung tissue (MucilAir), whereas the ePen3 device barely reached 50% toxicity at 1000 puffs. The ePen3 system is designed with an interchangeable cartomiser which contains the coil and wick for e-liquid aerosolisation. This closed system is more tightly controlled and less prone to consumer misuse or dry wicking, which ultimately results in a cleaner and less toxic aerosol, as confirmed by the study.

It is clear that *in vitro* techniques need to evolve alongside new and emerging products, and that assessment of these products cannot rely on traditional cigarette smoke testing approaches. These new categories are chemically and physically different and should be treated as such. Future aerosol testing strategies should consider the

following in study design to ensure the most appropriate conclusions/interpretation of data. Aerosol delivery (dose being delivered) can ensure cross platform and category comparisons, and contextualisation of data through human consumption and dose extrapolation. This approach has enabled more appropriate tailoring of experimental design, comparisons within category, bridging between different exposure platforms, and links to human consumption and daily use estimates. However, perhaps more importantly, using this refined approach investigators will be able to demonstrate the evolution of the ENDS category, and show that product evolution and innovation has taken an already simplified aerosol and made it significantly cleaner as demonstrated by reduced biological activity.

Summary and recommendations for testing whole smoke, aerosol and vapour samples

Following all of the presentations related to whole smoke, aerosols and single chemical vapours, the recommendations made by the individual speakers were discussed. The workgroup discussed specific wording for each of the statements, and agreed on the summary provided in Table 5.

Workshop summary and overall recommendations

The third IIVS Workshop focused on outlining the challenges involved in evaluating tobacco products, particularly the NGPs, in *in vitro* toxicological test systems, primarily for standard regulatory genetic toxicology assays. Prior to the workshop, a subgroup developed draft graphics and terminology which were presented to the full working group. These were discussed and consensus reached. This terminology and a series of graphics illustrating the various types of samples that can be prepared and used to evaluate tobacco products are included in this publication. The research and perspectives of the expert speakers are described in the above sections. Each of the speakers contributed recommendations that were discussed by the full workgroup, with the outcome of these discussions summarised below.

Overall Workshop Recommendations

1. There is a need for greater standardisation in aerosol generation and collection processes.
2. Methods for testing the NGPs need to be developed and/or optimised since simply mirroring cigarette smoke testing approaches for NGPs may be insufficient.
3. Understanding and quantitating the delivered dose is fundamental to the interpretation of data and conclusions from the study.
4. Whole smoke/aerosol approaches need to be better contextualised with regard to key criteria, which includes controls, environmental conditioning, analytical monitoring, verification and performance criteria.

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Author contributions

Dr Moore and Dr Curren, the co-chairs of the workshop series, facilitated the workshop discussion, managed between-meeting workgroup activities, and coordinated the first draft of the manuscript. Specific authors, as identified in the manuscript, provided text for individual sections of the manuscript. All authors contributed to the discussion, activities of the workgroup and the content of the manuscript. All authors read and approved the final manuscript.

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