Regulatory Toxicology and Pharmacology 68 (2014) 222–230

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Investigating predictability of *in vitro* toxicological assessments of cigarettes: Analysis of 7 years of regulatory submissions to Canadian regulatory authorities

M. Belushkin [a,*], J.-J. Piadé [a], S. Chapman [b], G. Fazekas [b]

[a] Philip Morris International R&D, Philip Morris Products S.A., Rue des Usines 90, 2000 Neuchâtel, Switzerland
[b] Rothmans, Benson & Hedges Inc., 174 Kennedy Road South, L6W 3G6 Brampton, Ontario, Canada

ARTICLE INFO

Article history:
Received 24 September 2013
Available online 30 December 2013

Keywords:
Regulatory submissions
In vitro results
Smoke constituent yields
Prediction

ABSTRACT

A wealth of *in vitro* toxicological information on different types of tobaccos and tobacco products has been acquired and published, although the link between *in vitro* data and impact on human health remains elusive. The present study investigates the possibility of establishing quantitative models for the *in vitro* toxicological endpoint responses to cigarette smoke. To this end, it relies on information submitted to Canadian health authorities during the period 2006–2012. To our knowledge, this is the first time that published results concerning the influence of such factors as cigarette blend, diameter and filter type on *in vitro* toxicity are confirmed at the level of a representative range of products on a market. Taking these cigarette design features into account and adding a limited amount of quantitative mainstream smoke composition information, it is shown that, within the boundaries of the considered cigarette design parameters, the *in vitro* toxicological response can be effectively predicted. *In vitro* tests of tobacco products are an invaluable initial comparative product assessment tool. The present results reveal the limited value of data from repeated tests on products which do not undergo significant modifications.

1. Introduction

A wealth of information with regards to *in vitro* toxicity of different types of tobaccos and tobacco products has been generated (National Cancer Institute, 1976; Paschke et al., 2002; Richter et al., 2010; Thayer and Kensler, 1964). In fact, *in vitro* tests have been included as a standard industry practice for product assessment (Baker et al., 2004; Bombick et al., 1997; Carmines, 2002; Dempsey et al., 2011; Doolittle et al., 1990; Roemer et al., 1998; Zenzen et al., 2012), and regulatory authorities have also come to use them as one of the sources of product information (Health Canada, 2005b). *In vitro* toxicity testing of tobacco products provides valuable information for the characterization of the relative toxicity of different products, and different types of assays are chosen to investigate the different types of cell and DNA responses to exposure to tobacco smoke (CORESTA In Vitro Toxicology Task Force, 2004; Dempsey et al., 2011). Nevertheless, the relationship between *in vitro* toxicity and human smoking-related disease remains inconclusive (Brownawell, 2007; Byrd, 2004; Johnson et al., 2009). Although relationships between certain *in vitro* and *in vivo* endpoints have been discussed (Vanparys et al., 2012), it is considered that even *in vivo* testing would not provide results readily generalizable to evaluating the health effects in humans (Klaassen and Eaton, 1991; SCHER/SCENIHR/SCCS, March 2013).

A number of factors have been identified as influencing the *in vitro* toxicological activity of cigarette smoke. The influence of tobacco type and blend (Tso, 1999) has been extensively studied (Bombick et al., 1998; DeMarini et al., 2008; Richter et al., 2010; Rickert et al., 2007; Roemer et al., 2009, 2004; Roeper et al., 2004; Schramke et al., 2006; Yauk et al., 2012). Design features such as cigarette diameter and presence of activated carbon in the filter (Browne, 1990) have also been demonstrated to impact certain *in vitro* endpoints (Coggins and Gaworski, 2008; Irwin, 1989). Even the stalk position of the tobacco leaf has emerged as a possible influencing factor (Bombick et al., 1998). Filter ventilation (Norman, 1999) has also been reported to impact *in vitro* toxicological endpoint response (Rickert et al., 2007), but is not considered in the present study because all included *in vitro* data were obtained under a smoking regimen which requires complete blocking of ventilation holes.

Through the analysis of a single large data set collected in the frame of regulatory submissions to Canadian authorities, the

* Corresponding author.
E-mail address: Maxim.Belushkin@pmi.com (M. Belushkin).

0273-2300
http://dx.doi.org/10.1016/j.yrtph.2013.12.009

combined effects of all these parameters which have been demonstrated to have an impact on *in vitro* endpoints were evaluated.

In 2000, Canadian authorities introduced the Tobacco Reporting Regulations (Health Canada, 2000). This federal law requires each tobacco manufacturer and importer to annually provide Health Canada with by-brand information on sales data, research activities, promotional activities, manufacturing information, product ingredients, over 20 tobacco constituents and more than 40 product emissions (smoke constituent yields). This information constitutes a very detailed description of each cigarette brand, from basic design features to end-performance.

In 2005, Canadian authorities extended the reporting requirements by introducing Regulations Amending the Tobacco Reporting Regulations (Health Canada, 2005b). These additionally required three toxicity tests to be performed annually on each brand. The three *in vitro* tests selected were the Ames bacterial mutagenicity test (Health Canada, 2004a; Maron and Ames, 1983), the neutral red uptake (NRU) cytotoxicity test (Babich and Borenfreund, 1992; Borenfreund and Puerner, 1985; Health Canada, 2004b), and the micronucleus (MN) clastogenicity test (Countryman and Heddle, 1976; Fenech, 2000; Health Canada, 2004c). Initially, the Canadian authorities introduced them as a change control method, to monitor for possible unintended consequences of the then-novel requirement of reduced cigarette ignition propensity (RCIP) (Health Canada, 2005a) which affected all cigarette brands on the market. The hypothesis that RCIP paper could increase the toxicity of cigarettes has since been refuted (e.g. (World Health Organization, 2008) and references therein), but the annual *in vitro* toxicity data reporting requirement remains.

The subject of the indefinite continuation of the requirement to provide *in vitro* toxicity data as part of the yearly submission was debated. *In vitro* tests are a valuable source of information to assess the relative toxicity of novel products or novel product design features (Dempsey et al., 2011). In a public consultation, tobacco industry members suggested to the Canadian Department of Health that *"re-testing is not necessary if a product has not been modified beyond conventional parameters ..."* (Health Canada, 2005a). Nonetheless, the Canadian Department of Health concluded that *"manufacturers often make changes to their cigarettes"* (Health Canada, 2005a) and maintained their requirement for annual toxicity testing (Health Canada, 2005a,b).

The present study investigates whether annual *in vitro* toxicological testing generates information which is not reflected in the other content of the submissions to Health Canada, when product changes are confined to conventional adjustments. A conventional adjustment is defined here as a change of cigarette design features and mainstream smoke deliveries (Appendix A) within ranges typical of the investigated product set. For example, a cigarette change achieved by modifying the cigarette filter design or paper while remaining within the ranges defined by the other products in the set is a conventional adjustment, whereas the original introduction of novel requirements such as RCIP (Health Canada, 2005a) is not.

The investigation is conducted using data submitted by Rothmans, Benson & Hedges – the Canadian affiliate of Philip Morris International – to Health Canada. The database includes information submitted on 88 unique brands during the period 2006–2012. It is demonstrated, in a proof-of-concept manner, that quantitative models with substantial predictive power can be built to link the *in vitro* data with the remainder of the content of the regulatory submissions when product changes are confined to conventional adjustments. This mathematical proof does not establish any causal relationship between toxicity and cigarette design features or mainstream smoke constituent yields; correlation does not equal causation. For example, cigarette smoke consists of more than 5000 constituents the interrelationships of which would be impossible to follow. Correlations observed at this level may be indicative of very complex associations in the cigarette smoke per se, and therefore cannot be assumed to be directly in the causation line.

2. Materials and methods

2.1. Data set

All data included in the present study correspond to, or are directly derived from, data submitted to Canadian authorities in the period 2006–2012. Data submitted in the period 2006–2011 are used throughout the whole analysis, and are referred to as the prediction set. Data submitted in 2012 only serve to validate the conclusions, and are referred to as the validation set.

2.1.1. Basic product information

Basic product information reported in accordance with year 2000 regulations (Health Canada, 2000) and considered in this study is summarized in Appendix A: Cigarette attributes. It includes the submission year, some basic cigarette design features and analytical data. Design features include the blend type (dark air-cured, blended or Virginia flue-cured), the filter type (regular or activated carbon), and a cigarette diameter classification (superslim or non-superslim, see Appendix A). The combination of these three design features defines the cigarette type. The cigarette blend type and the cigarette diameter classifications were derived from the information in the submissions. One brand reported as dark air-cured was classified as such in a group of one. Brands reported as American Blend, or as containing all three of burley, Virginia flue-cured and oriental tobacco varieties, were classified as blended. All remaining brands were reported as Virginia flue-cured and classified as Virginia flue-cured. Cigarette brand variants with a cigarette paper bobbin width (which is larger than the cigarette circumference by the width of the seam) of 19 mm were classified as superslim.

Analytical data include chemical analysis of the tobacco cut filler and mainstream smoke (MS) constituent yields as determined under the Health Canada Intense (HCI) smoking regime, which specifies a puff volume of 55 mL, a puffing frequency of 2 puffs per minute, a puff duration of 2 s and the requirement that filter ventilation holes are blocked (Health Canada, 1999).

2.1.2. In vitro toxicity data

In vitro test results submitted in accordance with year 2005 regulations are referred to as *in vitro* data, and each individual assay referred to as an endpoint. These endpoints are summarized in Appendix B: *In vitro* endpoints. They include results on 10 endpoints from the Ames bacterial mutagenicity assay (5 bacterial strains, each tested with and without S9 metabolic activation) (Health Canada, 2004a; OECD, 1997); results on 3 endpoints from the NRU cytotoxicity assay (exposures to the particulate phase (PP), gas–vapor phase (GVP), and both combined (PP+GVP)) (Health Canada, 2004b); and results on 2 endpoints from the MN assay (3-h exposure with and without S9 metabolic activation) (Health Canada, 2004c). As per the regulatory requirements, all results are obtained solely with smoke fractions generated under HCI smoking conditions (Health Canada, 2004a,b,c), which is also the reason why only mainstream smoke constituent yields obtained under HCI smoking conditions are considered for analysis among the basic product information.

Regulatory submissions of *in vitro* data are required to indiscriminately contain all the quantitative information obtained and, as per regulatory requirements, no statistical treatment is performed prior to data submission. However, not all *in vitro* assays

are equally responsive to the cigarettes tested. For example, the Ames bacterial mutagenicity assay is most frequently employed to simply determine whether the test substance is mutagenic or not, and the diversity of the possible effects is assessed using a standard set of bacterial strains that are not necessarily expected to all react positively. In its application to cigarette smoke condensate, a quantitative comparison of mutagenicity values is sought; in this case slopes of the number of revertant colonies per unit mass TPM added to the bacterial culture are compared. For the present data evaluation the test is considered non-responsive if the slope of revertants per unit total particulate matter (TPM) dose is not statistically different from zero (slope of linear region rule), and if no TPM dose gives at least a twofold increase over the negative control (solvent) substance (twofold rule). Note that this combination of the two requirements yields conservative conclusions, and is selected because the twofold rule alone has limitations (Kim and Margolin, 1999). Similarly, in the micronucleus assay the relationship is considered non-responsive if the slope of micronucleated cells per unit TPM dose is not statistically significantly different from zero. Conversely, no statistical test is performed on the results from the NRU cytotoxicity assay. It is thus always considered responsive in the present study.

2.1.3. Extent of the data set

The number of brands per year per cigarette type in the data set is provided in Table 1 (Supplementary Material). Because the testing was conducted by two laboratories, differentiation by laboratory facility is additionally made. HCI 'tar' values for the brands range from 17 mg to 42 mg per cigarette, and the corresponding ISO 'tar' values range from 0.7 mg to 15 mg per cigarette. 'Tar' is calculated as the TPM yield minus the sum of the nicotine and the water yields (International Organization for Standardization, 1991).

2.2. Data normalization

In vitro toxicological data are normalized to total particulate matter (TPM) mass, a commonly used procedure to obtain *in vitro* results from mainstream smoke exposure. Indeed, Ames bacterial mutagenicity assay results are typically reported as the number of revertants observed per unit mass of TPM added to the bacterial plate (Roemer et al., 2004, 2009). The NRU cytotoxicity assay results can be reported in terms of the inverse of the 50% inhibitory concentration – mL per unit mass of TPM added to the cell culture in the case of particle-phase exposure. When assaying gas-phase components, the reporting convention is to relate the results to the mass of TPM collected alongside the tested GVP. The micronucleus clastogenicity assay results are typically reported as the number of micronucleated cells per unit mass of TPM added to the cell culture.

In vitro data reported as above are an intensive rather than an extensive property ranking the quality rather than the quantity of the TPM. When relating the *in vitro* data to MS constituent yields, the latter should also be normalized accordingly to ensure comparability. Since the MS TPM values were unavailable in the present study, the yield of each of the MS constituents was normalized to 'tar' for the purpose of all the analyses. It is noted that due to the high variability of water yield under HCI smoking conditions (Purkis et al., 2011a), normalization to 'tar' may be expected to provide more pertinent results for mechanistic investigations than normalization to TPM.

2.3. Source laboratory selection

The data set contains *in vitro* results obtained in two different laboratories: Labstat International ULC (Kitchener, Ontario, Canada) and Arista Laboratories (Richmond, Virginia, USA). Although the methods used to perform the *in vitro* studies are the same, inter-laboratory variability generates extra noise in addition to the inherent variability of the biological assays and the methods (CORESTA *In Vitro* Toxicology Task Force, 2007; Oldham et al., 2012). Thus although the analysis approach in the present study could also be applied to data obtained in different laboratories, this may reduce the robustness of results. Consequently, because Labstat is the sole laboratory used since 2011 and contributes 87% of the total data (Table 1 in Supplementary Material), only Labstat results are included in the analysis.

3. Results and discussion

Three main questions are addressed in the present study. Which *in vitro* endpoints are responsive to cigarette smoke? Do previously reported relationships between selected cigarette design features and *in vitro* response hold at the level of a broad range of products? And can quantitative models specific to the considered product range be built to predict the *in vitro* response?

3.1. Assay response rates

The first part of the study investigates which *in vitro* endpoints yield meaningful data. The response rate of an assay can be defined as the percentage of responsive entries in the data set relative to the total number of entries for that assay. The criteria for defining an entry non-responsive are discussed in Section 2.1.

As Fig. 1 demonstrates, the cytotoxicity assay is 100% responsive by definition, and the micronucleus assay is highly responsive. In the Ames assay, on the other hand, the situation is different. Indeed, bacterial strains TA102 and TA1535 are essentially non-responsive both with and without S9 metabolic activation. Without metabolic activation, strain TA100 is non-responsive and strain TA1537 is only responsive in about a quarter of all cases. This is consistent with published results: most mutagens in TPM of MS have been reported to require S9 metabolic activation, and Ames endpoints without S9, even when responsive, have been reported to yield significantly lower responses than corresponding endpoints with S9 (DeMarini, 1983; DeMarini et al., 2008; Rickert et al., 2007).

In vitro endpoints with response rates at or below the few-percent-level will not be considered further, thus the Ames TA100, TA102, TA1535, TA102 +S9 and TA1535 +S9 endpoints are removed from the analysis due to absence of data to facilitate further statistical treatment (Fig. 1). The bacterial strain TA1537 without metabolic activation (Ames TA1537 endpoint), which appears to respond to mainstream smoke condensate in about a quarter of all cases, exhibits a response which is random over all categories of cigarette brand, blend type, filter type and cigarette diameter, i.e. the pattern of non-zero responses is neither stable over time, nor correlated with any of the aforementioned cigarette design features. Therefore, the Ames TA1537 endpoint can be described as consistently being on its "limit of detection" for all brands alike, and therefore the data generated by this endpoint are best described as analytical noise. It is therefore not considered in the model.

Thus only 9 endpoints – Ames TA98, Ames TA98 +S9, Ames TA100 +S9, Ames TA1537 +S9, NRU PP, NRU GVP, NRU PP+GVP, MN and MN +S9, provide meaningful data. Summary statistics of results obtained for these endpoints in each submission year are provided in the Supplementary Material.

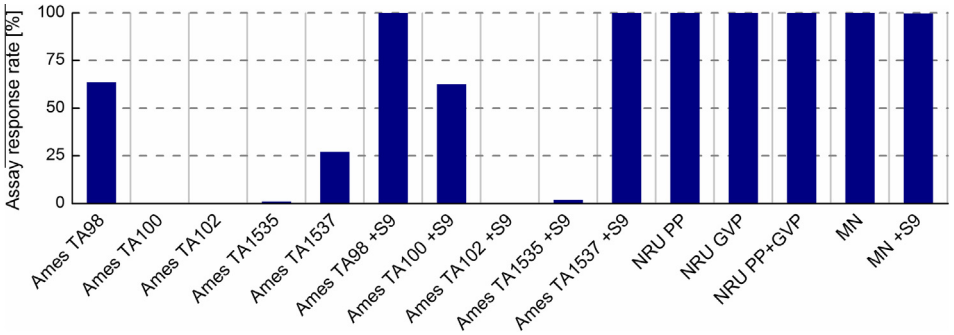

Fig. 1. Assay response rate defined here as the percentage of responsive entries relative to the total in the data set. All Ames and micronucleus assays are performed on particulate matter only. TA98, TA100, TA102, TA1535 and TA1537 are different Salmonella bacterial strains. +S9 refers to the presence of metabolic activation with the S9 enzyme fraction.

3.2. Variability of in vitro toxicity test results in time

The natural levels of variability of *in vitro* toxicity test results in Labstat have recently been reported in the form of coefficients of variance (CV) calculated over a 30-month period for the assays performed on a monitor cigarette (Wright and Rickert, 2007). These numbers are broadly consistent with the average coefficient of variance (CV) per brand found in the present study calculated over the period 2006–2011. The average CV per brand agrees within 1% with the reported 30-month CV for the Ames TA98 +S9 and MN +S9 endpoints, is lower by 5% for the Ames TA100 +S9 endpoint, and does not exceed the reported 30-month CV by more than 7% for the other endpoints.

Moreover, strong correlations exist between median values of different cigarette types across the years. This confirms that the annual differences are attributable to the long-term variability of the *in vitro* assays and methods, rather than changes in the products – it is difficult to imagine product changes affecting all brands in the same way across different cigarette types across all years. For example, the Pearson correlation coefficient between the median values of blended and Virginia flue-cured cigarette types is statistically significant and exceeds 0.8 for the TA98 +S9, TA1537 +S9, MN and MN +S9 endpoints, reaching a maximum value of 0.92 for the MN endpoint, and is at the level of 0.5–0.7 for the TA100 +S9, NRU PP, NRU GVP and NRU PP+GVP endpoints. Summary statistics for all endpoints are provided in Supplementary Material, and summary statistics for the blended and Virginia flue-cured cigarette types for three *in vitro* endpoints are additionally shown in Fig. 2.

These results together suggest that the considered product range has not undergone major changes, although it is noted that in 2009, a ban on all cigarette ingredients with the exception of processing aids such as humectants, was adopted in Canada (Health Canada, 2010). As a result, in 2010 the blended brands were reformulated, which normally results in a notable net reduction in the content of Burley and oriental tobacco varieties in the blends. The main impact of this ban was on blended cigarettes because in these cigarettes tobacco additives are mainly used to restore sugar levels of air-cured tobaccos which lose essentially all their sugar content during the curing process (Purkis et al., 2011b; Roemer et al., 2012). It is noteworthy that this reformulation does not appear to have impacted any of the *in vitro* endpoint responses to the blended cigarettes (Fig. 2 and Supplementary Material).

3.3. General relationships between in vitro data and cigarette design features

A number of studies have explored the individual influence of such cigarette design features as the blend type (Bombick et al., 1998; DeMarini et al., 2008; Richter et al., 2010; Rickert et al., 2007; Roemer et al., 2004, 2009; Roeper et al., 2004; Schramke et al., 2006; Yauk et al., 2012), filter type (Coggins and Gaworski, 2008) and cigarette diameter (Irwin, 1989) on *in vitro* toxicity. Here, they are confirmed within a broad product range where different combinations of all the influencing factors are present at the level of individual brands.

3.3.1. Discriminatory power of individual in vitro endpoints

Discriminatory power of a toxicological endpoint is defined here as that endpoint's ability to distinguish between different cigarette types. Fig. 3 presents a comparison of the magnitudes of the responses of each of the *in vitro* endpoints to the different cigarette types, averaging over all brands of a given type and all prediction years.

The Ames TA98 and MN +S9 endpoints both fail to differentiate between the basic cigarette design features. This does not imply that these two endpoints may not be useful in change control stud-

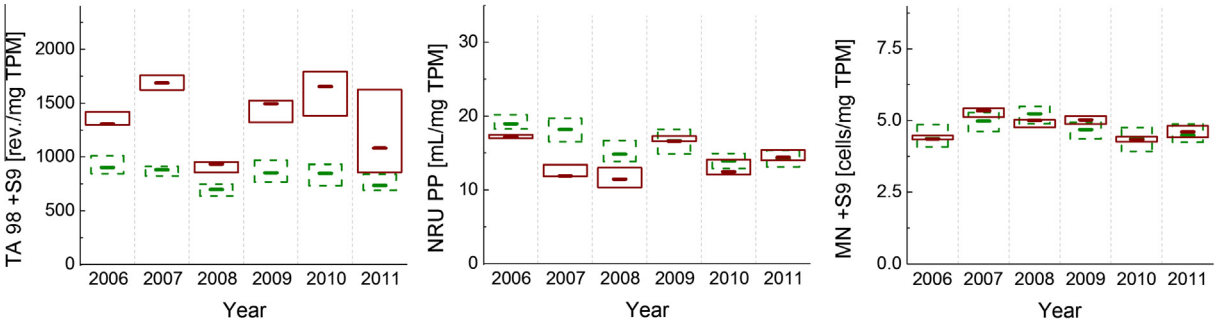

Fig. 2. *In vitro* endpoint response in different years averaged over all blended brands (solid red) and Virginia flue-cured brands (non activated carbon filter, non-superslim diameter) (dashed green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

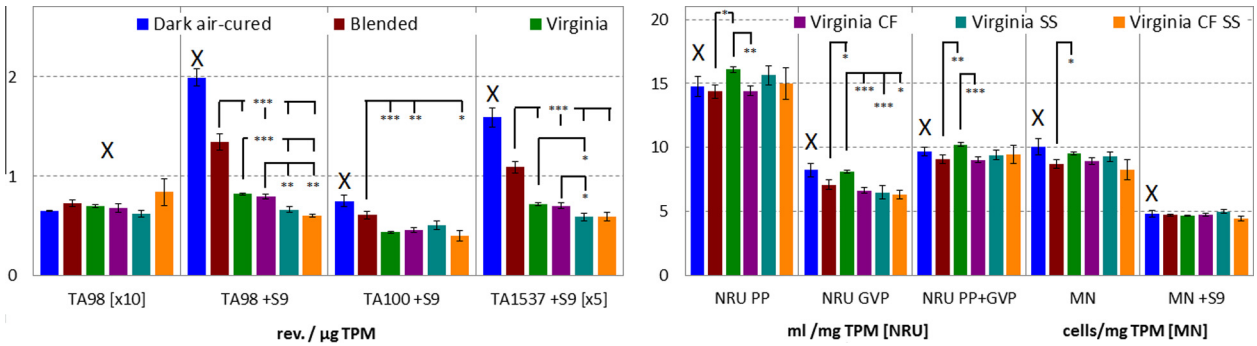

Fig. 3. Discriminatory power of the different *in vitro* assays in terms of cigarette blend and design features. Bars show values averaged over all brands and years 2006–2011 for the designated cigarette type, and error bars show the corresponding standard errors. Differences which are statistically significant in a *t*-test for differences are shown with a $^{*}(p < 0.05)$, $^{**}(p < 0.01)$ or $^{***}(p < 0.001)$ symbol, respectively. '*X*' denotes insufficient data for statistical treatment. CF stands for 'activated carbon filter'. SS stands for 'superslim diameter'.

ies, such as those regularly conducted at Philip Morris International (Dempsey et al., 2011). However, it is apparent that both endpoints hardly distinguish between the different cigarette types, and they are insensitive to any changes which may have been made on the brands over the 6-year period considered.

The results for the other Ames endpoints are consistent with published literature. The cigarette blend plays a major role in determining the test result, and mutagenicity is higher for blended cigarettes than for the Virginia flue-cured blend cigarettes (Fig. 3, left), in agreement with published data (Rickert et al., 2007; Roemer et al., 2004). Indeed, Burley cigarette tobacco smoke is reported to exhibit a higher mutagenicity than Virginia flue-cured tobacco smoke (Roeper et al., 2004; Schramke et al., 2006), with blended cigarettes in between. The superslim cigarette diameter also appears to have the expected (Irwin, 1989) reduction on the Ames results (Fig. 3, right). There is no observable effect of activated carbon filtration on the Ames bacterial mutagenicity results. Indeed, activated carbon largely affects the gas–vapor phase of smoke (Branton and Bradley, 2011; Gaworski et al., 2009), whereas Ames assay studies are conducted with the particulate phase of smoke.

The ability of the cytotoxicity assay to differentiate between different cigarette blends is also well-described in the literature. The ranking compared to Ames mutagenicity is reversed, namely Virginia flue-cured tobacco evokes a higher response in the NRU cytotoxicity assay than Burley tobacco (Bombick et al., 1998; Richter et al., 2010; Rickert et al., 2007; Roemer et al., 2004, 2009; Roeper et al., 2004). This is also reflected in the present study, whereby Virginia flue-cured cigarettes generally evoke a higher cytotoxicity assay response than blended cigarettes (Fig. 3, right). Activated carbon filtration has an impact on the composition of the gas–vapor phase, which affects results for activated carbon filter cigarettes assayed for NRU GVP and NRU PP+GVP endpoints (Fig. 3), consistent with previously reported results (Coggins and Gaworski, 2008).

The micronucleus assay has been reported to show very low by-blend specificity (DeMarini et al., 2008; Yauk et al., 2012). This is confirmed in the present study (Fig. 3, right). Indeed, the MN +S9 endpoint is essentially constant both in time (see Supplementary Material and Fig. 2) and across different cigarette types (Fig. 3, right), and the MN endpoint is barely an improvement. Furthermore, both endpoints appear sensitive neither to the effects of activated carbon filtration nor to cigarette diameter.

3.3.2. Correlations between in vitro results and mainstream smoke constituents

Authors have often pointed to strong correlations between certain smoke constituent classes and *in vitro* toxicity test results.

In the Ames assay, strong correlations between the *in vitro* results and levels of nonvolatile polycyclic nitrogen compounds in MS have been reported (DeMarini et al., 2008; Rickert et al., 2007; Yauk et al., 2012). These correlations are confirmed in the present study for Ames endpoints with S9 metabolic activation: the Pearson correlation coefficient between the *in vitro* response and levels of nitrogen oxides in mainstream smoke – a proxy for other nitrogen-containing compounds (Piadé et al., 2013), is 0.83 for the TA98 +S9 endpoint, 0.51 for the TA100 +S9 endpoint, and 0.73 for the TA1537 +S9 endpoint. Additionally, a previously reported negative correlation between mutagenicity in the Ames assay and formaldehyde (Rickert et al., 2011) is confirmed (Pearson correlation coefficients −0.75 for the TA98 +S9 endpoint, −0.53 for the TA100 +S9 endpoint, and −0.63 for the TA1537 +S9 endpoint). It can be attributed to reported negative correlations between the yields of nitrogen-containing compounds and formaldehyde (Piadé et al., 2013). These results are illustrated in Fig. 4.

In addition, the separation between non-superslim and superslim cigarettes in Ames results (Fig. 4) coincides with the reported lower deliveries of aromatic amines from superslim cigarettes compared to non-superslim cigarettes (Siu et al., 2013).

In the NRU cytotoxicity assay, a strong correlation between the levels of acrolein and NRU GVP endpoint has been reported (Tewes et al., 2003). However, the CV of acrolein (per mg 'tar') in the considered range of products is less than 10%, and consequently this correlation cannot be easily confirmed (Pearson correlation coefficient 0.14).

3.4. Predictive power

The previously reported influence of cigarette blend, diameter and filter type on the response of the considered toxicological endpoints has been confirmed at the level of a representative product range. Furthermore, a number of previously reported correlations between *in vitro* toxicity endpoints and smoke constituent yields are also confirmed. The remaining question is whether this information is sufficient to construct a quantitative model which would be capable of effectively predicting *in vitro* endpoint response. A modeling approach was thus performed to investigate to what extent *in vitro* toxicity response can be explained quantitatively from the remainder of the information present in the product information submissions.

It should already be noted that the purpose of the present study is not to provide quantitative models for all *in vitro* endpoints. Rather, it is to investigate the possibility of constructing useful models. In effect, the existence of a quantitative predictive model would provide evidence that *in vitro* data do not add further information on top of the other content of the mandatory submis-

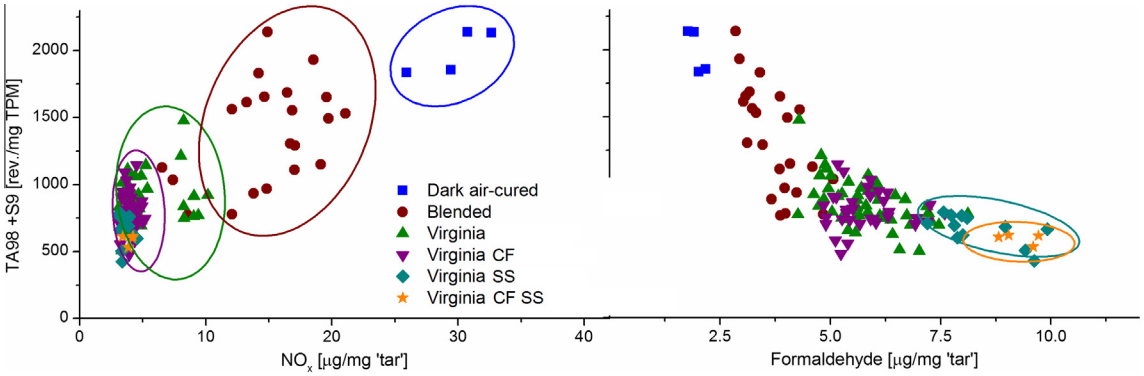

Fig. 4. (Left) correlations between mutagenicity in the Ames assay and nitrogen-containing compounds can be visualized by studying one of the Ames endpoints in terms of mainstream smoke yields of nitrogen oxides – a proxy for other nitrogen-containing compounds (Piadé et al., 2013). The clustering of dark air-cured, blended and Virginia flue-cured cigarettes (Rickert et al., 2007; Roemer et al., 2004; Roeper et al., 2004; Schramke et al., 2006) is confirmed. (Right) apparent negative correlation between formaldehyde and mutagenicity in the Ames assay reported previously (Rickert et al., 2011) can be explained by negative correlations between formaldehyde and nitrogen-containing compounds (Dempsey et al., 2011; National Cancer Institute, 1976; Piadé et al., 2013). CF stands for 'activated carbon filter'. SS stands for 'superslim diameter'.

sions to Health Canada, which would be used as the predictors of the model.

3.4.1. Quantitative models: proof of concept

According to the estimate of the natural variability of the biological assays and methods (Wright and Rickert, 2007) and average brand CV as discussed in Section 3.2, a good model of the data can be expected to explain up to 70–80% of the variance. This can indeed be achieved. For example, an analysis of variance (ANOVA) of the Ames TA98 +S9 endpoint shows that one factor, namely cigarette type (one of dark air-cured, blended, Virginia flue-cured, Virginia flue-cured activated carbon filter, Virginia flue-cured superslim diameter, Virginia flue-cured activated carbon filter superslim diameter) accounts for about 60% of variance in the data. Adding the MS yields of nitrogen oxides (NO_x) and the four aromatic amines (1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl and 4-aminobiphenyl) as predictors to the linear model accounts for about 80% of the variance, precisely in line with the expectations for a good model. A comparison of the values predicted from this linear model to the measured values is shown in Fig. 5 (left). The predictive set of data – *in vitro* toxicity results submitted in years 2006–2011, are the data on which the linear model is built. The year 2012 data serve to validate the model, as they are not included in the regression. To judge the goodness-of-fit of the model, R^2 values are calculated. In the case of the Ames TA98 +S9 endpoint, $R^2 = 0.83$ for the predictive set, and the corresponding adjusted R^2 that adjusts for the number of model parameters versus the number of data points is $R^2_{adj} = 0.75$. R^2 is 0.83 also for the validation set (Fig. 5, left), confirming that the variance unaccounted for is comparable to the expected natural variability.

The situation is different for *in vitro* endpoints where the assay and method variability is close to the total CV of all products, i.e. when the total variability of the response across all the different brands is comparable to the expected natural variability. This is, for example, the case for the NRU PP+GVP endpoint. A model of these data can be constructed by means of a mathematically-driven approach (see e.g. Seilkop et al., 2012) using different combinations of MS constituent yields representative of particle- and gas-phase compounds. For example, a model using the cigarette type and the MS yields of phenol, CO and 4-aminobiphenyl does capture the inherent trends, but the variability of the assay is larger than the observed effect. The results of this model are presented in Fig. 5, right. In this case, the adjusted R^2 value is only 0.3. It is stressed that these smoke constituents are chosen solely for their strong mathematical correlation with the NRU PP+GVP endpoint data, and that other combinations of smoke constituents can provide similar results.

Quantitative models for the other endpoints can be constructed in a similar fashion. As detailed above for the two extreme cases,

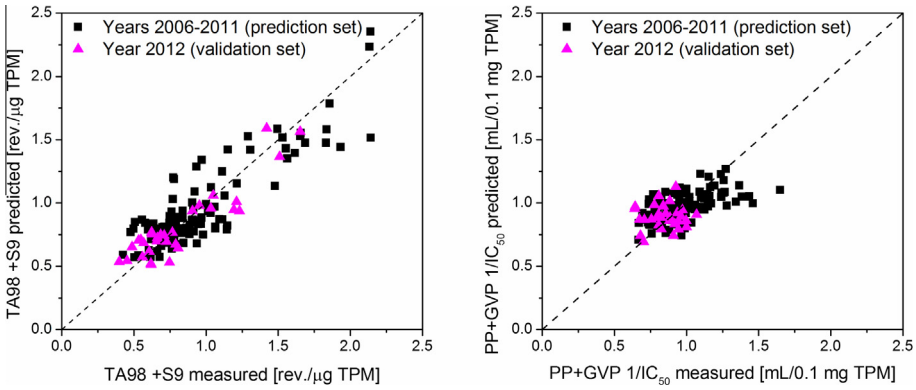

Fig. 5. Linear model results comparing predicted and measured values in the Ames TA98 +S9 toxicity endpoint (left) and NRU PP+GVP toxicity endpoint (right). The Ames linear model includes cigarette type (blend type, diameter and filter type) and the MS yields of nitrogen oxides (NO_x) and the four aromatic amines. The NRU linear model includes cigarette type and the yields of three constituents which are representative of gas-phase and particle-phase constituent yields: phenol, CO and 4-aminobiphenyl. Black squares show the year 2006–2011 data from which the linear model is built (predictive set). Magenta squares show the year 2012 data which were not included in the regression (validation set), and are thus a prediction. The limiting factor for modeling is the 20%-level relative variability of the assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the corresponding goodness-of-fit measures depend mainly on the magnitude of the overall variance of the corresponding *in vitro* endpoint.

In principle, such modeling makes quantitative predictions for each *in vitro* toxicity endpoint, and the typical error estimate is a direct consequence of the variance of the assay and method, at a level of about 20%. However, the model relies on correlations which should not be confused with causation. Specifically, the MS constituents selected for the models described above are not necessarily linked to any of the *in vitro* endpoints in a cause-and-effect manner. For example, the model for the TA98 +S9 endpoint is *"response = Const + 0.004*NO_x + 0.117*1AN + 0.166*2AN + 7.450*3-AB − 3.037*4AB"*, where NO_x, 1AN, 2AN, 3AB and 4AB stand for tar-normalized yields of nitrogen oxides, 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl and 4-aminobiphenyl expressed in μg/mg tar (NO_x) and ng/mg tar (aromatic amines), respectively, and the type-specific constant "Const" is 0.203469 for blended, −0.085512 for dark air-cured, 0.133976 for Virginia flue-cured, 0.119047 for Virginia flue-cured activated carbon filter, 0.012423 for Virginia flue-cured activated carbon filter superslim, and 0.076298 for Virginia flue-cured superslim cigarettes. The units of the model parameters are such that the resulting response has the units of revertants/μg TPM. However, it has been shown that the actual contribution to the overall mutagenicity of a broad set of compounds that includes those appearing in the equation above is smaller than 0.1% (Tewes et al., 2003). For the purpose of the present study, many other combinations of smoke constituents, none of which would be necessarily linked to mutagenicity in a cause-and-effect manner, could have been used to achieve comparable predictive power. Conversely, not all compounds which are known to cause a significant portion of the magnitude of the response of a given end-point are useful predictors. For example, acrolein which is known to contribute over 40% to the cytotoxicity of the gas–vapor phase (Tewes et al., 2003) is not a useful predictor of this endpoint in the present study. This is likely because the CV of acrolein yields (per mg 'tar') across all brands is very small, under 10%. A likely reason for this is the fact that the smoke data are obtained under the HCI smoking regime, whereby adsorbance effects in the cigarette filter are limited (Purkis et al., 2010a,b).

Fundamentally, the use of the model is restricted to the range of parameters on which it has been established.

4. Conclusions

Data for 15 *in vitro* toxicological endpoints mandated for reporting by Canadian regulatory authorities was considered in the present study for a representative product range comprised of 88 unique brands, over a 7-year period. It was demonstrated that only 7 of these *in vitro* endpoints (Ames TA98 +S9, Ames TA100 +S9, Ames TA1537 +S9, NRU PP, NRU GVP, NRU PP+GVP and MN) provide data which differentiates between some fundamental cigarette design features. It was further shown that in the absence of meaningful product changes, the response can be predicted up to the level of natural variability of the biological assays and methods.

In vitro toxicological studies are useful for the assessment of novel products or product modifications (Dempsey et al., 2011), but the 7 years of *in vitro* toxicity data investigated in the present study do not provide additional information further to that contained in submissions according to the original Health Canada requirements (Health Canada, 2000) because the products had been subjected only to conventional adjustments. The introduction of novel product design features would readily be reflected in the submissions according to the original Health Canada requirements.

It should be noted that the response in *in vitro* toxicity assays, in particular *in vitro* data for cigarette smoke, is generally not considered to be directly predictive of health outcome (Johnson et al., 2009), and thus also predictions of *in vitro* toxicological data cannot be related directly to predictions of health outcome. Consistent with the literature, in the present analysis there are differences in the response of blended (higher in Ames bacterial mutagenicity test) or Virginia flue-cured (higher in NRU cytotoxicity test) products in different *in vitro* assays, but this does not appear to be the case for health outcome at the population level. Epidemiological studies in predominantly Virginia flue-cured or predominantly blended cigarette markets have shown that the cigarette type does not appear to influence the risk of lung cancer or chronic obstructive pulmonary disease (Lee et al., 2009).

In summary, the present study demonstrates that when no novel design features are introduced within a range of cigarette products, e.g. when changes are limited to cigarette blend, filter design or cigarette format, *in vitro* toxicity re-testing does not provide any knowledge in addition to that contained already in the content of submissions to Health Canada (Health Health Canada, 2000).

Conflict of interest

Maxim Belushkin and Jean-Jacques Piadé are both employees of Philip Morris International. Steve Chapman and George Fazekas are both employees of Rothmans, Benson & Hedges Inc.

Appendix A. Cigarette attributes

Basic information, design features			
Year	Submission year	Cigarette format[a]	Non-superslim or superslim
Blend type[b]	Dark air-cured, blended, Virginia flue-cured	Filter type	Regular or activated carbon
Cut filler constituents (per gram of tobacco)			
Nicotine	μg/g	Arsenic	ng/g
Nornicotine	μg/g	Selenium	ng/g
Anabasine	μg/g	Mercury	ng/g
Myosmine	μg/g	Nitrate	mg/g
Anatabine	μg/g	NNN	ng/g
Ammonia	μg/g	NAT	ng/g
Propylene glycol	mg/g	NAB	ng/g
Glycerol	mg/g	NNK	ng/g
Triethylene glycol	mg/g	Sodium propionate	μg/g
Cadmium	ng/g	Triacetin	μg/g
Chromium	ng/g	Sorbic acid	μg/g
Nickel	ng/g	Benzo[a]pyrene	ng/g
Lead	ng/g		
Mainstream smoke constituents (Health Canada Intense smoking regime, per mg 'tar')			
Tar	mg	1-aminonaphthalene	ng/mg
Nicotine	mg/mg	2-aminonaphthalene	ng/mg
CO	mg/mg	3-aminobiphenyl	ng/mg
Formaldehyde	μg/mg	4-aminobiphenyl	ng/mg
Acetaldehyde	μg/mg	Ammonia	μg/mg

Acetone	µg/mg	1,3-Butadiene	µg/mg
Acrolein	µg/mg	Isoprene	µg/mg
Propionaldehyde	µg/mg	Acrylonitrile	µg/mg
Crotonaldehyde	µg/mg	Benzene	µg/mg
Methyl ethyl ketone	µg/mg	Toluene	µg/mg
Butylaldehyde	µg/mg	Pyridine	µg/mg
Hydroquinone	µg/mg	Quinoline	µg/mg
Resorcinol	µg/mg	Styrene	µg/mg
Catechol	µg/mg	NNN	ng/mg
Phenol	µg/mg	NAT	ng/mg
m,p-cresols	µg/mg	NAB	ng/mg
o-cresol	µg/mg	NNK	ng/mg
Benzo[a]pyrene	ng/mg	Mercury	ng/mg
NO	µg/mg	Cadmium	ng/mg
NO_x	µg/mg	Lead	ng/mg
HCN	µg/mg		

CO – carbon monoxide; NO – nitrogen oxide; NO_x – nitrogen oxides; HCN – hydrogen cyanide; NNN – N′-nitrosonornicotine; NNK – 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT – N′-nitrosoanatabine; NAB – N′-nitrosoanabasine.

[a] Brands with a cigarette paper bobbin width of 19 mm were classified superslim.

[b] One brand reported as dark air-cured was classified dark air-cured. Brands reported as American Blend, or containing all three of Burley, oriental and Virginia flue-cured tobacco varieties were classified as blended. All other brands were reported as Virginia flue-cured and thus classified as Virginia flue-cured.

Appendix B. *In vitro* endpoints

Ames bacterial mutagenicity assay (Ames)

Bacterial strain	S9 metabolic activation	Bacterial strain	S9 metabolic activation
TA98	No (Ames TA98)	TA98	Yes (Ames TA98 +S9)
TA100	No (Ames TA100)	TA100	Yes (Ames TA100 +S9)
TA102	No (Ames TA102)	TA102	Yes (Ames TA102 +S9)
TA1535	No (Ames TA1535)	TA1535	Yes (Ames TA1535 +S9)
TA1537	No (Ames TA1537)	TA1537	Yes (Ames TA1537 +S9)

Neutral red uptake cytotoxicity assay (NRU): smoke fraction

Particulate phase (NRU PP)
Gas–vapour phase (NRU GVP)
Particulate and gas–vapour phase (NRU PP+GVP)

Micronucleus assay (MN)

Without S9 metabolic activation (MN)
With S9 metabolic activation (MN +S9)

In principle, a requirement exists to report an additional endpoint in the micronucleus clastogenicity assay, namely 30-h exposure without S9 metabolic activation (Health Canada, 2004c). This endpoint is not included in the present analysis due to absence of data, since according to regulations it would only need to be reported if the 3-h period of treatment, both with and without S9 metabolic activation, would yield "negative or equivocal results" (Health Canada, 2004c).

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2013.12.009.

References

Babich, H., Borenfreund, E., 1992. Cytotoxic and morphological effects of phenylpropanolamine, caffeine, nicotine, and some of their metabolites studied *in vitro*. Toxicol. In Vitro 6, 493–502.

Baker, R.R. et al., 2004. An overview of the effects of tobacco ingredients on smoke chemistry and toxicity. Food Chem. Toxicol. 42, S53–S83.

Bombick, D.W. et al., 1997. Evaluation of the genotoxic and cytotoxic potential of mainstream whole smoke and smoke condensate from a cigarette containing a novel carbon filter. Fundam. Appl. Toxicol. 39, 11–17.

Bombick, D.W. et al., 1998. Comparative cytotoxicity studies of smoke condensates from different types of cigarettes and tobaccos. Toxicol. In Vitro 12, 241–249.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Branton, P., Bradley, R., 2011. Effects of active carbon pore size distributions on adsorption of toxic organic compounds. Adsorption 17, 293–301.

Brownawell, A., 2007. The LSRO Report on Biological Effects Assessment in the Evaluation of Potential Reduced-Risk Tobacco Products. Life Sciences Research Office, Bethesda, MA.

Browne, C.L., 1990. Design of Cigarettes. Hoechst Celanese.

Byrd, D., 2004. The LSRO Report on Review of Ingredients Added to Cigarettes: Phase One: The Feasibility of Testing Ingredients Added to Cigarettes. Life Sciences Research Office, Rockville, MD.

Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 1: cigarette design, testing approach, and review of results. Food Chem. Toxicol. 40, 77–91.

Coggins, C.R.E., Gaworski, C.L., 2008. Could charcoal filtration of cigarette smoke reduce smoking-induced disease? A review of the literature. Regul. Toxicol. Pharmacol. 50, 359–365.

CORESTA In Vitro Toxicology Task Force, 2004. The rationale and strategy for conducting *in vitro* toxicology testing of tobacco smoke. Available from: <http://www.coresta.org/Reports/IVT_TF_Rationale-IVT-Testing-Tob.-Smoke_Report_Jun04.pdf> (last accessed 06/11/2013).

CORESTA In Vitro Toxicology Task Force, 2007. Interlaboratory study of the *in vitro* toxicity of particulate matter from four cigarettes. Available from: http://www.coresta.org/Reports/Reports.htm (last accessed 19/09/2013), CORESTA.

Countryman, P.I., Heddle, J.A., 1976. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. Mutat. Res. 41, 321–332.

DeMarini, D.M., 1983. Genotoxicity of tobacco smoke and tobacco smoke condensate. Mutation Res. 114, 59–89.

DeMarini, D.M. et al., 2008. Genotoxicity of 10 cigarette smoke condensates in four test systems: comparisons between assays and condensates. Mutation Res. 650, 15–29.

Dempsey, R. et al., 2011. Toxicological assessment of cigarette ingredients. Regul. Toxicol. Pharmacol. 61, 119–128.

Doolittle, D.J. et al., 1990. Comparative studies on the genotoxic activity of mainstream smoke condensate from cigarettes which burn or only heat tobacco. Environ. Mol. Mutagen. 15, 93–105.

Fenech, M., 2000. The *in vitro* micronucleus technique. Mutation Res. 455, 81–95.

Gaworski, C.L. et al., 2009. Effect of filtration by activated charcoal on the toxicological activity of cigarette mainstream smoke from experimental cigarettes. Inhalation Toxicol. 21, 688–704.

Health Canada, 2000. Tobacco reporting regulations (SOR/DORS/2000-273). Can. Gazette Part II 134, 1761–1792.

Health Canada, 2004a. Official method T-501, bacterial reverse mutation assay for mainstream tobacco smoke.

Health Canada, 2004b. Official method T-502, neutral red uptake assay for mainstream tobacco smoke.

Health Canada, 2004c. Official method T-503, *in vitro* micronucleus assay for mainstream tobacco smoke.

Health Canada, 2005a. Cigarette ignition propensity regulations (SOR/2005-178). Can. Gazette Part II 139, 1505–1522.

Health Canada, 2005b. Regulations amending the tobacco reporting regulations (SOR/DORS/2005-179). Can. Gazette Part II 139, 1523–1526.

Health Canada, 2010. Bill C-32 (Chapter 27). Can. Gazette III 32.

Health Canada, 1999. Determination of "tar", nicotine and carbon monoxide in mainstream tobacco smoke (Health Canada method T-115. Available from: <http://www.hc-sc.gc.ca/hc-ps/alt_formats/hecs-sesc/pdf/tobac-tabac/legislation/reg/indust/method/_main-principal/nicotine-eng.pdf>, (last accessed 08/02/2013).

International Organization for Standardization, Geneva, Switzerland, ISO 4387: determination of total and nicotine-free dry particulate matter using routine analytical smoking machine, 1991.

Irwin, W.D.E., 1989. The effects of circumference on mainstream deliveries and composition: progress report 2. British American Tobacco Report No: RD.2135.

Johnson, M.D. et al., 2009. Evaluation of *in vitro* assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol. Biomark. Prev. 18, 3263–3304.

Kim, B.S., Margolin, B.H., 1999. Statistical methods for the Ames Salmonella assay: a review. Mutat. Res. 436, 113–122.

Klaassen, C.D., Eaton, D.L., 1991. Principles of toxicology. Casarett Doull's Toxicol. 4, 12–49.

Lee, P.N. et al., 2009. Does use of flue-cured rather than blended cigarettes affect international variation in mortality from lung cancer and COPD? Inhalation Toxicol. 21, 404–430.

Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173–215.
National Cancer Institute, Toward less hazardous cigarettes: Report No. 1. The first set of experimental cigarettes, U.S. Government Printing Office: Washington DC., 1976.
Norman, A., 1999. Cigarette design and materials. Tobacco. Production, Chemistry and Technology. 353–387.
OECD, 1997. Test No. 471: Bacterial Reverse Mutation Test. OECD Guidelines for the Testing of Chemicals/Section 4: Health Effects. OECD Publishing.
Oldham, M.J. et al., 2012. Discriminatory power of standard toxicity assays used to evaluate ingredients added to cigarettes. Regul. Toxicol. Pharmacol. 62, 49–61.
Paschke, T. et al., 2002. Effects of ingredients on cigarette smoke composition and biological activity: a literature overview. Beiträge zur Tabakforschung Int. 20, 107–247.
Piadé, J.J. et al., 2013. Formation of mainstream cigarette smoke constituents prioritized by the World Health Organization – Yield patterns observed in market surveys, clustering and inverse correlations. Food Chem. Toxicol. 55, 329–347.
Purkis, S.W. et al., 2010a. The influence of cigarette designs and smoking regimes on vapour phase yields. Beitrage zur Tabakforschung Int. Contribut. Tobacco Res. 24, 33–46.
Purkis, S.W. et al., 2010b. Limitations in the characterisation of cigarette products using different machine smoking regimes. Regul. Toxicol. Pharmacol. 58, 501–515.
Purkis, S.W. et al., 2011a. Some consequences of using cigarette machine smoking regimes with different intensities on smoke yields and their variability. Regul. Toxicol. Pharmacol. 59, 293–309.
Purkis, S.W. et al., 2011b. The fate of ingredients in and impact on cigarette smoke. Food Chem. Toxicol. 49, 3238–3248.
Richter, P.A. et al., 2010. Cytotoxicity of eight cigarette smoke condensates in three test systems: comparisons between assays and condensates. Regul. Toxicol. Pharmacol. 58, 428–436.
Rickert, W.S. et al., 2007. Effect of smoking conditions and methods of collection on the mutagenicity and cytotoxicity of cigarette mainstream smoke. Toxicol. Sci. 96, 285–293.
Rickert, W.S. et al., 2011. Mutagenic, cytotoxic, and genotoxic properties of tobacco smoke produced by cigarillos available on the Canadian market. Regul. Toxicol. Pharmacol. 61, 199–209.
Roemer, E. et al., 1998. Discrimination of cigarette mainstream smoke condensates with the Salmonella reverse mutation assay. Toxicol. Sci. 42, 295.
Roemer, E. et al., 2004. Chemical composition, cytotoxicity and mutagenicity of smoke from US commercial and reference cigarettes smoked under two sets of machine smoking conditions. Toxicology 195, 31–52.
Roemer, E. et al., 2009. Cytotoxicity, mutagenicity, and tumorigenicity of mainstream smoke from three reference cigarettes machine-smoked to the same yields of total particulate matter per cigarette. Food Chem. Toxicol. 47, 1810–1818.
Roemer, E. et al., 2012. Scientific assessment of the use of sugars as cigarette tobacco ingredients: a review of published and other publicly available studies. Crit. Rev. Toxicol. 42, 244–278.
Roeper, W. et al., 2004. 'Hoffmann' analytes and cigarette smoke *in vitro* toxicity revisited–how do the data compare? CORESTA, Kyoto Congress.
SCHER/SCENIHR/SCCS, 2013. Scientific Committee on Health and Environmental Risks/Scientific Committee on Emerging and Newly Identified Health Risks/Scientific Committee on Consumer Safety, Addressing the New Challenges for Risk Assessment. Available from: <http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_131.pdf> (last accessed 06/11/2013).
Schramke, H. et al., 2006. The mouse lymphoma thymidine kinase assay for the assessment and comparison of the mutagenic activity of cigarette mainstream smoke particulate phase. Toxicology 227, 193–210.
Seilkop, S.K. et al., 2012. Identification of chemical components of combustion emissions that affect pro-atherosclerotic vascular responses in mice. Inhalation Toxicol. 24, 270–287.
Siu, M. et al., 2013. The analysis of mainstream smoke emissions of Canadian 'super slim' cigarettes. Tobacco Control 22, e10.
Tewes, F.J. et al., 2003. Toxicological evaluation of an electrically heated cigarette. Part 3: genotoxicity and cytotoxicity of mainstream smoke. J. Appl. Toxicol. 23, 341–348.
Thayer, P.S., Kensler, C.J., 1964. Cigarette smoke: charcoal filters reduce components that inhibit growth of cultured human cells. Science 146, 642–644.
Tso, T.C., 1999. Seed to Smoke. Blackwell Science Ltd.
Vanparys, P. et al., 2012. Application of *in vitro* cell transformation assays in regulatory toxicology for pharmaceuticals, chemicals, food products and cosmetics. Mutation Res. 744, 111–116.
World Health Organization, 2008. The Scientific Basis of Tobacco Product Regulation. WHO Technical Report Series. 951.
Wright, W., Rickert, W.S., Stability of responses of Canadian mandated toxicological assays from the Kentucky reference cigarette 2R4F: a time series analysis. In: 61st Tobacco Science Research Conference, 22 – 27 September 2007, Charlotte, North Carolina, U.S.A, 2007.
Yauk, C.L. et al., 2012. Genetic toxicology and toxicogenomic analysis of three cigarette smoke condensates *in vitro* reveals few differences among full-flavor, blonde, and light products. Environ. Mol. Mutagen. 53, 281–296.
Zenzen, V. et al., 2012. Reduced exposure evaluation of an Electrically Heated Cigarette Smoking System. Part 2: smoke chemistry and *in vitro* toxicological evaluation using smoking regimens reflecting human puffing behavior. Regul. Toxicol. Pharmacol. 64, S11–S34.