Mainstream Smoke Chemistry and *In Vitro* and *In Vivo* Toxicity of the Reference Cigarettes 3R4F and 2R4F *

by

Ewald Roemer [1], Heike Schramke [1], Horst Weiler [2], Ansgar Buettner [3], Sandra Kausche [2], Susanne Weber [2], An Berges [4], Markus Stueber [2], Monja Muench [2], Edgar Trelles-Sticken [2], Jan Pype [4], Karola Kohlgrueber [2], Hartmut Voelkel [2], and Sandra Wittke [2]

[1] *Philip Morris International Operations, Philip Morris Products S.A., Rue des Usines 90, 2000 Neuchâtel, Switzerland*
[2] *Philip Morris International R&D, Philip Morris Research Laboratories GmbH, Fuggerstr. 3, 51149 Cologne, Germany*
[3] *Histovia GmbH, Schoene Aussicht 5, 51491 Overath, Germany*
[4] *Philip Morris International R&D, Philip Morris Research Laboratories bvba, Grauwmeer 14, 3001 Leuven, Belgium*

SUMMARY

A new reference cigarette, the 3R4F, has been developed to replace the depleting supply of the 2R4F cigarette. The present study was designed to compare mainstream smoke chemistry and toxicity of the two reference cigarettes under the International Organization for Standardization (ISO) machine smoking conditions, and to further compare mainstream smoke chemistry and toxicological activity of the 3R4F cigarette by two different smoking regimens, i.e., the machine smoking conditions specified by ISO and the Health Canada intensive (HCI) smoking conditions.

The *in vitro* cytotoxicity and mutagenicity was determined in the neutral red uptake assay, the *Salmonella* reverse mutation assay, and the mouse lymphoma thymidine kinase assay. Additionally, a 90-day nose-only inhalation study in rats was conducted to assess the *in vivo* toxicity. The comparison of smoke chemistry between the two reference cigarettes found practically the same yields of total particulate matter (TPM), 'tar', nicotine, carbon monoxide, and most other smoke constituents. For both cigarettes, the *in vitro* cytotoxicity, mutagenicity, and *in vivo* toxicity showed the expected smoke-related effects compared to controls without smoke exposure. There were no meaningful differences between the 2R4F and 3R4F regarding these toxicological endpoints. The assessments for the 3R4F cigarette by smoking regimen found as a trivial effect, due to the higher amount of smoke generated per cigarette under HCI conditions, an increased yield of toxicant and higher toxicological activity per cigarette. However, per mg TPM, 'tar', or nicotine, the amounts of toxicants and the *in vitro* toxicity were generally lower under HCI conditions, but the *in vivo* activity was not different between the two machine smoking conditions.

Overall, as the main result, the present study suggests equivalent smoke chemistry and *in vitro* and *in vivo* toxicity for the 2R4F and 3R4F reference cigarettes. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

ZUSAMMENFASSUNG

Eine neue Referenzzigarette 3R4F ist entwickelt worden, die die 2R4F Zigarette ersetzen soll, da deren Vorrat erschöpft ist. In der vorliegenden Studie sollten die Chemie des Hauptstromrauches und die Toxizität beider Referenzzigaretten unter den maschinellen Abrauchbedingungen der Internationalen Organisation für Normung (ISO) verglichen werden. Des Weiteren sollten die Chemie des Hauptstromrauches und dessen Toxizität für die 3R4F Zigarette unter den ISO-Abrauchbedingungen mit denen unter den Bedingungen, wie sie Health Canada (HCI) spezifiziert, verglichen werden.

Die *in vitro*-Zytotoxizität und -Mutagenität wurden im Neutralrot-Aufnahme-Test, dem *Salmonella*-Rückmutations-Test und dem Maus-Lymphoma-Thymidin-Kinase-Test bestimmt. Zusätzlich wurde in einer 90-Tage-Inhalationsstudie an Ratten die *in vivo*-Toxizität

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bestimmt. Der Vergleich beider Referenzzigaretten ergab keine wesentlichen Unterschiede in der Ausbeute an Partikelphase (TPM), Teer, Nikotin, Kohlenmonoxid und den meisten anderen Rauchbestandteilen. Die *in vitro*-Zytotoxizität und -Mutagenität und *in vivo*-Toxizität zeigten, verglichen mit den Kontrollen ohne Rauchexposition, für beide Zigaretten die erwarteten Raucheffekte. Es ergaben sich keine erwähnenswerten Unterschiede zwischen der 2R4F und der 3R4F Zigarette bezüglich dieser toxikologischen Endpunkte. Beim Vergleich der Abrauchbedingungen ergaben sich, bedingt durch die größere Rauchmenge, die unter HCI-Bedingungen generiert wurde, als trivialer Befund auch größere Mengen an toxischen Rauchbestandteilen und eine größere Toxizität pro Zigarette. Hingegen pro mg TPM, Teer oder Nikotin waren die Mengen an toxischen Rauchbestandteilen und die *in vitro*-Toxizität unter den HCI-Bedingungen im Allgemeinen geringer. Die *in vivo*-Toxizität war jedoch nicht unterschiedlich unter beiden Abrauchbedingungen.
Insgesamt, als Hauptergebnis, legt diese Untersuchung nahe, dass die Referenzzigaretten 2R4F und 3R4F bezüglich ihrer Rauchchemie sowie *in vitro*- und *in vivo*-Toxizität als gleichartig zu betrachten sind. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

RESUME

Une nouvelle cigarette de référence, la 3R4F, a été développée pour remplacer la 2R4F dont le stock s'épuise. La présente étude vise à comparer le courant principal de la fumée produite par ces deux cigarettes en termes de composition chimique et en termes de toxicité. Dans le cas de la 3R4F la fumée a été produite en suivant le protocole de fumage sur machine préconisé par l'Organisation Internationale de Normalisation (ISO) mais aussi suivant le protocole de fumage sur machine plus intense (HCI) spécifié par Santé-Canada. Ceci a permis de comparer la composition chimique et l'activité toxicologique du courant principal de la fumée de la 3R4F obtenu suivant chacun de ces deux régimes de fumage.
La cytotoxicité et la mutagenicité ont été mesurées *in-vitro* suivant le test de fixation du colorant rouge neutre, le test de mutation réverse de salmonelles et l'épreuve sur cellules de lymphome murin à gène TK. En outre, une étude d'inhalation de 90 jours a été effectuée chez le rat, par voies nasales uniquement, pour évaluer la toxicité *in-vivo*. Les deux cigarettes de référence donnent des fumées ayant des rendements équivalents en masse totale des particules (TPM), goudron et monoxyde de carbone, ainsi que la majorité des autres analytes. Les mesures de toxicité *in-vitro*, cytotoxicité et mutagénicité, ainsi que les tests *in-vivo* donnent les résultats attendus pour une exposition à la fumée. Aucune différence persuasive n'est observée entre 2R4F et 3R4F pour ce qui est de ces mesures de toxicité.
Comparé à un fumage selon ISO, dans le cas d'un fumage selon le protocole HCI les rendements en produits toxiques ainsi que l'activité toxique de la fumée de la 3R4F sont plus élevés lorsqu'ils sont calculés par cigarette. Ceci est trivial compte tenu de l'accroissement considérable de la quantité de fumée générée. Toutefois, exprimés par unité de masse (mg TPM, mg goudron ou mg nicotine) les rendements en composés toxiques et la toxicité *in-vitro* sont plus bas que ceux obtenus selon ISO.
Il n'y a pas de différence entre les résultats des mesures de toxicité *in-vivo* obtenus selon les 2 protocoles de fumage sur machine.
En conclusion, la présente étude suggère principalement une équivalence de la 2R4F et de la 3R4F en termes de leurs compositions chimiques et de leurs toxicités *in-vitro* et *in-vivo*. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

INTRODUCTION

Reference cigarettes play an important role in the identification and assessment of cigarette smoke-related effects. These cigarettes allow the replication and comparison of experiments performed in other laboratories. Such comparisons can be performed by setting the values from reference cigarette data to 100% and expressing the values from other experimental cigarette as a percentage of the reference value. In order to provide reference cigarettes that are easily available for all laboratories, all over the world, working in this field of research, the United States (U.S.) cigarette industry, on request of the Scientific Advisory Board of the Council for Tobacco Research, has provided such cigarettes since 1969. They resemble typical prototypes of certain market segments. Historically, these cigarettes have also provided the basis for both qualitative and quantitative comparisons of different cigarettes types (1), and they will continue to be necessary in the evaluation of future reduced harm products (2).
The University of Kentucky has provided the organizational structure for the design, development and distribution of reference cigarettes (http://www.ca.uky.edu/refcig/). These reference cigarettes differ in their design and characteristics, e.g., with and without filter, blend composition, and smoke delivery. The cigarettes are constructed to represent typical segments of the American market. One of these reference cigarettes is a "full flavor", filtered, American blended cigarette with a total particulate matter (TPM) yield of approximately 11 mg/cigarette under ISO machine smoking conditions, which is currently in its third version. The first version of this reference, called 1R4F was produced in 1983. It was later replaced in 2003 by its successor the 2R4F cigarette, which was chemically characterized and compared to the 1R4F (3, 4). Additional smoke chemistry data for the 2R4F can be found in studies by ADAM *et al.* (5) and INTORP *et al.* (6). The biological activity of its smoke has also been characterized in detail (4, 7–15). In 2008, due to diminishing stock of 2R4F, a replacement was made available. This replacement, the 3R4F cigarette has not yet been compared in the literature to the 2R4F. Evidence that these cigarettes are essentially the same regarding both their smoke chemistry and biological activities would allow for them to be used interchangeably as a comparison basis and would facilitate comparison of a larger number of studies as was the case with the previous versions, 1R4F and 2R4F (3).
In addition to reference cigarettes, the existence of generally accepted machine smoking protocols is essential to allow for the comparison of results from cigarette smoke obtained in different laboratories. Machine smoking

Table 1. Cigarette specifications.

Parameter	Cigarette	
	2R4F	3R4F
Physical data		
Cigarette length (mm)	84.0	84.0
Filter length (mm)	27.0	27.0
Circumference (mm)	24.9	24.5
Cigarette weight (g)	1.06	1.05
Filter ventilation (%)	28.0	29.0
Paper permeability (sec/50 mL)	24.0	24.0
Resistance to draw (cm H_2O)	13.4	12.8
Blend composition (%)		
Flue cured	32.5	35.4
Burley	19.9	21.6
Maryland	1.2	1.4
Oriental	11.1	12.1
Reconstituted (Schweitzer process)	27.1	29.6
Sugar (Isosweet™)	5.3	6.4
Glycerol	2.8	2.7
Filler analysis (%)		
Total alkaloids	2.3	2.1
Reducing sugars	10.7	8.7
Glycerol	2.4	2.4
Yield data from supplier		
Puff count	9.2	9.0
TPM (mg/cig)	11.7	11.0
'Tar' (mg/cig)	9.7	9.4
Nicotine (mg/cig)	0.9	0.7
Carbon monoxide (mg/cig)	13.0	12.0

protocols such as those defined by the International Organization for Standardization (16) and Health Canada (17) have been implemented by various regulatory authorities to provide consumers and regulators with data on cigarette smoke yields. It should be noted that standardized machine smoking protocols that apply either more- or less-intense smoking parameters can provide only one specific combination of possible settings of characteristics, like certain fixed puff volume, puff duration, and puff frequency, and are not meant to mimic human smoking behavior, nor could they be expected to do so, as each smoker smokes differently and as such, there is no typical human smoker (18–20).

Therefore, the objective of the present study was to compare the 3R4F cigarette with its predecessor the 2R4F cigarette in smoke chemistry and biological activity under the ISO machine smoking regimen for their interchangeability/similarity, and to further characterize the 3R4F between two smoking protocols, the ISO and HCI regimens in smoke chemistry and biological activity.

EXPERIMENTAL

Cigarettes and mainstream smoke (MS) generation

The reference cigarettes 3R4F and 2R4F were obtained from the University of Kentucky, Kentucky Tobacco Research and Development Center. Both are American blended filter cigarettes (for further details see Table 1). They were conditioned following ISO standard 3402 (21), i.e., at least 48 hours at target conditions of 22 °C ± 1 °C and a relative humidity of 60% ± 3%. MS was generated under ISO machine smoking conditions following ISO Standard 3308 (16), and under HCI smoking conditions (17). Minor deviations were necessary for technical reasons. The cigarettes were smoked on a 20-port Borgwaldt smoking machine (RM20H, Hamburg, Germany) for the *in vitro* tests, and on 30-port rotary smoking machines (15 ports blocked for HCI protocol) with an active sidestream smoke exhaust (type Philip Morris Research Laboratories (PMRL), SM2000, equipped with a programmable dual-syringe pump (22) for the *in vivo* studies. In short, puff volume, puff duration, and puff frequency for the ISO smoking conditions were 35 mL, 2 s, and 1/min. For the HCI smoking conditions, the respective values were 55 mL, 2 s, and 2/min. Under HCI smoking conditions, all cigarette filter ventilation holes were completely covered by tape.

Mainstream smoke chemistry

MS was generated for both 2R4F and 3R4F cigarettes under the ISO conditions as described above. In addition, the 3R4F was smoked according the HCI specifications.

Analytes in smoke were quantified and compared for both cigarettes according to established methodology (17, 23) as previously described (9). Total particulate matter (TPM) was determined gravimetrically from the smoke trapped on Cambridge glass fiber filters (23) which were also used for sample collection of individual particle phase analytes (see below). Nicotine was determined by gas chromatography (GC) with flame ionization detection from a 2-propanol extract of the TPM filter. Water was determined from the same 2-propanol extract by Karl Fischer titration (24). Carbon monoxide was determined by non-dispersive infra-

red photometry (25). 'Tar' yield was calculated as the TPM yield minus the nicotine and water yields (23). Aldehydes, derivatized with 2,4-dinitrophenylhydrazine and stabilized with pyridine, were determined by high-performance liquid chromatography with ultraviolet (HPLC/UV) detection using water/acetonitrile (9:1) and methanol as solvents (26). Vinyl chloride, 1,3-butadiene, isoprene, benzene, toluene, acrylonitrile, and styrene in the gas phase were trapped in three impingers containing methanol at approx. – 78 °C cooled with 2-propanole and dry ice and analyzed after addition of internal standards by GC using a CP PoraBond Q column (25 m x 0.25 mm, 3 µm) coupled to a mass spectrometer (GC-MS) with electron impact ionization in single ion monitoring mode (27). Styrene and acetamide in TPM were extracted from a glass fiber filter using acetone and analyzed after addition of internal standards by GC using a DB-WAX column (30 m x 0.25 mm, 0.25 µm) coupled to a mass spectrometer (GC/MS) with electron impact ionization in single ion monitoring mode. The analysis of acrylamide after extraction from a glass fiber filter was performed as described (28). Ethylene oxide in the gas phase was trapped in an impinger containing toluene at approx. – 78 °C (cooled with 2-propanole and dry ice) which was connected in series with a glass fiber filter as first trap. After addition of the internal standard propylene oxide-d_6, the toluene solution was analyzed by GC using a CP PoraPlot U column (25 m x 0.25 mm, 8 µm) and hydrogen as carrier gas coupled to a mass spectrometer (GC-MS) with electron impact ionization in single ion monitoring mode (29). 2-nitro-propane was determined from mainstream smoke trapped on a silica cartridge by adding 2-methyl-2-nitro-propane as internal standard, washing the cartridge with pentane and eluting the target analyte using 15% diethyl ether in n-pentane. 2-nitropropane was analyzed by GC-MS/MS in chemical ionization mode using iso-butane as ionization gas, helium as carrier gas and argon as collision gas. Aromatic amines were determined by extracting TPM-filters with dilute hydrochloric acid, followed by back extraction, derivatization, clean-up by solid phase extraction, and analysis by GC with a triple quadrupole mass spectrometer (30). Nitrogen oxides were determined by online gas phase chemiluminescence according to the CORESTA recommended method (31). Hydrogen cyanide was trapped in two impingers with sodium hydroxide solution connected in series. An aliquot was analyzed by headspace GC with nitrogen sensitive detection after acidification of the samples with phosphoric acid. Ammonia was trapped on a glass fiber filter and a wash bottle connected in series. The glass fiber filter was extracted with the content of the wash bottle, derivatized with dansyl chloride, and analyzed by HPLC with a tandem mass spectrometer (HPLC/MS-MS) (32).

Volatile *N*-nitrosamines were collected on a glass fiber filter and in two wash bottles containing a citrate/phosphate buffer solution with ascorbic acid to inhibit artificial generation of *N*-nitrosamines. The glass fiber filter was extracted with citrate/phosphate buffer solution with ascorbic acid and combined with the buffer solution of the wash bottles. The combined buffer solution was three times extracted with dichloromethane and the concentrated chloromethane phase was eluted through an alumina column. After elution with dichloromethane and another concentration step, the extract was analyzed by GC with a thermal energy analyzer. Tobacco-specific *N*-nitrosamines (TSNAs) were analyzed as published (33). TSNAs were extracted with ammonium acetate solution from TPM trapped on a glass fiber filter pad, and analyzed by HPLC/MS-MS. Phenols were extracted from a TPM filter with trichloromethane/acetone after addition of the internal standards phenol-d_6, catechol-d_6 and hydroquinone-d_6. An aliquot of the extract was derivatized with *N,O*-bis-(trimethylsilyl)-trifluoracetamide / 1% trimethyl-chloro-silane and the trimethylsilyl ethers of the phenols were analyzed by GC-MS using electron impact ionization in single ion monitoring mode. Polycyclic aromatic hydrocarbons were extracted from TPM filters with pentane/isooctane (9:1) after addition of the labeled internal standards. The sample clean-up was performed by a 2-step solid phase extraction using aminopropyl cartridges eluted with n-hexane, and octadecyl cartridges eluted with methanol. After concentration of the eluate by solvent evaporation and dissolving in isooctane, the 13 target analytes were determined by GC-MS using electron impact ionization in single ion monitoring mode. Arsenic, cadmium, chromium, nickel, lead, and selenium were trapped in quartz glass tubes using electrostatic precipitation. The condensate was dissolved with dichloro-methane/methanol mixture, and after addition of nitric acid, hydrogen peroxide, and water, the samples were subjected to microwave digestion and analyzed with atomic absorption spectroscopy. In the case of matrix inter-ferences, selenium was reanalyzed with the flow injection analysis system furnace technique. Mercury, after electrostatic precipitation of the particle phase, was trapped in 2 impingers containing potassium permanganate in sulfuric acid. For microwave digestion hydrogen peroxide was added. The digest was made up with water and an aliquot was analyzed with a mercury analyzer.

In vitro toxicity

Assessments of cytotoxicity and mutagenicity were carried out for evaluations of 3R4F and 2R4F cigarettes under ISO machine smoking conditions and the 3R4F reference cigarette was also assessed according to HCI machine smoking conditions. Cytotoxicity of TPM, and the gas vapor phase (GVP) from the 3R4F and 2R4F reference cigarettes, was assessed with the neutral red uptake (NRU) assay with mouse embryo BALB/c 3T3 cells as previously described (8). Briefly, 1.6×10^4 cells were seeded and cultivated in culture medium containing 10% fetal bovine serum (FBS; 100 µL per well). Approximately 24 hours after seeding, the cells were exposed for 24 hours to the smoke fractions, suspended or dissolved in culture medium containing 5% FBS (100 µL per well) resulting in a final concentration of 1.6% dimethyl sulfoxide (DMSO) and 8.4% phosphate buffered saline (PBS), or to solvent control (100 µL per well; culture medium containing 5% FBS, 1.6% DMSO, and 8.4% PBS). Following exposure, cells were incubated for 3 hours in culture medium containing 5% FBS and neutral red dye (100 µL per well). Cells were washed with PBS, and the neutral red dye taken up by viable cells was extracted with a destaining solution

(ethanol/acetic acid; 100 µL per well). The optical density of the neutral red, a measure for the number of viable cells, was determined photometrically at 540 nm. Cytotoxicity assessments were performed in triplicate for each smoke fraction, from both cigarettes, using 8 equidistant smoke fraction concentrations with 2–16 cig/L (TPM) and 3–24 cig/L (GVP) for the reference cigarettes smoked under ISO machine smoking conditions, and 0.7–5.6 (TPM) cig/L and 1–8 cig/L (GVP) for the 3R4F smoked according to the HCI machine smoking conditions.

The Ames *Salmonella typhimurium* reverse mutation assay was performed in general accordance to the Organization for Economic Co-operation and Development, OECD, guideline no. 471 (1997) using the five tester strains TA98, TA100, TA102, TA1535, and TA1537, with and without the S9 fraction, as previously reported (34). Three TPM concentrations per strain were used, ranging from 0.4–2.5 mg TPM/plate depending on the strain. The S9 fraction was purchased from Cytotest Cell Research (CCR, Rossdorf, Germany), and was prepared from the livers of male Sprague-Dawley rats injected with Aroclor 1254. Bacterial mutagenicity was determined for two independent TPM batches of both reference cigarettes. The number of revertants with and without the metabolic activation system was determined for each mutagenicity assay with an automatic colony counter.

The mouse lymphoma assay (MLA) for the mutagenicity of TPM was performed using L5178Y/tk+/--3.7.2C mouse lymphoma cells essentially according to OECD guideline no. 476 (1997) in the microtiter plate version (35) as previously described (10). Cells were obtained from LGC Standards, Germany (in partnership with American Tissue Culture Collection, Manassas, VA, USA). The assays were performed with two independent TPM batches, at three TPM doses with S9 metabolic activation (80, 140/150, 200/220 µg/mL TPM) and without S9 metabolic activation (25, 40, 55 µg/mL TPM). S9 metabolic activation mix was obtained from CCR, Rossdorf, Germany.

Inhalation / in vivo toxicity

Two 90-day nose-only inhalation studies with male and female Sprague-Dawley rats were performed to determine the biological activity of diluted MS. The first study was conducted using an exposure regimen of 6 h/day, 7 days/week at 200 µg/L TPM of the 2R4F or 3R4F cigarette. The second study was conducted at an exposure regimen of 6 h/d, 5 days/week at three increasing concentrations of 100, 150, and 200 µg/L TPM of the 3R4F cigarette and compared between the ISO and HCI machine smoking protocols.

Generally, 10 rats/sex/group were exposed nose-only to MS or to filtered, conditioned fresh air (sham-exposure group). General conditions and animal health, as well as smoke exposure and uptake were monitored. Local effects in the respiratory tract along with systemic effects were investigated after 13 weeks of smoke exposure as previously described (36). Endpoints included all parameters specified in the OECD Guideline for Testing of Chemicals 413 (37) with an extended histopathological assessment of irritation in the respiratory tract. Histopathological changes were scored according to a defined severity scale from 0 to 5. Mean severity scores were calculated based on all rats in a group. Hereby, special histological sections were prepared for the nose according to the method of YOUNG (38), and for the larynx according to LEWIS (39). The trachea was cut frontally (at the bifurcation). One frontal section passing through the main bronchus for the left lung and one frontal section passing through a maximum number of lobes for the right lung were prepared (40). A 42-day post-inhalation period was included for the high-dose smoke exposure and the sham-exposure groups, to assess reversibility, persistence, or delayed occurrence of smoke-exposure effects (data not shown).

Statistical analysis

All tests were conducted without correction for multiple comparisons. The significance level is $\alpha \leq 0.05$, with the exception of smoke chemistry tests which were conducted at the significance levels of 0.05, 0.01, and 0.001. Generally, the mean and the standard error were given as descriptive statistics. Comparisons were made on a per cigarette, a per milligram TPM, a per milligram 'tar', and a per milligram nicotine basis.

For smoke chemistry, comparisons were performed using the t-test. For the NRU assay, the reciprocal EC_{50} ($1/EC_{50}$) was determined separately for each smoke fraction (TPM and GVP) and for each of the three batches. The mean reciprocal EC_{50} values were compared by t-test. The Ames assay data were evaluated based on the slopes of the linear dose-response curves using linear regression analysis with Poisson-weighted data excluding the 0-dose as previously described (8). Analysis of covariance (ANCOVA) was used to compare slopes. MLA assay data was compared using the dose-response curves for the mutant frequency values as calculated by nonlinear regression analysis with the power function $y = a + bx^c$ from which the smoke concentrations were calculated that resulted in a mutagenic response as high as three times the background (spontaneous) mutant frequency (C_{3B}). In addition, non-linear dose-response curves were examined using the sum of square reduction test (comparison 3R4F ISO/HCI only). In the 90-day rat inhalation studies, MS exposure groups were compared with the sham-exposure group using one-way ANOVA, followed by the Dunnett post-hoc test to confirm exposure effects. Differences in biological activity due to the different smoking protocols for all endpoints were compared on a per milligram TPM basis using two-way ANOVA. Histopathological findings from non-respiratory organs were evaluated with a scoring system from 0 to 5 and analyzed either with the Cochran Mantel Haenszel test for overall and pair-wise comparisons or by ANOVA. Incidences were analyzed with χ^2 statistics.

RESULTS

Cigarette smoke chemistry

In the comparisons of the 3R4F with the 2R4F reference cigarette analyte, yields smoked according to ISO machine smoking conditions, the mean difference over all calculation bases was less than 3% between both cigarettes

Table 2. Relative smoke constituent yields of the 3R4F versus the 2R4F reference cigarette, when smoked according to ISO machine smoking conditions.

Parameter	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
ISO Parameter				
TPM	105**	100	99	101
'Tar'	105***	101	100	101
Nicotine	104*	99	99	100
Water	100	95	95	96
CO	105**	100	99	100
Aldehydes				
Formaldehyde	109	104	103	104
Acetaldehyde	105	100	99	100
Acrolein	104	99	98	99
Propionaldehyde	103	98	98	99
Crotonaldehyde	112*	107	106	108
Aliphatic dienes				
1,3-Butadiene	99	94	94	95
Isoprene	96	92*	91*	92*
Acid derivatives				
Acetamide	102	97	97	98
Acrylamide	87**	83***	83***	84***
Acrylonitrile	100	95	95	96
Epoxides				
Ethylene oxide	97	92	92	93
Nitro compounds				
2-Nitropropane	90	86	85	86
Aromatic amines				
o-Toluidine	101	96	96	97
o-Anisidine	104	99	99	100
2-Naphthylamine	98	93*	93*	94
4-Aminobiphenyl	97	93*	92*	93
Halogen compounds				
Vinyl chloride	87*	83*	82*	83*
Inorganic compounds				
Nitrogen oxides	98	94*	93*	94*
Hydrogen cyanide	102	97	96	97
Ammonia	90	85	85	86
Monocyclic aromatic hydrocarbons				
Benzene	98	94*	93*	94
Toluene	100	95	95	96
Styrene	99	94	93	94
Volatile N-nitrosamines				
NDMA	<LOQ	<LOQ	<LOQ	<LOQ
NMEA	<LOQ	<LOQ	<LOQ	<LOQ
NDEA	<LOQ	<LOQ	<LOQ	<LOQ
NPRA	<LOQ	<LOQ	<LOQ	<LOQ
NBUA	<LOQ	<LOQ	<LOQ	<LOQ
NPY	<LOQ	<LOQ	<LOQ	<LOQ
NPI	<LOQ	<LOQ	<LOQ	<LOQ
Tobacco-specific N-nitrosamines				
NNN	84***	80***	79***	80***
NNK	78***	74***	74***	75***
NAB	103	98	98	99
NAT	106	101	100	101

Table 2. (cont.).

Parameter	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Phenols				
Phenol	108*	103	102	104
Catechol	102	98	97	98
Hydroquinone	106***	101	101	102
Polycyclic aromatic hydrocarbons				
Pyrene	108*	103	102	103
Benz[*a*]anthracene	105	101	100	101
Benzo[*b*]fluoranthene	113***	107*	107*	108*
Benzo[*j*]fluoranthene	113**	108*	107*	109*
Benzo[*k*]fluoranthene	116**	111*	110*	111*
Benzo[*a*]pyrene	108**	103	103	104*
Dibenz[*a,h*]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,e*]pyrene	108	103	103	104
Dibenzo[*a,h*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,i*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,l*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Indeno[*1,2,3-cd*]pyrene	113***	107**	107**	108**
5-Methylchrysene	<LOQ	<LOQ	<LOQ	<LOQ
Elements				
Cadmium	99	95	94	95
Chromium	105	100	100	101
Nickel	<LOQ	<LOQ	<LOQ	<LOQ
Lead	93	89***	89***	89**
Arsenic	109***	104*	103*	104*
Selenium	102	98	97	98
Mercury	90**	86	85	86

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N'*-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
Statistical significances: *: $0.01<p \leq 0.05$, **: $0.001<p \leq 0.01$, ***: $p \leq 0.001$.

(Table 2; Figure 1; Annex, Table A). The maximum differences were + 16% (benzo[*k*]fluoroanthene, per cigarette) and – 26% (NNK, per mg TPM and 'tar'). Statistically significant lower yields for the 3R4F cigarettes were found for acrylamide, ammonia, vinyl chloride, and the TSNA *N*-nitrosonornicotine (NNN) and 4-(methyl-nitro-samino)-1-(3-pyridyl)-1-butanone (NNK) using all calculation bases (per cigarette, per mg 'tar', TPM or nicotine). Statistically significant higher yields for the 3R4F reference cigarette were found for 4 PAHs and arsenic for all calculation bases. Other statistically significant differences were well within the inherent variability of the analytes yields and not observable under all calculation bases.

Further comparisons of smoke chemistry for the 3R4F reference cigarette were performed under the ISO and the HCI machine smoking conditions. Yield ratios for each calculation basis are presented in Table 3. As a trivial finding, due to the higher amount of smoke produced under the HCI machine smoking conditions, statistically significant higher yields per cigarette were found for all constituents under the HCI compared to the ISO machine smoking conditions ($p \leq 0.001$). However, when expressed per mg TPM, per mg 'tar', and per mg nicotine the yields of TSNAs, phenols, nitrogen oxide, aromatic amines, and benzene, were generally lower under HCI machine smoking conditions, as compared to when using the ISO regimen ($p \leq 0.05$; for all calculation bases). Polycyclic aromatic hydrocarbon levels were lower under the HCI compared to the ISO machine smoking conditions ($p \leq 0.01$) on a per mg TPM basis and on a per mg 'tar' basis

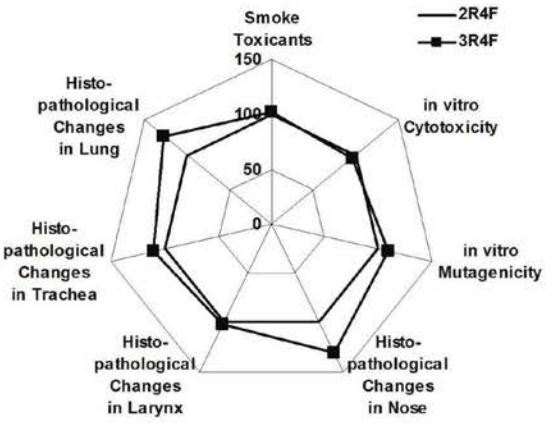

Figure 1. Summary of comparisons of the 3R4F to 2R4F reference cigarettes - means from Tables 2, 4, and 6.

Table 3. Relative smoke constituent yields of the 3R4F reference cigarette when smoked according to ISO and HCI machine smoking conditions.

Parameter	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
ISO Parameter				
TPM	367*** [a]	100	119***	136***
'Tar'	308***	84***	100	114***
Nicotine	271***	74***	88***	100
Water	796***	217***	258***	294***
CO	279***	76***	91***	103
Aldehydes				
Formaldehyde	338***	92***	110	125***
Acetaldehyde	274*** [a]	75***	89**	101
Acrolein	298*** [a]	81***	97	110***
Propionaldehyde	267*** [a]	73***	87***	98
Crotonaldehyde	415***	113*	135***	153***
Aliphatic dienes				
1,3-Butadiene	272***	74**	88	100
Isoprene	280***	76**	91	103
Acid derivatives				
Acetamide	407***	111*	132***	150***
Acrylonitrile	273***	74***	89*	101
Nitro compounds				
2-Nitropropane	244***	67***	79**	90
Aromatic amines				
o-Toluidine	223***	61***	72***	82***
o-Anisidine	231*** [a]	63***	75***	85**
2-Naphthylamine	203***	55***	66***	75***
4-Aminobiphenyl	248***	68***	81**	92
Halogen compounds				
Vinyl chloride	249***	68***	81**	92
Inorganic compounds				
Nitrogen oxides	246***	67***	80***	91**
Hydrogen cyanide	427***	116*	139***	158***
Monocyclic aromatic hydrocarbons				
Benzene	241***	66***	78***	89*
Toluene	257***	70***	83**	95
Styrene	418*** [a]	114*	136**	154***
Volatile N-nitrosamines				
NDMA	<LOQ	<LOQ	<LOQ	<LOQ
NMEA	<LOQ	<LOQ	<LOQ	<LOQ
NDEA	<LOQ	<LOQ	<LOQ	<LOQ
NPRA	<LOQ	<LOQ	<LOQ	<LOQ
NBUA	<LOQ	<LOQ	<LOQ	<LOQ
NPY	<LOQ	<LOQ	<LOQ	<LOQ
NPI	<LOQ	<LOQ	<LOQ	<LOQ
Tobacco-specific N-nitrosamines				
NNN	249***	68***	81***	92**
NNK	268***	73***	87**	99
NAB	231***	63***	75***	85**
NAT	250***	68***	81***	92*
Phenols				
Phenol	203***	55***	66***	75***
Catenol	251***	68***	81***	93**
Hydroquinone	269*** [a]	73***	87***	99
o-Cresol	196***	53***	64***	72***
m-Cresol	196***	53***	64***	72***

Table 3. (cont.).

Parameter	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Phenols				
p-Cresol	207***	56***	67***	76***
Resorcinol	286***	78***	93*	106
Polycyclic aromatic hydrocarbons				
Pyrene	248***	67***	80**	91
Benz[*a*]anthracene	260***	71***	84***	96
Benzo[*b*]fluoranthene	263***	72***	85**	97
Benzo[*j*]fluoranthene	264*** [a]	72***	86**	97
Benzo[*k*]fluoranthene	262***	71***	85**	97
Benzo[*a*]pyrene	250*** [a]	68***	81***	92*
Dibenz[*a,h*]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,e*]pyrene	253***	69***	82***	93*
Dibenzo[*a,h*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,i*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,l*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Indeno[1,2,3-*cd*]pyrene	250***	68***	81***	92**
5-Methylchrysene	<LOQ	<LOQ	<LOQ	<LOQ
Elements				
Arsenic	378***	103	123**	139***
Cadmium	<LOQ	<LOQ	<LOQ	<LOQ
Chromium	<LOQ	<LOQ	<LOQ	<LOQ
Nickel	332***	90	108	122*
Lead	314***	85*	102	116

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NAB = *N*-nitrosoanabasine; NAT = *N*-nitrosoanatabine; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N'*-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
[a]: assumption of equality of variances not reasonable, Satterthwaites approximation of the t-test used,
Statistical significances: *: $0.01<p\leq0.05$, **: $0.001<p\leq0.01$, ***: $p\leq0.001$.

but similar on a per mg nicotine basis. Yields of benzo-[*a*]pyrene, dibenzo[*a,e*]pyrene, and indeno-[1,2,3-*c,d*]-pyrene were only significantly lower on a per mg nicotine basis ($p \leq 0.05$).

Water, crotonaldehyde, acetamide, hydrogen cyanide and styrene levels were higher for the 3R4F reference cigarette smoked under HCI machine smoking conditions compared to when smoked under ISO conditions ($p \leq 0.05$) regardless of the calculation basis.

In vitro cytotoxicity (NRU assay)

For all samples there was a reproducible dose-dependent decrease in cell viability for both TPM and GVP exposure of both reference cigarettes. There were no statistically significant differences in the cytotoxicity of either the TPM or GVP smoke fractions of the 3R4F cigarette when compared to that of the 2R4F cigarette smoked under the same ISO machine smoking conditions, regardless of the calculation basis (i.e. per cigarette, per unit mass of TPM, 'tar', or nicotine). The mean difference was – 5% and the maximum difference was – 9% for GVP on a per cigarette basis (Table 4).

There were significant differences, however, in the cytotoxicity values of the TPM and GVP smoke fractions from the 3R4F reference cigarette when smoked under HCI machine smoking conditions, as compared to the ISO conditions. The expected statistically significant increase in cytotoxicity per cigarette was + 183% for the TPM and + 198% for the GVP. A statistically significant decrease in cytotoxicity of – 18% was observed for the TPM on a per mg TPM basis. The cytotoxicity of the GVP on the same calculation basis was reduced to the same extent, but the difference was not statistically significant. The other calculation bases did not reveal statistical significances (Table 5).

In vitro bacterial mutagenicity (Ames assay)

Dose-dependent increases in the number of revertants was observed in strains TA98, TA100, and TA1537, with and without S9 metabolic activation, following TPM-exposure from either reference cigarette, compared to solvent control. For these tester strains that, according to the literature (9), have been proven to be responsive to TPM and discriminative, the dose-dependent increases were in most cases statistically significant. There were no statistically significant differences, however, in the observed mutagenicity between the 2R4F and 3R4F reference cigarettes, when smoked under ISO machine smoking conditions, with and without S9 activation, regardless of the calculation basis (Table 4). On a per cigarette basis, a significantly higher mutagenicity of TPM from the 3R4F cigarette (+ 98% to + 263%) when smoked

Table 4. Relative in *vitro* toxicity of the 3R4F versus the 2R4F reference cigarette, when smoked according to ISO machine smoking conditions.

Assay, measure, smoke fraction	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Cytotoxicity, 1/EC_{50}				
TPM	96	97	98	99
GVP	91	93	93	94
Bacterial mutagenicity, TPM, Revertants per calculation basis				
TA 98, +S9	98	94	94	99
TA 100, +S9	109	104	104	106
TA1537, +S9	122	117	117	119
TA 100, -S9	125	121	119	94
Mammalian cell mutagenicity, TPM, 1/C_{3B}				
+S9	101	109	107	104
-S9	101	107	107	103

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. Statistical significances: *: $0.01<p \leq 0.05$, **: $0.001<p \leq 0.01$, ***: $p \leq 0.001$.

under HCI machine smoking conditions was observed, as compared to TPM obtained under ISO smoking conditions, reflecting the higher smoke yields produced under the HCI smoking conditions. With metabolic activation all sensitive tester strains showed decreases in the mutagenic activity per mg TPM, 'tar', or nicotine. The decreases, up to –45%, were statistically significant for the calculations based on per mg TPM.

In vitro mammalian mutagenicity (ML assay)

Under ISO machine smoking conditions, dose-dependent increases in TPM mutant frequencies from both the 2R4F and 3R4F reference cigarettes were observed, with and without metabolic activation, compared to solvent control. No significant differences in mutagenicity were observed between the reference cigarettes, regardless of the calculation basis. Comparisons of the TPM mutagenicity from the 3R4F cigarette by both machine smoking regimens found an increase in total activity of approximately + 150% per cigarette, with and without metabolic activation. On a per mg TPM basis, statistically significant decreases in activity were observed with (– 34%) and without (– 23%) metabolic activation. When expressed on a per mg 'tar' basis, the decreases were less distinct. On a per mg nicotine basis, the activities were not different between both machine smoking regimens.

Ninety-day rat inhalation

- *In life* observations and body weights

Following MS-exposure to 2R4F and 3R4F cigarette smoke generated under ISO machine smoking conditions, significant reductions in body weight development in male rats were observed, compared to sham-exposed rats ($P \leq 0.01$). In addition, Harderian gland secretion and wet fur were more frequently observed for MS-exposed groups, compared to the sham-exposed groups. The same magnitude of effects was observed for both cigarettes. There were no other significant differences observed between the smoke-exposed groups and sham-exposure groups. 3R4F reference cigarette mainstream smoke was generated under both the ISO and the HCI machine smoking regimens showed the same effect on body weight reduction, Harderian gland secretion, and the occurrence of wet fur.

- Clinical chemistry, hematology and organ weights

Following MS-exposure to both the 2R4F and 3R4F cigarettes smoked according to ISO machine smoking conditions, only expected alterations in clinical chemistry (41) were observed (e.g., decreased serum concentrations of proteins, triglycerides, cholesterol), hematology (increased hemoglobin) and organ weights (e.g., decreased thymus weight). There were no further consistent, or significant, differences for these parameters between both reference cigarettes (data not shown). Following exposure to MS of the 3R4F reference cigarette under either machine smoking condition, none of the above parameters was affected by the smoking regimen in a meaningful and consistent way (data not shown).

- Histopathology

The histopathological evaluation of the respiratory tract organs revealed qualitatively similar findings for the 2R4F and 3R4F reference cigarettes when smoked under ISO machine smoking conditions that are comparable to expected results following MS exposure reported in the literature (41). Quantitatively, there was no consistent trend (male and female rats, response at various sites) for a difference in toxicity (Table 6), although, numerically, the 3R4F reference cigarette smoke-exposed female rats showed effects that were slightly more pronounced than those in the 2R4F group.

MS exposure to the 3R4F reference cigarette smoked according to ISO and HCI machine smoking conditions did not reveal consistent and meaningful differences. A higher response in the trachea in the male rats exposed to the smoke generated under HCI conditions relative to the ISO groups is due to the fact that at very low incidence rates differences that are small on an absolute basis translate into high relative differences in percent (Table 6, Figure 2).

Table 5. Relative *in vitro* toxicity of the 3R4F reference cigarette when smoked according ISO and HCI machine smoking conditions.

Assay, measure, smoke fraction	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Cytotoxicity, 1/EC_{50}				
TPM	283***	82**	98	106
GVP	298***	83	102	112
Bacterial mutagenicity, TPM, Revertants per calculation basis				
TA 98, +S9	267***	75***	90	99
TA 100, +S9	258***	72**	86	96
TA1537, +S9	198***	55**	66*	74
TA 100, -S9	363***	99	120	133
Mammalian cell mutagenicity, TPM, 1/C_{3B}				
+S9	242***	66***	79***	90
-S9	276***	77**	92	103

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. Statistical significances: *: $0.01 < p \leq 0.05$, **: $0.001 < p \leq 0.01$, ***: $p \leq 0.001$.

Comparisons performed on the rats after the post-inhalation period did not show differences between the toxicity of the smoke from the 3R4F and the 2R4F reference cigarettes or between the two smoking regimens (data not shown). With the exception of thymus atrophy, tissues from non-respiratory organs were not significantly affected by the exposure to cigarette smoke.

Nineteen animals showed histo-morphological alterations that are indicative for a rat respiratory virus (RRV) infection. More sham-exposed rats than MS-exposed rats (16 vs. 3, respectively) were affected. The histopathological evaluation of the lung tissue did not appear to be compromised as the infection-related changes can be discriminated from the morphological alterations associated with MS exposure (2). Therefore, the RRV infections were not considered to interfere with the objective of the study.

DISCUSSION

In the evaluation of smoke chemistry under ISO machine smoking conditions, similar results for TPM, 'tar', and carbon monoxide were obtained for the 2R4F and 3R4F reference cigarettes. For some toxicants including the carcinogenic TSNAs and acrylamide, somewhat lower yields were observed for the 3R4F, compared to the 2R4F reference cigarette.

As previously reported from comparisons of the 2R4F and the 1R4F reference cigarette, some variation in smoke constituent yields could be expected due to year-to-year differences in the tobacco crop (3). Overall, however, the objective to produce a new reference cigarette as close as possible to the previous 2R4F reference cigarette was met. Thus, for practical purposes, smoke chemistry data of the 2R4F and the 3R4F are equivalent. Both cigarettes can be used interchangeably as references, although it is recognized that some smoke constituents as, e.g., acrylamide, NNN, and NNK, show statistically significant differences.

The two reference cigarettes also displayed similar *in vitro* cytotoxicity and mutagenicity. In addition, there were no meaningful differences in biomarkers or histopathological changes obtained in the inhalation studies in rats that would suggest any significant differences in toxicity from exposure to the smoke of the 3R4F reference cigarette, as compared to the 2R4F reference cigarette. This also holds true when the 3R4F data are compared to published data for the 2R4F reference cigarette (2, 8, 15). Accordingly, the toxicological profiles of the 2R4F and the 3R4F reference cigarettes are considered to be equivalent.

As expected e.g. (7, 8), due to the significantly higher amount of 3R4F smoke generated under the intense HCI machine smoking conditions, when expressed on a per cigarette basis, toxicants yields, *in vitro* mutagenicity and cytotoxicity, and inhalation toxicity increased compared to when smoked according to ISO machine smoking conditions.

However, as with other cigarettes, comparisons on a per mg TPM, per mg 'tar', or per mg nicotine basis, were reduced under the HCI machine smoking conditions. This observation has been previously reported or can be deduced from data presented in similar investigations of machine smoking protocols for other cigarettes (8, 9, 42, 43). One of the potential explanations for this seemingly counterintuitive observation is based on the increase in the flow of air and thus oxygen that is drawn through the burning zone of the cigarette. Specifically, the higher puff volume that, due to occluded filter ventilation, cannot bypass the burning zone of the cigarette, resulting in higher oxygen supply to the burning zone and thus to higher combustion temperatures when smoked under HCI machine smoking conditions.

It can be hypothesized that higher combustion temperatures obtained under the HCI machine smoking conditions might result in more complete combustion and thus to a lower yield of cytotoxic and mutagenic toxicants. This is consistent with the increase in water in the TPM under intense machine smoking conditions (8).

It is noteworthy that the same authors have demonstrated that these reductions in toxicant yields and cytotoxic and mutagenic activity under intense machine smoking conditions, when normalized to TPM, 'tar', or nicotine, are less apparent between different cigarettes. Differences observable for different cigarettes under ISO machine smoking conditions are also not as apparent when smoked

Table 6. Relative *in vivo* toxicity of the 3R4F versus the 2R4F reference cigarette when smoked according to ISO machine smoking conditions, and the 3R4F reference cigarette when smoked under both the HCI and ISO machine smoking conditions.

Organ, Epithelium, Finding	3R4F to 2R4F ratio (%)		3R4F HCI to 3R4F ISO ratio (%)					
	Male rats	Female rats	Male rats			Female rats		
	200 µg/L	200 µg/L	100 µg/L	150 µg/L	200 µg/L	100 µg/L	150 µg/L	200 µg/L
Nose level 1, respiratory epithelium,								
Reserve cell hyperplasia	100	100	106	100	100	100	100	100
Nasal septum, loss of goblet cells	112	108	98	100	93	100	102	98
Squamous epithelial metaplasia	100	100	111	123	100	118	103	103
Lamina propria, inflammatory cell infiltrates	95	161*	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Lumen								
Exudate	125	406**	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Nose level 2, respiratory region								
Reserve cell hyperplasia	78	139	76	124	95	77	108	100
Olfactory region								
Atrophy	128	111	30	102	100	171	100	91
Squamous epithelial metaplasia	123	100	n.r.	94	115	600*	96	90
Loss of nerve bundle	96	117	30	116	97	200	102	85
Nose level 3, olfactory epithelium								
Atrophy	129	103	n.r.	81	117	n.r.	125	94
Squamous epithelial metaplasia	138	111	n.r.	77	120	n.r.	130	82
Lamina propria, inflammatory cell infiltration	263*	142	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Loss of nerve bundle	150	111	n.r.	87	117	n.r.	126	100
Nose level 4 , olfactory epithelium								
Atrophy	129	107	n.r.	91	105	n.r.	129	93
Squamous epithelial metaplasia	131	100	n.r.	44	105	n.r.	144	85
Larynx, ventral depression								
Squamous epithelial metaplasia	103	100	126	139	152	55	138	109
Floor of larynx								
Squamous epithelial metaplasia	100	100	102	100	100	100	100	100
Cornification	100	88	106	100	100	107	100	100
Vocal cords, lower medial region								
Squamous epithelium hyperplasia	80	100	121	88	104	108	100	100
Cornification	103	100	195	59	143	89	110	100
Vocal cords, upper medial region								
Pseudostratified epithelium hyperplasia	89	147	171	64	117	n.r.	73	65
Cornification	85	147	n.r.	21	67	n.r.	356	156
Vocal folds								
Pseudostratified epithelium Cornification	124	96	197	94	94	100	124	98
Trachea, bifurcation								
Squamous epithelial metaplasia	50	123	n.r.	254	285	<100	n.r.	>100
Lung, alveoli								
Goblet cell hyperplasia	108	212*	93	92	96	211	125	77
Lumen								
Alveolar macrophages	81	105	79	133	100	107	110	93

Values are derived from mean scores for histopathological changes after a 90-day inhalation period. Statistical significances: *:0.01<p ≤0.05, **: 0.001<p ≤0.01, ***: p ≤0.001, n.r.: not recorded.

under more intense machine smoking regimens (44). While notable effects were observed between different machine smoking conditions for smoke chemistry and *in vitro* assessments, there were no such differences noted in the *in vivo* investigations. The results are qualitatively identical to previous results observed in smoke-exposed rats (2, 15, 41, 45, 46). There were also no quantitative differences in toxic effects detected in our study between both machine smoking conditions. Recently, distinct differences have been published regarding the *in vivo* toxicity of cigarette smoke generated under smoking regimens that were rather extreme in intensity (8). It can be argued that the *in vivo* endpoints were different from those reported here. The study published by ROEMER *et al.* deals with dermal

tumorigenicity (skin painting) in mice and not with irritative effects as presented in this publication. Due to their study design (2-stage protocol: mice initiated by dermal application of a carcinogen and subsequent application of cigarette smoke condensate) they put their emphasis of their assessment on the promoting activity (47) of cigarette smoke condensate. However, the promoting, non-genotoxic activity of cigarette smoke has been linked to its irritative capacity (48). As such, one might expect similarities in response between effects in the inhalation toxicity and the 2-stage dermal tumorigenicity study. This apparent discrepancy might be explainable by the fact, that by inhalation exposure both, the particulate as well as the gas phase constituents can exert their action. The mouse skin painting assay, in contrast, investigates only the particulate phase of cigarette smoke. Further research on the possible differences behavior of the gas/vapor phase and the particulate phase under different intensities of smoking regimens might contribute to the understanding of the inherent toxicity of cigarette smoke.

There were two exposure regimens, i.e., seven days per week at 200 µg/L TPM and five days per week at 100, 150, and 200 µg/L, all with a daily exposure duration of six hours. The smoke induced effects were for the same exposure concentration of 200 µg/L quantitatively and even qualitatively approximately the same. The group with 5 days exposure scored, as a mean, 0.6 points lower than the group with 7 days per week. The group with 150 µg/L and 5 days per week scored slightly lower, i.e., 0.8 points. This can be interpreted that the toxicity may be somewhat more dependent on the concentration than on the weekly exposure time, which is in line with results obtained for 21 inhalation toxicants (49) or with cigarette smoke (50). However, both exposure regimens, i.e., 7 days or 5 days per week, are obviously suitable methods to assess the toxicity of cigarette smoke.

Expressing smoke chemistry or toxicity data on a calculation basis other than on a per cigarette basis, i.e., a normalization to account for different yield data, as it is performed here for the comparison of the ISO and HCI machine smoking conditions, always needs to be interpreted cautiously due to nonlinear relationships and possibly unidentified measurement biases. Both complications are especially important for low delivery cigarette data (51). Nevertheless, interpretations of normalized data using all bases of calculation are useful for evaluating human exposure, even for cigarettes with low smoke deliveries, as they allow at least a meaningful estimate of the amount of toxicants or biological activity accompanied with a certain amount of nicotine, 'tar', etc. For the 2R4F and 3R4F reference cigarette data presented here, these precautions do not apply, as both cigarettes have high yields under machine smoking conditions. Standardized machine smoking conditions that apply either more or less intense smoking parameters can provide only one specific combination of possible settings of characteristics, such as fixed puff volume, puff duration, and puff frequency, and are not meant to mimic human smoking behavior, nor could they be expected to do so, as each smoker smokes differently. Accordingly, there is no typical human smoker and no typical human smoking regimen (18–20). However, the results obtained under different machine smoking conditions of different intensity can give insight into the possible spectrum of different smoke qualities and the underlying mechanisms.

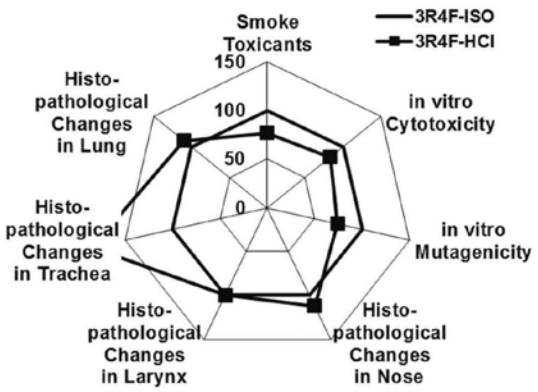

Figure 2. Summary of comparisons of the 3R4F reference cigarettes HCI to ISO machine smoking conditions - means from Tables 3, 5, and 6.

CONCLUSIONS

Overall, the results of the present study suggest equivalent smoke chemistry and toxicity for the 3R4F and 2R4F reference cigarettes when smoked under the same smoking regimen. As observed already for other cigarettes, 3R4F mainstream smoke generated under intense smoking conditions is generally less cytotoxic and mutagenic *in vitro* than the smoke generated under less intense conditions. The *in vivo* inhalation toxicity, however, seems not to be different.

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Corresponding author:

Dr. Ewald Roemer
c/o Philip Morris Intl.
Rue des Usines 90
2000 Neuchâtel
Switzerland
ewald.roemer@pmi.com

APPENDIX

Table A. Smoke constituent yields for the 3R4F and the 2R4F reference cigarettes.

Analyte	Unit per cig	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
ISO Parameter							
TPM	mg	9.77	0.04	9.32	0.08	37.7	0.3
'Tar'	mg	7.98	0.03	7.57	0.05	25.5	0.2
Nicotine	mg	0.707	0.005	0.678	0.007	1.90	0.02
Water	mg	1.08	0.04	1.08	0.02	10.3	0.2
CO	mg	11.2	0.1	10.7	0.1	32.7	0.2
Aldehydes							
Formaldehyde	µg	20.0	0.7	18.4	0.4	68.1	1.4
Acetaldehyde	µg	567	10	542	5	1534	32
Acrolein	µg	56.7	1.4	54.7	0.7	155	3
Propionaldehyde	µg	48.4	0.9	47.0	0.4	124	3
Crotonaldehyde	µg	10.1	0.3	9.00	0.21	43.1	0.9
Aliphatic dienes							
1,3-Butadiene	µg	38.5	1.2	38.9	1.6	76.5	2.1
Isoprene	µg	395	11	411	5	863	27
Acid derivatives							
Acetamide	µg	4.46	0.12	4.37	0.05	15.2	0.2
Acrylamide	µg	1.37	0.02	1.57	0.03	-	-
Acrylonitrile	µg	26.4	0.7	26.5	0.5	67.0	0.8
Epoxides							
Ethylene oxide	µg	9.24	0.18	9.56	0.36	-	-
Nitro compounds							
2-Nitropropane	µg	18.3	0.3	20.4	1.7	35.9	0.6
Aromatic amines							
o-Toluidine	ng	54.1	0.7	53.5	0.7	99.3	1.4
o-Anisidine	ng	2.32	0.04	2.23	0.03	4.25	0.12
2-Naphthylamine	ng	5.69	0.07	5.83	0.12	10.1	0.4
4-Aminobiphenyl	ng	1.01	0.01	1.04	0.02	2.24	0.06
Halogen compounds							
Vinyl chloride	ng	49.7	2.0	57.2	2.1	69.7	2.1
Inorganic compounds							
Nitrogen oxides	µg	265	3	270	5	626	6
Hydrogen cyanide	µg	70.9	1.9	69.8	1.5	319	9
Ammonia	µg	11.1	0.2	12.4	0.2	-	-
Monocyclic aromatic hydrocarbons							
Benzene	µg	45.7	0.9	46.6	0.6	104	1
Toluene	µg	73.6	1.4	73.7	1.0	208	5
Styrene	µg	6.00	0.22	6.09	0.09	24.9	0.9
Volatile N-nitrosamines							
NDMA	ng	<5.00	-	<5.00	-	<10.0	-
NMEA	ng	<10.0	-	<10.0	-	<20.0	-
NDEA	ng	<7.00	-	<7.00	-	<14.0	-
NPRA	ng	<11.0	-	<11.0	-	<22.0	-
NBUA	ng	<9.00	-	<9.00	-	<18.0	-
NPY	ng	<7.00	-	<7.00	-	<14.0	-
NPI	ng	<8.00	-	<8.00	-	<16.0	-
Tobacco-specific N-nitrosamines							
NNN	ng	92.1	1.5	110	2	276	3
NNK	ng	85.5	1.8	110	2	243	6
NAB	ng	9.60	0.46	9.33	0.40	24.0	0.5
NAT	ng	92.9	4.4	87.9	3.6	251	5

Table A. (cont.).

Analyte	Unit per cig	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
Phenols							
Phenol	µg	7.04	0.10	6.52	0.13	14.8	0.3
Catenol	µg	37.1	0.2	36.2	0.3	89.3	0.5
Hydroquinone	µg	29.1	0.1	27.4	0.2	75.7	1.0
Polycyclic aromatic hydrocarbons							
Pyrene	ng	38.0	0.8	35.3	0.2	92.5	1.9
Benz[*a*]anthracene	ng	11.8	0.3	11.2	0.1	29.8	0.6
Benzo[*b*]fluoranthene	ng	5.09	0.08	4.52	0.04	13.2	0.3
Benzo[*j*]fluoranthene	ng	3.24	0.07	2.86	0.04	8.35	0.19
Benzo[*k*]fluoranthene	ng	2.02	0.05	1.74	0.02	5.38	0.11
Benzo[*a*]pyrene	ng	6.73	0.11	6.21	0.07	16.2	0.4
Dibenz[*a,h*]anthracene	ng	<0.970	-	<0.970	-	<2.42	-
Dibenzo[*a,e*]pyrene	ng	0.173	0.005	0.160	0.004	0.858	0.015
Dibenzo[*a,h*]pyrene	ng	<0.230	-	<0.230	-	<0.575	-
Dibenzo[*a,i*]pyrene	ng	<0.220	-	<0.220	-	<0.550	-
Dibenzo[*a,l*]pyrene	ng	<0.190	-	<0.190	-	<0.475	-
Indeno[*1,2,3-cd*]pyrene	ng	2.87	0.04	2.55	0.02	7.37	0.10
5-Methylchrysene	ng	<0.400	-	<0.400	-	<1.00	-
Elements							
Cadmium	ng	38.3	0.3	38.5	1.2	146	3
Chromium	ng	2.48	0.09	2.36	0.12	<6.40	-
Nickel	ng	<2.10	-	<2.10	-	<8.40	-
Lead	ng	9.89	0.13	10.6	0.1	32.2	1.1
Arsenic	ng	2.81	0.01	2.58	0.01	8.62	0.38
Selenium	ng	0.621	0.018	0.606	0.017	-	-
Mercury	ng	2.81	0.1	3.13	0.15	-	-

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N'*-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
The 3R4F reference cigarette values obtained under HCI machine-smoking conditions (3R4F-HCI) were derived in separate studies to the 2R4F (2R4F-ISO) and 3R4F (3R4F-ISO) values obtained under ISO machine smoking conditions. The calculation of 3R4F-HCI/3R4F-ISO ratios may result in slightly different values as presented in Tables 3, 5, and 6 as concurrently determined 3RF4-ISO values (not presented) are used.

Table B. *In vitro* toxicity of the 3R4F and the 2R4F reference cigarettes.

Assay, measure, smoke fraction	Unit	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
Cytotoxicity, 1/EC_{50}	mL/mg TPM						
TPM		10.3	0.2	10.6	0.4	8.6	0.3
GVP		7.8	0.3	8.4	0.3	6.2	0.3
Bacterial mutagenicity, slope, TPM	Revertants/ mg TPM						
TA 98, +S9		2343	85	2507	52	1991	116
TA 100, +S9		1285	72	1232	106	986	74
TA1537, +S9		395	34	337	43	281	24
TA 100, -S9		100	17	83	19	130	25
Mammalian cell mutagenicity, 1/C_{3B}, TPM,	mL/mg TPM						
+S9		6.3	0.3	5.8	0.3	4.1	0.5
-S9		27.7	2.9	26.0	1.8	23.2	1.6

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. The 3R4F reference cigarette values obtained were obtained under HCI machine smoking conditions (3R4F-HCI). 3R4F (3R4F-ISO) and 2R4F (2R4F-ISO) values obtained under ISO machine smoking conditions. Three replicates per assay in the cytotoxicity and two in the bacterial and mammalian mutagenicity assay. M = mean, SE = standard error.

Table C. *In vivo* toxicity of the 3R4F and the 2R4F reference cigarettes when smoked under ISO machine smoking conditions.

Organ, Epithelium, Finding	3R4F ISO				2R4F ISO			
	Male rats		Female rats		Male rats		Female rats	
	200 µg/L		200 µg/L		200 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE
Nose level 1								
Respiratory epithelium								
Reserve cell hyperplasia	4.0	0.0	4.0	0.0	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.8	0.1	4.3	0.2	3.4	0.2	4.0	0.3
Squamous epithelial metaplasia	4.0	0.0	4.0	0.0	4.0	0.0	4.0	0.0
Lamina propria inflammatory cell infiltrates	2.1	0.2	2.9*	0.4	2.2	0.4	1.8	0.2
Lumen								
Exudate	1.5	0.3	2.8	0.6	1.2	0.5	0.6	0.2
Nose level 2								
Respiratory region								
Reserve cell hyperplasia	2.8	0.4	3.2	0.4	3.6	0.4	2.3	0.5
Olfactory region								
Atrophy	4.6	0.3	4.2	0.4	3.6	0.5	3.8	0.5
Squamous metaplasia	3.2	0.5	3.0	0.6	2.6	0.6	3.0	0.5
Loss of nerve bundle	2.2	0.6	2.8	0.6	2.3	0.5	2.4	0.5
Nose level 3								
Olfactory epithelium								
Atrophy	3.6	0.4	3.1	0.6	2.8	0.6	3.0	0.5
Squamous epithelial metaplasia	3.6	0.4	3.1	0.6	2.6	0.6	2.8	0.6
Lamina propria inflammatory cell infiltration	2.1	0.3	1.7	0.4	0.8	0.3	1.2	0.4
Loss of nerve bundle	3.6	0.4	3.1	0.6	2.4	0.7	2.8	0.6
Nose level 4								
Olfactory epithelium								
Atrophy	3.6	0.4	3.1	0.6	2.8	0.6	2.9	0.6
Squamous epithelial metaplasia	3.4	0.4	2.8	0.6	2.6	0.6	2.8	0.6
Larynx								
Ventral depression								
Squamous epithelial metaplasia	3.8	0.6	4.9	0.1	3.7	0.5	4.9	0.1
Cornification	5.0	0.0	2.8	0.7	5.0	0.0	5.0	0.0
Floor of larynx								
Squamous metaplasia	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region								
Squamous epithelial hyperplasia	1.7	0.2	1.9	0.1	2.1	0.2	1.9	0.1
Cornification	4.0	0.6	5.0	0.0	3.9	0.5	5.0	0.0
Vocal cords, upper medial region								
Pseudostratified epithelial hyperplasia	4.0	0.4	5.0	0.0	4.5	0.2	4.9	0.1
Cornification	2.3	0.5	4.7	0.3	2.7	0.5	3.2	0.4
Vocal folds								
Squamous epithelium Cornification	4.2	0.3	4.8	0.1	3.4	0.5	5.0	0.0
Trachea								
Bifurcation								
Squamous epithelial metaplasia	1.1	0.4	1.6	0.5	2.2	0.6	1.2	0.5
Lung								
Bronchioli								
Goblet cell hyperplasia	2.6	0.7	3.6	0.5	2.4	0.7	1.7	0.5
Lumen								
Alveolar macrophages	1.7	0.2	2.2	0.2	2.1	0.1	2.1	0.2
Pigmented macrophages	4.5	0.4	4.7	0.3	4.0	0.5	3.8	0.5

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.

Table D1. *In vivo* toxicity of the 3R4F reference cigarette when smoked under both the ISO and HCI machine smoking conditions, male rats.

Organ, Epithelium, Finding	ISO						HCI					
	100 µg/L		150 µg/L		200 µg/L		100 µg/L		150 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Nose level 1												
Respiratory epithelium												
Reserve cell hyperplasia	3.5	0.2	4.0	0.0	4.0	0.0	3.7	0.2	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.2	0.3	3.9	0.2	4.4	0.2	3.1	0.3	3.9	0.1	4.1	1.2
Squamous epithelial metaplasia	2.3	0.3	3.1	0.3	3.7	0.2	2.6	0.4	3.8	0.1	3.7	0.2
Nose level 2												
Respiratory region												
Reserve cell hyperplasia	1.8	0.1	2.5	0.2	3.8	0.1	1.4	0.3	3.1	0.1	3.6	0.2
Olfactory region												
Atrophy	0.4	0.2	2.4	0.6	3.6	0.5	0.1	0.1	2.4	0.6	3.6	0.6
Squamous metaplasia	0.0	0.0	1.3	0.5	2.0	0.5	0.0	0.0	1.2	0.5	2.3	0.5
Loss of nerve bundle	0.4	0.2	2.5	0.7	3.8	0.6	0.1	0.1	2.9	0.7	3.7	0.6
Nose level 3												
Olfactory epithelium												
Atrophy	0.0	0.0	1.6	0.7	2.4	0.7	0.0	0.0	1.3	0.6	2.8	0.6
Squamous epithelial metaplasia	0.0	0.0	1.3	0.6	2.0	0.6	0.0	0.0	1.0	0.5	2.4	0.5
Loss of nerve bundle	0.0	0.0	1.5	0.6	2.4	0.7	0.0	0.0	1.3	0.6	2.8	0.6
Nose level 4												
Olfactory epithelium												
Atrophy	0.0	0.0	1.1	0.5	2.0	0.6	0.0	0.0	1.0	0.4	2.1	0.5
Squamous epithelial metaplasia	0.0	0.0	0.9	0.4	1.9	0.6	0.0	0.0	0.4	0.3	2.0	0.5
Larynx												
Ventral depression												
Squamous epithelial metaplasia	0.9	0.3	1.9	0.5	2.7	0.6	1.1	0.4	2.6	0.7	4.1	0.5
Cornification	0.0	0.0	0.9	0.6	1.5	0.8	0.0	0.0	1.1	0.7	2.7	0.9
Floor of larynx												
Squamous metaplasia	4.9	0.1	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	4.7	0.3	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region												
Squamous epithelial hyperplasia	3.1	0.3	4.0	0.0	3.0	0.3	3.8	0.2	3.5	0.3	4.0	0.0
Cornification	1.2	0.4	3.6	0.6	3.0	0.7	2.4	0.6	2.1	0.7	4.3	0.2
Vocal cords, upper medial region												
Pseudostratified epithelial hyperplasia	1.3	0.5	4.0	0.5	3.5	0.5	2.1	0.5	2.6	0.5	4.1	0.1
Cornification	0.0	0.0	2.7	1.0	1.5	0.9	0.0	0.0	0.6	0.6	1.0	0.6
Vocal folds												
Squamous epithelium												
Cornification	1.22	0.5	3.0	0.5	4.1	0.3	2.4	0.7	2.8	0.8	3.9	0.3
Trachea												
Bifurcation												
Squamous epithelial metaplasia	0.0	0.0	0.1	0.1	0.2	0.1	0.0	0.0	0.3	0.3	0.6	0.3
Lung												
Bronchioli												
Goblet cell hyperplasia	1.2	0.3	2.2	0.5	3.1	0.5	1.1	0.3	2.0	0.4	3.0	0.3
Lumen												
Alveolar macrophages	1.9	0.2	1.8	0.1	2.6	0.3	1.5	0.2	2.4	0.2	2.6	0.3
Pigmented macrophage nests	0.5	0.2	0.5	0.3	1.3	0.4	0.5	0.3	0.9	0.3	1.3	0.4

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.

Table D2. *In vivo* toxicity of the 3R4F reference cigarette when smoked under both the ISO and HCI machine smoking conditions, female rats.

Organ, Epithelium, Finding	ISO						HCI					
	100 µg/L		150 µg/L		200 µg/L		100 µg/L		150 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Nose level 1												
Respiratory epithelium												
Reserve cell hyperplasia	3.5	0.2	4.0	0.0	4.0	0.0	3.5	0.2	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.5	0.2	4.1	0.2	4.3	0.2	3.5	0.2	4.2	0.1	4.2	0.1
Squamous epithelial metaplasia	2.2	0.3	3.5	0.2	3.9	0.1	2.6	0.3	3.5	0.3	4.0	0.0
Nose level 2												
Respiratory region												
Reserve cell hyperplasia	1.3	0.2	2.5	0.2	3.2	0.2	1.0	0.2	2.7	0.2	3.2	0.2
Olfactory region												
Atrophy	0.7	0.3	3.6	0.2	4.4	0.2	1.2	0.4	3.6	0.2	4.0	0.5
Squamous metaplasia	0.1	0.1	2.5	0.4	2.9	0.2	0.6	0.4	2.4	0.3	2.6	0.3
Loss of nerve bundle	0.8	0.4	4.1	0.1	4.7	0.2	1.6	0.6	4.2	0.1	4.0	0.5
Nose level 3												
Olfactory epithelium												
Atrophy	0.0	0.0	2.4	0.5	3.6	0.4	0.3	0.2	3.0	0.4	3.4	0.4
Squamous epithelial metaplasia	0.0	0.0	2.0	0.6	3.4	0.4	0.2	0.1	2.6	0.5	2.8	0.4
Loss of nerve bundle	0.0	0.0	2.7	0.6	3.6	0.4	0.2	0.2	3.4	0.4	3.6	0.4
Nose level 4												
Olfactory epithelium												
Atrophy	0.0	0.0	1.4	0.4	2.7	0.3	0.0	0.0	1.8	0.4	2.5	0.5
Squamous epithelial metaplasia	0.0	0.0	0.9	0.4	2.7	0.4	0.0	0.0	1.3	0.5	2.3	0.5
Larynx												
Ventral depression												
Squamous epithelial metaplasia	2.0	0.7	3.3	0.6	3.9	0.6	1.1	0.5	4.6	0.4	4.3	0.5
Cornification	1.3	0.8	2.1	0.8	3.3	0.7	0.1	0.1	4.4	0.6	3.8	0.8
Floor of larynx												
Squamous metaplasia	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	4.7	0.2	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region												
Squamous epithelial hyperplasia	3.3	0.3	4.0	0.0	4.0	0.0	3.5	0.2	4.0	0.0	4.0	0.0
Cornification	2.4	0.7	4.1	0.2	4.2	0.3	2.1	0.6	4.5	0.2	4.2	0.3
Vocal cords, upper medial region												
Pseudostratified epithelial Hyperplasia	2.1	0.6	4.0	0.2	3.8	0.4	1.0	0.3	4.3	0.4	3.8	0.6
Cornification	0.6	0.6	1.0	0.6	1.6	0.8	0.0	0.0	3.6	0.8	2.5	0.9
Vocal folds												
Squamous epithelium												
Cornification	2.1	0.5	3.9	0.3	4.3	0.4	2.1	0.4	4.8	0.1	4.2	0.3
Trachea												
Bifurcation												
Squamous epithelial metaplasia	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2
Lung												
Bronchioli												
Goblet cell hyperplasia	0.6	0.4	2.0	0.2	2.9	0.3	1.3	0.5	2.5	0.4	2.2	0.3
Lumen												
Alveolar macrophages	1.5	0.2	2.1	0.2	3.0	0.2	1.6	0.2	2.3	0.2	2.8	0.2
Pigmented macrophage nests	0.3	0.2	1.4	0.4	2.5	0.3	0.6	0.2	1.5	0.5	1.7	0.4

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.