



# Evaluation of the Tobacco Heating System 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with mentholated and non-mentholated cigarette smoke

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## ABSTRACT

The toxicity of a mentholated version of the Tobacco Heating System (THS2.2M), a candidate modified risk tobacco product (MRTP), was characterized in a 90-day OECD inhalation study. Differential gene and protein expression analysis of nasal epithelium and lung tissue was also performed to record exposure effects at the molecular level.

Rats were exposed to filtered air (sham), to THS2.2M (at 15, 23 and 50 µg nicotine/l), to two mentholated reference cigarettes (MRC) (at 23 µg nicotine/l), or to the 3R4F reference cigarette (at 23 µg nicotine/l). MRCs were designed to meet 3R4F specifications.

Test atmosphere analyses demonstrated that aldehydes were reduced by 75%–90% and carbon monoxide by 98% in THS2.2M aerosol compared with MRC smoke; aerosol uptake was confirmed by carboxyhemoglobin and menthol concentrations in blood, and by the quantities of urinary nicotine metabolites.

Systemic toxicity and alterations in the respiratory tract were significantly lower in THS2.2M-exposed rats compared with MRC and 3R4F. Pulmonary inflammation and the magnitude of the changes in gene and protein expression were also dramatically lower after THS2.2M exposure compared with MRCs and 3R4F.

No menthol-related effects were observed after MRC mainstream smoke-exposure compared with 3R4F.

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## 1. Introduction

The U.S. Family Smoking Prevention and Tobacco Control Act (FSPTCA) defines a Modified Risk Tobacco Product (MRTP) as “any tobacco product that is sold or distributed for use to reduce harm or

the risk of tobacco related disease associated with commercially marketed tobacco products” (Family Smoking Prevention and Tobacco Control Act). This publication is part of a series of nine publications describing the nonclinical and part of the clinical assessment of a candidate MRTP, THS2.2 regular and a mentholated version (THS2.2M). The series of publications provides part of the overall scientific program to assess the potential for THS2.2 to be a reduced risk product. The first publication in this series describes THS2.2 and the assessment program for MRTPs (Smith et al., 2016). This is followed by six publications, including this one, that describe the nonclinical assessment of THS2.2 regular and THS2.2M

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## Abbreviations

THS2.2	Tobacco Heating System 2.2
HPHCs	harmful and potentially harmful constituents
M RTP	modified risk tobacco product
THS2.2M	mentholated version of the Tobacco Heating System
MRC	mentholated reference cigarettes
MA	mainstream aerosol
MS	mainstream smoke
OECD	Organization for Economic Cooperation and Development
GLP	Good Laboratory Practice
RNE	respiratory nasal epithelium
EHC	Electrically Heated Cigarette
TPM	total particulate matter
CO	carbon monoxide
AAALAC	American Association for the Accreditation of Laboratory Animal Care
AVA	Agri-Food & Veterinary Authority of Singapore
NACLAR	National Advisory Committee for Laboratory Animal Research
BALF	bronchoalveolar lavage fluid
PDSP	Programmable Dual-port Syringe Pump
GC	gas chromatograph
COHb	carboxyhemoglobin
PBS	phosphate-buffered saline

BSA	bovine serum albumin
EGAFS	ethanol glycerol acetic acid formaldehyde saline
H&E	hematoxylin and eosin
FC	fold-changes
MMAD	mass median aerodynamic diameter
GSD	geometrical standard deviation
NNIC	nicotine
NCOT	nicotine
3'HOCOT	3'-hydroxycotinine
COT	cotinine
NN'O	nicotine-N'-oxide
HPMA	3-hydroxypropylmercapturic acid
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
SPMA	S-phenylmercapturic acid
CEMA	2-cyanoethylmercapturic acid
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
M-I	p-menthane-3,8-diol
BW	Body weight
CCL	Chemokine (C-C motif) ligand
M-CSF-1	macrophage colony-stimulating factor
MMP9	metalloproteinase 9
DEGs	differentially expressed genes
DEPs	differentially expressed proteins
HED	Human Equivalent Doses
LOD	Limit of Detection
LOQ	Limit of Quantification

(Kogel et al., 2016 (in this issue); Oviedo et al., 2016 (in this issue); Schaller et al., 2016a (in this issue); Schaller et al., 2016b (in this issue); Sewer et al., 2016 (in this issue); Wong et al., 2016 (in this issue)). The eighth publication in the series describes a clinical study to assess whether the reduced formation of Harmful and Potentially Harmful Constituents (HPHC) for THS2.2 regular also leads to reduced exposure to HPHCs when the product is used in a clinical setting (Haziza et al., 2016 (in this issue)). A final publication utilizes data gathered from the reduced exposure clinical study on THS2.2 regular to determine if a systems pharmacology approach can identify exposure response markers in peripheral blood of smokers switching to THS2.2 (Martin et al., 2016 (in this issue)). Here we report on the toxicological data generated in a 90-day inhalation study on rats using THS2.2M, the mentholated version of THS2.2, and compare it with mentholated reference cigarettes (MRC). The MRCs were designed and manufactured by Philip Morris Products S.A. to be similar to the 3R4F reference cigarette from the University of Kentucky (<http://www.ca.uky.edu/refcig/>) in terms of general smoke parameters, but with menthol added to match menthol levels achieved in the aerosol from THS2.2M. The smoke chemistry analysis and *in vitro* toxicity of TH2.2M is summarized in Schaller et al. (2016a (in this issue)) further details on the MRC are described here.

Menthol is commonly used as a flavor in cigarettes. Although the toxicological properties of menthol are well described (World Health and Expert Committee on Food, 2015), the clinical effects of applying menthol to cigarettes are less well understood. It has been suggested that menthol in cigarettes may increase smoke exposure by affecting smoking behavior and topography (as reviewed by Wickham, (2015)), increasing the cigarettes' nicotine impact, or increasing exposure to toxic smoke constituents. On the other hand, results from clinical studies showed that smokers of menthol and nonmenthol cigarettes exhibit similar levels of biomarkers of smoke exposure (urinary nicotine and metabolites as

well as NNAL, and carboxyhemoglobin) (Heck, 2009). Another study, from the Center for Tobacco Products at the Food and Drug Administration (Rostron, 2013) showed that upon analyzing urinary NNAL concentrations in a large group of U.S. smokers, NNAL concentrations were lower among menthol smokers compared with nonmenthol smokers among smokers overall and White smokers. Finally, a small study assessing the impact of menthol on smoking behaviors, biomarkers of exposure and subjective responses concluded a minimal impact for menthol (Strasser et al., 2013). This issue is subject of ongoing debate and out of scope for the work presented, focusing on the toxicological properties of mentholated THS2.2, compared with those elicited by mentholated reference cigarettes. However, exposure levels have been assessed through the quantification of biomarkers of exposure in the present study, comparing these biomarkers in rats exposed to smoke from mentholated reference cigarettes and the nonmentholated reference 3R4F.

The main objective of the current study was to characterize the toxicological effects of MA from THS2.2M, including pulmonary inflammation, and to compare them with those elicited by MS from MRC in a 90-day sub-chronic rat inhalation study, to assess whether THS2.2M causes additional toxicity compared with the smoke from mentholated cigarettes. Additional endpoints, such as differential gene and protein expression, were added to better describe potential changes on the molecular level.

In the current study, the reference cigarette 3R4F was included as an internal reference. Results from MRCs and 3R4F were compared to determine any significant alterations of smoke-related biological effects resulting from the addition of menthol to cigarettes.

Smoke from lit-end cigarettes contains more than 8000 chemical compounds (Rodgman and Perfetti, 2013), whereas the heat-not-burn tobacco product design lowers the extent of pyrolysis and quantity of combustion products (Coggins et al., 1989; Werley

et al., 2008). Studies on earlier versions of electrically heated tobacco products have been previously reported (Moennikes et al., 2008; Schorp et al., 2012; Werley et al., 2008), and the THS2.2M tested in this study is an evolution of these products. THS2.2M tobacco sticks are heated in a stick holder in an electronic device (Smith et al., 2016 (in this issue)). The aerosol generated contains mainly water, glycerin, nicotine, and tobacco flavors, while it has markedly lower quantities of harmful and potentially harmful chemical constituents because of the absence of combustion of tobacco, compared with 3R4F (Schaller et al., 2016a (in this issue)).

The 90-day rat repeated exposure nose-only inhalation toxicity study was performed following the Organization for Economic Cooperation and Development (OECD) guideline 413 (OECD, 2009), supplemented with additional endpoints and the following deviations for the reference cigarettes for practical limitations: (1) Since no mentholated reference cigarettes were available from the University of Kentucky, MRCs were designed and manufactured by Philip Morris International to meet the tar, nicotine, carbon monoxide (CO), and cigarette diameter specifications of 3R4F. (2) Rats were exposed to three concentrations (with nicotine as dosing parameter) of aerosol from THS2.2M and to MS from only two MRC controls with menthol concentration in the test atmosphere to match menthol levels similar to those in THS2.2M. (3) MS from both MRCs had the same nicotine concentration in the test atmosphere to match the medium nicotine concentration of the MA from THS2.2M. Higher nicotine test atmosphere concentrations for the MRCs were not achievable because of the toxicity related to CO levels in MS.

Since previous studies with candidate modified risk tobacco products (MRTP) showed reduced toxicological effects compared with 3R4F exposure on OECD endpoints (Kogel et al., 2014; Patskan and Reininghaus, 2003; Terpstra et al., 2003; Werley et al., 2008), systems toxicological endpoints were included in this study to add a level of granularity in the description of the changes that may be induced by MA from THS2.2M. Gene and protein expression data were generated from the respiratory tract tissues, i.e. nasal epithelium and lung tissue, that were expected to be affected by MS and MA exposure and showed histological changes (Moennikes et al., 2008; Terpstra et al., 2003; Vanscheeuwijck et al., 2002). While emphasis is placed in this manuscript on the global molecular response profiles measured using omics technologies, more extensive analyses of these endpoints are provided in our accompanying manuscript by Kogel et al. (Kogel et al., 2016 (in this issue)).

Detailed THS2.2M smoke chemistry and *in vitro* assay data on mutagenicity/genotoxicity and cytotoxicity are presented in Schaller et al. (2016a (in this issue)).

## 2. Materials and Methods

### 2.1. Experimental design

This 90-day inhalation study was designed to characterize potential adverse toxicological effects of exposure to mainstream aerosol (MA) from the mentholated MRTP THS2.2 and to compare them with those elicited by mainstream smoke (MS) from mentholated reference cigarettes (MRC) in Sprague–Dawley rats following the OECD Test Guideline 413 (OECD, 2009). The Test Guideline has been designed to fully characterize test article toxicity by the inhalation route for a subchronic duration (90 days), and to provide robust data for quantitative inhalation risk assessments. The study was conducted according to the OECD Principles on Good Laboratory Practice (GLP) (as revised in 1997). Exposure concentrations were based on the nicotine content in the test atmospheres, which was 23 µg/l for MRCs and 15, 23, and 50 µg/l for THS2.2M. The reference cigarette 3R4F (exposure concentration of

23 µg nicotine/l) was used as an internal reference in this study.

To assess the effects of THS2.2M in terms of additional toxicity that would not be seen in the endpoints prescribed by OECD TG 413, gene and protein expression investigations were performed on the molecular level. As this analysis is performed on rats exposed according to the same protocol as the rats used for the determination of the OECD-recommended endpoints, and because exposure was performed concomitantly, this additional analysis is therefore referred as the “OECD plus” part. Gene and protein expression data were generated from tissues at anatomical sites where histological changes are expected (Terpstra et al., 2003; Vanscheeuwijck et al., 2002), and from which sufficient tissue can be obtained for gene expression analysis, i.e. the respiratory nasal epithelium (RNE) and lung parenchyma tissues.

### 2.2. THS2.2 tobacco sticks and reference cigarettes

THS2.2M blend FR1/Menthol containing different elements (tobacco plug, transfer section, and mouth piece) assembled with an over-wrapping of cigarette paper with a menthol yield in smoke condensate of 2.09 mg/cig when smoked according to ISO 3308 (3308:2012, 2012), were provided by Philip Morris Products S.A., Neuchâtel, Switzerland. THS2.2 is an evolution of the Electrically Heated Cigarette (EHC) and heats the tobacco in tobacco sticks, thus lowering the quantity of pyrolysis and combustion products (Schaller et al., 2016a (in this issue)). THS2.2M contains natural menthol applied to a cellulose acetate yarn included in the polymer-film filter and to the inner liner paper included in the tobacco stick pack. Natural menthol contains mainly L-menthol. The device includes a battery, controlling electronics, a heating element, and the stick extractor, and was provided by Philip Morris Products S.A. At the operating temperature of THS2.2M, pyrolysis of menthol can be excluded (Jenkins et al., 1970).

Two MRC versions were designed to match the nicotine, total particulate matter (TPM), and carbon monoxide (CO) levels of the 3R4F reference cigarette with a menthol yield in smoke condensate of 2.04 mg/cig (MRC(LM)) and 2.58 mg/cig (MRC(HM)) when smoked according to the ISO 3308 (3308:2012, 2012) standard. These menthol levels were chosen to be in the range of those in the aerosol from THS2.2M. MRC have been manufactured by Philip Morris Products S.A., Neuchâtel, Switzerland for the purpose of the present study. Smoke tar levels were 9.9 and 10.5 mg/cig, nicotine 0.64 and 0.64 mg/cig and CO 11.4 and 11.3 mg/cig for MRC(LM) and MRC(HM) respectively. For comparison, in smoke from 3R4F tar is 9.4 mg/cig, nicotine 0.7 mg/cig and CO 12.0 mg/cig. For MRC(LM), L-menthol (CAS 2216-51-5) was added to 11.1 mg/cig (9.0 mg/cig in the filter, 2.1 mg/cig pack inner liner), for MRC(HM) 18.4 mg/cig (9.0 mg/cig in the filter, 3.9 mg/cig pack inner liner, 5.5 mg/cig cut filler or tobacco rod). The L-menthol used had a purity >99%.

The 3R4F reference research cigarettes were purchased from the University of Kentucky (<http://www2.ca.uky.edu/refcig/>).

### 2.3. Animals and treatment

All procedures involving animals were performed in an AAALAC (American Association for the Accreditation of Laboratory Animal Care) accredited, AVA (Agri-Food & Veterinary Authority of Singapore) licensed facility with approval from an Institutional Animal Care and Use Committee, and performed in compliance with guidelines set by the National Advisory Committee for Laboratory Animal Research (NACLAR). All procedures were conducted in compliance with GLP, with the exception of bronchoalveolar lavage fluid (BALF) cytokine analysis and transcriptomics and proteomics investigations.

Outbred male and female Sprague–Dawley rats [CrI:CD(SD)],

bred under specific pathogen-free conditions, were obtained from Charles River, USA (breeding area Raleigh R04, Raleigh, NC, USA). Upon arrival, the health status of a group of six animals per sex was verified by histopathological examination and serological screening (as described in Vanscheeuwijck et al. (2002)), and by examining the health report from the sourced breeding area. The rats were kept and exposed in an animal laboratory with restricted access under controlled conditions of good hygiene. The laboratory air was filtered fresh air, and positive pressure was maintained inside the laboratory. The room temperature was maintained at  $22 \pm 3^\circ\text{C}$ , and the relative humidity at  $54 \pm 4\%$ . The light/dark cycle was 12 h/12 h, the light period starting at 7 a.m. Identification, housing, feeding, and watering were performed as described previously (Vanscheeuwijck et al., 2002).

For the OECD and OECD plus part of the study, 102 male and 102 nulliparous and non-pregnant female rats were individually identified by means of subcutaneous transponders, and were randomized to the different treatment groups stratified by sex and body weight the day before the first exposure. The relative standard deviation of body weight for each group and sex was smaller than 10%. For the OECD part, 10 rats per sex were allocated to either the filtered, conditioned air group (sham) or 5 aerosol-exposure groups: MRC(LM) 23  $\mu\text{g}$  nicotine/l, MRC(HM) 23  $\mu\text{g}$  nicotine/l, low THS2.2M (THS2.2M Low, 15  $\mu\text{g}$  nicotine/l), medium THS2.2M (THS2.2M Medium, 23  $\mu\text{g}$  nicotine/l), or high THS2.2M (THS2.2M High, 50  $\mu\text{g}$  nicotine/l). In addition, 8 rats per sex from the sham, 3R4F, MRC(HM), and THS2.2M High groups were kept for a 42 day recovery period. For the molecular endpoints (OECD plus part), 6 male and 6 female rats were allocated to each 90-day exposure group, and 6 female rats to the recovery group. The rats from the recovery groups and OECD plus groups were exposed concomitantly and in the same exposure chambers as the rats from the main study. The age of the rats at the start of the inhalation period was 8–10 weeks. The body weights were within 20% of the mean weight for each sex (172 g for females and 190 g for males). In-life observations were recorded.

#### 2.4. Test atmosphere generation and exposure

Before test atmosphere generation, mentholated tobacco sticks and MRCs were unpacked, placed in sealed boxes in order to minimize menthol losses, and conditioned in sealable climate chambers for 1–3 days prior to smoking, at a temperature of  $22.5 \pm 0.2^\circ\text{C}$  and  $58.6 \pm 0.3\%$  relative humidity. Test atmosphere was generated in basic conformity with the Health Canada Intense smoking protocol, i.e. puff duration, 2.0 s; puff volume, 55 ml; puff frequency, 2/60 s (Burns et al., 2008; Health Canada, 1999), with some minor deviations (e.g. smoking whole puffs instead of rounding to the nearest tenth of a puff) necessary for technical reasons.

MS from 3R4F and MRCs was generated on 30-port rotary smoking machines (15 ports blocked) equipped with a Programmable Dual-port Syringe Pump (PDSP) with active side stream exhaust (type PMRL-G, SM2000) (Kogel et al., 2014). Smoking machines were designed by Philip Morris Products S.A. and manufactured by Burghart Messtechnik, Weidel, Germany (<http://www.burghart-mt.de/>). MA from THS2.2M was generated using 30-port carousel smoking machines with a smoking device docking station equipped with stick holders and a PDSP. The docking station served as an interface to ensure synchronization between the cigarette holder and the smoking machine. The THS smoking machines also included a temperature-controlled insulation kit (tube warming system) in the undiluted aerosol pathway, to reduce aerosol condensation prior to dilution to the targeted aerosol concentration.

Test atmosphere was diluted with filtered, conditioned air to obtain the target nicotine concentrations (8, 15, or 23  $\mu\text{g}$  nicotine/l). The rats were nose-only exposed to the test atmosphere for 6 h/d, 5 d/week for a period of 90 days (13 weeks) using flow pass inhalation chambers as previously described (Wong et al., 2016 (in this issue)). A post-inhalation period of 42 days was included to investigate reversibility, persistence, and delayed occurrence of exposure effects. A 5-day dose-adaptation regimen was applied at the start of the inhalation period. On study days 1 and 2, the rats were exposed to one third of the target concentrations. On study days 3 and 4, they were exposed to two thirds of the target smoke concentrations. From day 5 onward, they were exposed to target concentrations. The 90-day exposure period is defined in OECD TG 413 (OECD, 2009).

The 3R4F reference cigarette was used as an internal reference to classify the severity of effects of MRCs and to monitor consistency with historical data of the laboratory.

#### 2.5. Analytical characterization of the test atmospheres

To monitor the stability and reproducibility of test atmosphere generation, nicotine, TPM, CO, and aldehydes (formaldehyde, acetaldehyde, and acrolein) were determined at the breathing zone of the rats, as well as the particle size distribution and the temperature in the exposure chambers. Relative humidity was measured in the sham exposure chamber only. All evaluations were performed according to previously described analytical methods (Hausmann et al., 1998; Stabbert et al., 2003).

For the determination of menthol in the diluted test atmosphere, samples were collected using an ethanol-impregnated diatomaceous earth column (EXTrelut<sup>®</sup> NT 3, Merck, USA). The EXTrelut<sup>®</sup> NT 3 column was impregnated with 2 ml of analytical grade ethanol (min. 99.8%) 15 min before sampling. Sample collection was carried out for 30 min at a flow of 0.7 l/min. After sample collection, the column was sealed at both ends to prevent evaporation of menthol during transfer to laboratory for analysis. All collected samples were processed immediately by adding 250  $\mu\text{l}$  of a 4 mg/ml N-heptadecane standard in ethanol to each column prior to elution with 25 ml of ethanol for a total of five cycles (5 ml per cycle). One  $\mu\text{l}$  of the eluent was analyzed using an Agilent Technologies gas chromatograph (GC) fitted with a flame ionization detector and HP-INNOWAX GC column with 1  $\mu\text{m}$  film thickness, 30 m long, 0.53 mm I.D. (J&W Scientific, Folsom, CA, USA). The GC temperature program was as follows: After equilibration at  $100^\circ\text{C}$ , the temperature was held for 2 min, subsequently increased by  $5^\circ\text{C}/\text{min}$  to  $150^\circ\text{C}$ , held for 3 min, increased by  $20^\circ\text{C}/\text{min}$  to  $200^\circ\text{C}$ , then held again for another 3 min.

All quantification was based on a series of calibration standards containing N-heptadecane as the internal standard along with analytical grade L-Menthol ( $\geq 99.0\%$ , Sigma-Aldrich, St. Louis, MO, USA) at 0.5–6 ng/ $\mu\text{L}$ . All quantitated extracts were within this range and diluted if necessary.

#### 2.6. Biological parameters

All parameters specified in OECD TG 413 were assayed with a focus on local effects in the respiratory tract in the OECD groups only (see 2.3). To provide an estimate of the test atmosphere concentration and bio-monitoring, nicotine and cotinine in plasma, as well as menthol and its metabolite p-menthane-3,8-diol (M-I) in plasma, were analyzed by Analytisch-biologisches Forschungslabor (ABF, Munich, Germany); Nicotine and cotinine in plasma was determined according to Piller et al. (2014); Menthol was determined from plasma after mixing with internal standard solution (menthol-d4). Enzymatic hydrolysis with 10  $\mu\text{l}$  glucuronidase was

performed overnight at 37 °C in PBS. After extraction by a Headspace-SPME procedure for 20 min at 80 °C, GC-MS analysis was performed in selected ion monitoring (SIM) mode. Menthol was quantified using a GC-MS system with an electron impact (EI) ionization source. For the determination of M-I, internal standard ((+)-cis-p-Menthan-3,8-diol) was added to plasma and hydrolyzed as mentioned above. After protein precipitation, supernatants are injected onto the LC-MS/MS system. Furthermore, the steady-state blood carboxyhemoglobin (COHb) concentration, respiratory physiology parameters, and representative urine nicotine metabolites, as well as biomarkers of exposure, were determined (as described in Wong et al. (2016 (in this issue)). Food consumption and body weight, as well as hematological, clinical-chemical, gross pathological (as described in Wong et al. (2016 (in this issue)), histopathological, and pulmonary inflammation parameters (described below), were determined to characterize the biological activity of the aerosols.

#### 2.6.1. Lung lavage and analysis

BALF was collected from the right lung at the end of the 90-day inhalation period using five consecutive cycles of filling and emptying. For the first cycle, pre-warmed calcium- and magnesium-free phosphate-buffered saline (PBS) was used. For cycles 2–5, bovine serum albumin (BSA) was added to the PBS. The lavage fluid of the first cycle was collected separately, centrifuged, and the supernatant fraction was frozen in aliquots (below –60 °C) and submitted to Myriad Rules-Based Medicine (Austin, TX, USA) for exploratory (non-GLP) analyses of a panel of selected proteins (RodentMAP® v3.0).

Cell pellets from the first lavage cycle were re-suspended in ice-cold PBS/BSA and pooled into the BALF from cycles 2–5. The number and viability of the free lung cells were determined, and they were further differentiated by flow cytometry (FACSCanto II, Becton, Dickinson and Company, USA) into alveolar macrophages, neutrophils, lymphocytes, and eosinophils after staining with cell-type specific antibodies; for details, see (Friedrichs et al., 2006).

#### 2.6.2. Necropsy, gross pathology, and organ weights

Full necropsy was performed without prior fasting the day after the last exposure, according to previously described methods (Vanscheeuwijck et al., 2002). The weight of the spleen, thymus, lung with larynx and trachea, heart, kidneys, adrenal glands, testis, brain, and liver was determined. Paired organs were weighed separately.

#### 2.6.3. Histopathology

To assess systemic effects, including inflammation, histological examinations of the following non-respiratory tract organs were performed (sham, 3R4F, MRC(HM), and THS2.2M(H)): Liver, kidney, adrenal glands, heart, brain, testes, spleen, thymus, lymph nodes (bronchial, mediastinal, and mesenteric), sternum with bone marrow, jejunum (including Peyer's patches), and skin. Non-respiratory tract organs were fixed in 4% formaldehyde, except for the sternum and testes, which were fixed in Schaffer's and Bouin solutions, respectively.

Histopathological evaluation was performed to investigate local effects in respiratory tract organs, i.e. nasal passages with nasal-associated lymphoid tissue, larynx, trachea, and lungs. After weight determination, the right lung was separated for BALF extraction. Fixation and histoprocessing of the left lung was performed as described previously (Vanscheeuwijck et al., 2002), although the fixative was replaced by ethanol glycerol acetic acid formaldehyde saline (EGAFS) solution. Histological sections of respiratory tract organs were prepared at defined levels (see below) and stained with hematoxylin and eosin (H&E) (Terpstra et al.,

2003). Sections from the nose at levels 1 to 4, the trachea at level 4 (bifurcation), and the left lung stained with Alcian blue/periodic acid-Schiff reagent were evaluated histopathologically. The laryngeal epithelial thickness was determined at the floor of the larynx and at the lower medial region of the vocal cords (level of arytenoid projections).

Histopathological evaluation of the respiratory tract was performed according to a defined grading system (0 = no finding; 1 = slight; 2 = slight/moderate; 3 = moderate; 4 = moderate/ marked; 5 = marked) (Terpstra et al., 2003). The nature of some of the endpoints only allowed the evaluation of incidences, which were recorded separately. Slides were evaluated in a blinded manner (without knowledge of treatment groups) by a board-certified veterinary pathologist with experience in CS-related changes in the respiratory tract of rodents.

#### 2.6.4. Statistical evaluation

Various comparisons between groups were performed. This was made separately per sex (comparisons between sexes were thus not performed). For each sex, all exposed groups were compared to the sham group. In addition, all THS2.2M-exposed groups were compared to MRC-exposed groups and, finally, MRC-exposed groups were compared against the 3R4F-exposed group to investigate whether the designed MRCs had a comparable toxicological effect in rats as the classical 3R4F reference cigarette. Both MRC groups were also compared with the 3R4F-exposed group to investigate whether the designed MRCs had a comparable toxicological effect in rats as the classical 3R4F reference cigarette.

Descriptive statistics (N, mean or median, standard deviation or geometric standard deviation) of the main aerosol parameters (TPM, CO, nicotine, formaldehyde, acetaldehyde, acrolein, and particle size distribution) were computed for each exposure chamber. For the parameters of bio-monitoring and exposure (blood COHb, selected nicotine and aerosol metabolites in urine, and respiratory physiology), descriptive statistics (number of valid measurements, mean, standard deviation or standard error of the mean) were computed for each exposure group and sex, and when relevant, for various time points.

For biological endpoints (body weight, food consumption, lung inflammation, hematology, clinical chemistry, organ weight and pathology), descriptive statistics were computed for each exposure group and sex, and when relevant, for various time points. For parameters evaluated on a continuous scale (e.g. body weight, hematology), basic statistics (number of valid measurements, mean, standard deviation, standard error of the mean) were computed. For ordinal parameters (e.g. histopathological scores), the number of valid measurements and a frequency table (absolute and relative to the number of valid observations) were computed, as well as the mean and standard deviation or standard error of the mean. For incidence parameters, the number of valid measurements and a frequency table (absolute and relative to the number of valid observations) were computed.

Additionally and separately for each sex, pairwise differences between groups were estimated. For continuous variables, this was done by means of a *t*-test (potentially accounting for variance heterogeneity) associated with 95% confidence intervals or, depending on the normality of the data, either a parametric test (ANOVA) or a non-parametric test was performed. For incidences, this was done using Fisher's exact test. For ordinal variables, this was done by the Cochran-Mantel-Haenszel test, using the row mean scores statistic (with the alternative hypothesis that mean responses differ across compared groups, the scores being defined as integer scores). Finally, for data from multi-analyte profiles in BALF, as the number of values below the limit of detection/quantification was high for some analytes, a Mann-Whitney-Wilcoxon

rank sum test was performed.

### 2.7. Tissue preparation for systems toxicology endpoints (“OECD plus”)

Dissection took place 16–24 h after the last exposure. Prior to organ removal, a whole body perfusion with cold saline was performed. In short, after setting the flow rate of a peristaltic pump at 60 ml/min, a needle was inserted in the left ventricle and then a small incision was performed at the right atrium of the heart. Perfusion was performed for approximately 8 min until organs turned pale. For transcriptomics analysis, RNE was isolated from the left side of the nose. For proteomics analysis, the RNE was isolated from the right side. The left lung lobe was cryo-sectioned into 40  $\mu$ m thick slices, and the slices were collected alternating for transcriptomics and proteomics (and backup) analysis.

### 2.8. Transcriptomics

RNA from RNE and lung samples was isolated using RNeasy Mini extraction kit (Qiagen, Hilden, Germany). RNA was processed for hybridization on GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) by using the High Throughput 3' In Vitro Transcription PLUS kit (Affymetrix). Further details can be found in our accompanying manuscript (Kogel et al., 2014). Raw CEL files were background-corrected, normalized, and summarized using frozen Robust Multiarray Analysis (fRMA) (McCall et al., 2010). To identify genes that were differentially expressed, a linear model was fitted for each exposure condition and its respective sham group, and *p*-values from a moderated *t*-statistic were calculated with the empirical Bayes approach (Gentleman et al., 2004). The Benjamini-Hochberg False Discovery Rate (FDR) method was then used to correct for multiple testing effects. Genes with an adjusted *p*-value <0.05 were considered differentially expressed.

A heatmap of the log<sub>2</sub> transformed gene expression fold-changes (FC) between samples exposed to MA and the corresponding samples exposed to sham was generated, using the heatmap.2 function in the gplots R package (Warnes et al., 2012). A gene was defined as differentially expressed if its FDR <0.05. Fold-change was set to zero for all the non-differentially expressed genes.

### 2.9. Proteomics

Proteome alterations were assessed by isobaric tag-based quantification using the iTRAQ® approach. The full details of the method are given in our accompanying manuscript (Sewer et al., 2016 (in this issue)). Briefly, two multiplexed iTRAQ analysis sets were prepared to allow for all relevant comparisons: iTRAQ set 1 contained all samples of the 90-day time point, and iTRAQ set 2 was focused on the exposure and 90 + 42d recovery effects for female rats only. RNE samples were homogenized and sonicated in tissue lysis buffer (Bio-Rad, Hercules, CA, USA) in random order, and the proteins were precipitated with acetone. Frozen rat lung tissue was homogenized with a bead-assisted procedure in TissueLyser II (Qiagen, Hilden, Germany) in tissue lysis buffer before acetone precipitation. Protein precipitates were processed for the iTRAQ 8-plex labeling procedure according to the manufacturer's instructions (SCIEX, Framingham, MA, USA).

Samples were analyzed in random order using an Easy-nanoLC 1000 liquid chromatograph (Thermo Scientific, Waltham, MA, USA) connected online to a Q Exactive (Thermo Scientific) mass analyzer. Mass spectrometry runs were processed and quantified using Proteome Discoverer version 1.4.0.288 software (Thermo Scientific) and custom software. For the detection of differentially

expressed proteins, a linear model was fitted for each exposure condition and its respective sham group, and *p*-values from a moderated *t*-statistic were calculated with the empirical Bayes approach (Gentleman et al., 2004). The Benjamini-Hochberg FDR method was then used to correct for multiple testing effects. Proteins with an adjusted *p*-value <0.05 were considered differentially expressed.

## 3. Results

### 3.1. Test atmosphere composition

Analysis of the test atmospheres throughout the 90-day inhalation period indicated that the target nicotine concentration for all groups was achieved, and was within  $\pm 10\%$  of target values. Daily concentrations of TPM and CO were also reproducible and within a variation of  $\pm 10\%$  throughout the entire exposure period. MA generated from THS2.2M contained approximately only 50% of the TPM and only 2% of the CO measured in MS from MRCs at the same nicotine concentration.

The particle size distribution measurements indicated that particles were equally respirable in all MS- and MA-exposed groups: The mass median aerodynamic diameter (MMAD) ranged from 0.62 to 0.71  $\mu$ m (geometrical standard deviation (GSD) from 1.33 to 1.52) in the test atmosphere from MRCs and THS2.2M.

At equal nicotine concentrations, the quantities of formaldehyde and acetaldehyde present in THS2.2M MA were approximately 85% and 78% lower, respectively, compared with MS from MRCs; acrolein levels in THS2.2M MA were lower by more than 90%.

The menthol concentration in diluted MS from the low MRC matched the concentration from the medium THS2.2M, whereas the menthol concentration in MS from the high MRC reached approximately 75% of the high THS2.2M concentration.

A detailed analysis of the test atmospheres generated from THS2.2M and MRCs, is given in Table 1.

Test atmosphere characteristics for the 3R4F reference cigarette (Table 1) were in accordance with those determined in other studies (Wong et al., 2016 (in this issue)).

### 3.2. Bio-monitoring

#### 3.2.1. Respiratory physiology

Because of a technical error in the equipment setup, it was only possible to measure respiratory frequencies. Data are shown in Tables 2a and 2b.

The respiratory frequencies of the male and female rats exposed to MRC(LM) and MRC(HM) were statistically significantly lower than those in sham-exposed male and female rats (*p* < 0.05 and *p* < 0.01, respectively). In contrast, the respiratory frequencies of rats exposed to THS2.2M MA remained unaffected compared with sham, and were statistically significantly higher in the female THS2.2M Medium group compared with the MRC(LM) group, indicating that MS from MRC may have a higher irritant power than MA from THS2.2M. No menthol-related differences were observable in respiratory frequency in MRC-groups compared with the 3R4F group.

#### 3.2.2. Carboxyhemoglobin

The steady-state concentration of COHb in blood was up to 26.5% in rats exposed to MRCs and up to 4.6% in rats exposed to THS2.2M (Tables 2a and 2b). COHb levels in MRC MS-exposed animals were comparable to 3R4F, and are in line with our historical data for 3R4F MS inhalation (Wong et al. (in this issue)), indicating efficient uptake of the aerosols by the animals. COHb in male THS2.2M MA-exposed rats showed no statistically significant

**Table 1**

Characterization of test atmospheres – determinations performed in the exposure chambers.

Group	TPM ( $\mu\text{g/l}$ )	Particle size distribution		Nicotine ( $\mu\text{g/l}$ )	Carbon monoxide (ppm)	Formaldehyde ( $\mu\text{g/l}$ )	Acetaldehyde ( $\mu\text{g/l}$ )	Acrolein ( $\mu\text{g/l}$ )	Menthol in diluted aerosol ( $\mu\text{g/l}$ )
		MMAD ( $\mu\text{m}$ )	GSD						
Sham	0	0	0	0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3R4F	303.9 $\pm$ 16.5	0.670–0.751	1.23–1.96	22.67 $\pm$ 1.65	362.59 $\pm$ 21.80	0.6 $\pm$ 0.11	17.47 $\pm$ 2.25	1.8 $\pm$ 0.18	<LOQ
MRC (LM)	335.8 $\pm$ 14.6	0.669–0.728	1.22–1.77	22.92 $\pm$ 1.20	375.19 $\pm$ 23.88	0.89 $\pm$ 0.18	15.7 $\pm$ 3.2	1.73 $\pm$ 0.31	56.12 $\pm$ 2.24
MRC(HM)	342.5 $\pm$ 14.2	0.670–0.718	1.23–2.53	22.17 $\pm$ 1.03	372.15 $\pm$ 15.96	0.89 $\pm$ 0.25	14.95 $\pm$ 4.46	1.66 $\pm$ 0.46	80.22 $\pm$ 1.64
THS2.2M Low	88.6 $\pm$ 4.7	0.601–0.666	1.16–2.78	14.5 $\pm$ 0.54	4.76 $\pm$ 0.25	0.09 $\pm$ 0.01	2.25 $\pm$ 0.15	0.08 $\pm$ 0.01	32.68 $\pm$ 1.9
THS2.2M Medium	143.3 $\pm$ 8.7	0.604–0.787	1.40–4.94	22.60 $\pm$ 1.10	6.80 $\pm$ 0.60	0.14 $\pm$ 0.02	3.48 $\pm$ 0.3	0.14 $\pm$ 0.01	48.87 $\pm$ 2.08
THS2.2M High	370.4 $\pm$ 26.7	0.606–0.626	1.20–2.18	49.26 $\pm$ 2.57	16.8 $\pm$ 0.92	0.34 $\pm$ 0.04	8.04 $\pm$ 0.71	0.36 $\pm$ 0.04	109.14 $\pm$ 8.74

Results represent mean  $\pm$  standard deviation. Abbreviations: TPM, total particulate matter; MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; LOQ, lower limit of quantification.

differences compared with sham-exposed rats. Slightly higher proportions of COHb that were statistically significant were observed in females in the THS2.2M Medium and THS2.2M High groups. Similarly higher proportions of COHb were found in the male rats exposed to these test atmospheres, though without reaching statistically significant differences. Measurements repeated at the end of the recovery period showed that all COHb values were at basal levels.

### 3.2.3. Nicotine metabolites in urine

The quantities of nicotine metabolites (nornicotine (NNIC), norcotinine (NCOT), 3'-hydroxycotinine (3'HOCOT), cotinine (COT), and nicotine-N'-oxide (NN'O)) were determined in 24-h urine (Tables 2a and 2b). As controls, nicotine metabolites were determined in urine samples collected before the 90-day exposure phase and during the 42-day recovery phase. The results obtained were all below the limit of quantification, and were as follows (in  $\mu\text{mol/l}$ ): NNIC: 1.746, NCOT: 0.906, 3'HOCOT: 1.734, COT: 1.307, NN'O: 5.225 (data not shown). The total quantities of nicotine metabolites excreted were comparable between the two MRC groups, and did not differ from the 3R4F group. In THS2.2M-exposed rats, the total quantities of nicotine metabolites excreted were comparable between male and female rats, and proportional to the nicotine test atmosphere concentrations, except for the THS2.2M Medium female group. At equal nicotine concentrations in the test atmosphere, the total quantities of nicotine metabolites excreted were higher in the THS2.2M Medium group than in the MRC MS-exposed rats. This suggests an overall higher uptake of aerosol constituents in the THS2.2M MA-exposed rats.

The relative distribution of the five nicotine metabolites measured (data not shown) was similar in all groups, indicating similar metabolism, in line with results observed in previous studies (Vanscheeuwijck et al., 2002).

There was no menthol effect on the level of nicotine metabolites observed in the MRC groups compared with 3R4F.

Biomarkers of exposure, including 2-cyanoethylmercapturic acid (CEMA) (metabolite of acrylonitrile), 3-hydroxypropylmercapturic acid (HPMA) (metabolite of acrolein), total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)), and S-phenylmercapturic acid (SPMA) (metabolite of benzene), were quantified in 24-h urine (Tables 2a and 2b). The concentrations of biomarkers in sham animals during exposure and in the urine of rats collected before the start of the exposure period were very low, with the exception of HPMA due to endogenous production of acrolein (as a metabolic product) in normal rats (Stevens and Maier, 2008). During exposure, the quantity of HPMA was at least 2-fold lower in the THS2.2M-exposed groups than in the MRC groups at equal nicotine exposure concentrations. The quantities of CEMA, SPMA, and NNAL were 40-fold, 46-fold, and 4-

fold lower, respectively, in the THS2.2M-exposed groups than in the reference groups at equal nicotine exposure concentrations.

No menthol-related differences were observed for any of the biomarkers in urine in MRC groups compared with the 3R4F group.

### 3.2.4. Biomarkers of exposure in blood

Biomarkers of exposure in blood included nicotine and cotinine, as well as menthol and its metabolite p-menthane-3,8-diol (M-I) in plasma (Tables 3a and 3b). In THS2.2 MA-exposed groups, a trend of higher nicotine and cotinine levels in plasma was observed, in function of higher test atmosphere nicotine concentrations. The concentration of nicotine and cotinine in plasma in the THS2.2M Medium groups was increased 1.5-fold and 1.2-fold, respectively, in male rats, and 1.2-fold in female rats compared with the THS2.2M Low groups. In the THS2.2M High groups, nicotine and cotinine levels were 3.3-fold and 1.8-fold higher, respectively, in male rats, and 2.8-fold and 2.0-fold higher, respectively, in female rats, compared with the THS2.2M Low groups.

Female rats showed higher levels of nicotine and cotinine in serum relative to male rats.

The uptake of menthol was verified by quantification of menthol and M-I in plasma. The observed serum menthol and M-I concentrations showed a concentration-dependent response in the THS2.2M MA- and MRC MS-exposed rats. In line with the test atmosphere data, menthol and M-I levels in blood were lower in the MRC(HM) group compared with the THS2.2M High group.

The levels of menthol in the MRC had no observable influence on the nicotine or cotinine levels in serum, and values were comparable to those in 3R4F.

### 3.3. In-life observations, clinical observations

Clinical observations after daily exposure included harderian gland secretion and wet fur, which were frequently observed in sham and all MA/MS-exposed groups, whereas nasal discharge and closed eyelids were seen in MS-exposed groups only. Overall, similar clinical observations have been noted in previous rat inhalation studies with cigarette smoke (Schramke et al., 2014) and are known to be related to tube restraint and/or the irritant capacity of cigarette smoke.

Body weight (BW) development revealed an exposure-related reduction for MRC-exposed male rats during the 90-day inhalation period (Fig. 1); BW was statistically significantly lower by 18% at the end of the 90-day exposure period for all MRC MS-exposed groups, as well as for the 3R4F group (17% lower), indicating that there was no menthol-related effect on BW development.

BW development of THS2.2M-exposed male rats started to differ from the sham group on study day 50; the BW of all THS2.2M groups were clustered together, and were statistically significantly lower by 9–11% compared with sham. At equivalent nicotine

**Table 2a**  
Body weights and bio-monitoring parameters measured during the 90-day exposure period to test atmosphere.

Parameter	Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High
Body weight (day 89) [g]	M 448 ± 12.7 [9] F 249 ± 6.2 [10]	374 ± 8.5 [10] *** 270 ± 5.5 [8]**	370 ± 13.1 [10]*** 272 ± 9.2 [10]	366 ± 10.7 [10] *** 263 ± 6.6 [9]*	403 ± 8.5 [10]* 277 ± 8.9 [10]*	409 ± 12.5 [10] 283 ± 3.2 [10]***	404 ± 15.8 [8] 282 ± 8.6 [10]**
Respiratory frequency [breaths min <sup>-1</sup> ]	M 142.34 ± 8.38 [9] F 140.44 ± 10.2 [10]	116.16 ± 3.89 [9] * 102.05 ± 5.25 [10] **	120.21 ± 6.08 [10]* 101.16 ± 5.88 [9] **	117.38 ± 2.74 [10] * 105.48 ± 4.33 [10] **	133.21 ± 7.06 [10] 134.3 ± 5.42 [10]	141.3 ± 9 [10] 145.9 ± 7.8 [8]	127 ± 6.9 [8] 116.5 ± 5.6 [10]
COHb [%]	M 3.65 ± 0.22 [10] F 3.12 ± 0.03 [6]	27 ± 0.82 [8] *** 26.2 ± 1 [3] **	22.31 ± 0.8 [8] *** 22.54 ± 1.35 [7] ***	26.49 ± 0.68 [7] *** 24.07 ± 1.68 [7] ***	3.44 ± 0.05 [10] 2.94 ± 0.15 [8]	3.7 ± 0.1 [10] 3.6 ± 0.1 [10] **	4.3 ± 0.05 [9] 4.26 ± 0.1 [7] ***
CEMA in urine [ng/ml]	M 5.49 ± 0.56 [10] F 178.4 ± 115.8 [10]	802.28 ± 114.6 [10] *** 858.22 ± 95.18 [10] ***	715.62 ± 112.7 [10] *** 812.9 ± 116.01 [10] ***	765.21 ± 111.39 [10] *** 436.46 ± 116.59 [10] ***	11.32 ± 1.21 [10] ** 6.36 ± 0.6 [10] ***	12.2 ± 1.1 [10] ** 9 ± 0.9 [10] **	17.65 ± 2.8 [10] *** 362.4 ± 248.4 [10] **
SPMA in urine [ng/ml]	M 3.81 ± 0.46 [10] F 91.74 ± 59.72 [10]	478.13 ± 53.94 [10] *** 391.81 ± 52.95 [10] **	505.35 ± 62.06 [10] *** 370.72 ± 36.68 [10] **	610.83 ± 89.85 [10] *** 302.81 ± 81.12 [10] **	6.56 ± 0.89 [10] * 3.31 ± 0.33 [10]	7.1 ± 0.6 [10] *** 5.3 ± 0.6 [10]	11.23 ± 1.75 [10] ** 116.13 ± 73.6 [10] *
NNAL in urine [pg/ml]	M 0.85 ± 0 [10] F 253.9 ± 170.9 [10]	715.5 ± 95.4 [10] *** 803 ± 117.5 [10] *	509.2 ± 89.3 [10] *** 465.7 ± 58.9 [10] *	610.4 ± 93 [10] *** 295.3 ± 64.1 [10] *	21.9 ± 5.3 [10] ** 30.8 ± 8.2 [10]	35.1 ± 8.4 [10] ** 47.4 ± 10.2 [10] *	134.4 ± 40.5 [10] *** 380.4 ± 241.7 [10] *
HPMA in urine [ng/ml]	M 2932.3 ± 307.2 [10] F 4544.6 ± 1439 [10]	12098.7 ± 1912 [10] *** 11749.5 ± 1442 [10] **	13058.7 ± 1486 [10] *** 11315.4 ± 1182 [10] **	13297.7 ± 2557.8 [10] ** 10086.7 ± 1583 [10] *	4373.2 ± 480.1 [10] 3078.3 ± 463 [10]	5235.5 ± 794.5 [10] 4883.8 ± 969 [10]	5762.4 ± 804.9 [10] 6586.5 ± 2205 [10]
Total nicotine metabolites in urine [nmol]	M 9.02 ± 1.4 [10] F 7.7 ± 1.1 [10]	1982.2 ± 97.3 [10] *** 2437.5 ± 168 [10] ***	2852.9 ± 161.6 [10] *** 3107.4 ± 333.5 [10] ***	2367.5 ± 211.6 [10] *** 2280.2 ± 114.6 [10] ***	2752.9 ± 182.3 [10] *** 2656.1 ± 342.8 [10] ***	4618.8 ± 539.1 [10] *** 3228.6 ± 293.3 [10] ***	7934.9 ± 328.4 [10] *** 8186.7 ± 707.7 [10] ***
Nicotine in plasma [ng/ml]	M 1.92 ± 0.48 [10] F 2.46 ± 0.33 [10]	306.9 ± 22.75 [10] *** 509.26 ± 64.61 [10] ***	281.04 ± 25.04 [10] *** 441.2 ± 29.79 [10] ***	263.09 ± 17.89 [10] *** 486.96 ± 81.26 [10] ***	231 ± 17 [10] *** 431 ± 40.2 [10] ***	340.5 ± 18.3 [10] *** 590.6 ± 46.3 [10] ***	762 ± 70.7 [10] *** 1207.7 ± 81.4 [10] ***
Cotinine in plasma [ng/ml]	M 1.51 ± 0.13 [10] F 2.06 ± 0.49 [10]	509.26 ± 20.97 [10] *** 591.88 ± 41.82 [10] ***	569.01 ± 19.98 [10] *** 608.9 ± 27.9 [10] ***	526.02 ± 29.95 [10] *** 603.39 ± 41.44 [10] ***	737.9 ± 31.9 [10] *** 705.3 ± 35.3 [10] ***	875.2 ± 36.8 [10] *** 828.6 ± 29.2 [10] ***	1335.5 ± 53.2 [10] *** 1398.5 ± 106.3 [10] ***
Menthol in plasma [ng/ml]	M 17.75 ± 0 [10] F 17.75 ± 0 [10]	17.75 ± 0 [10] - 17.75 ± 0 [10] -	327.52 ± 34.51 [10] *** 609.35 ± 106 [10] ***	453.88 ± 35.62 [10] *** 850.06 ± 179.9 [10] ***	116.3 ± 10.3 [10] *** 169.3 ± 11.4 [10] ***	229.1 ± 24.8 [10] 317.7 ± 33.3 [10]	732.25 ± 75.6 [10] 971.5 ± 113. [10]
M-I in plasma [ng/ml]	M 0.85 ± 0 [10] F 0.85 ± 0 [10]	0.85 ± 0 [10] - 0.85 ± 0 [10] -	150.84 ± 18.54 [10] *** 242.6 ± 39.4 [10]***	217.49 ± 24.4 [10] *** 358.28 ± 84.7 [10] ***	126.6 ± 17.4 [10] *** 211.9 ± 26.1 [10] ***	192.4 ± 46.6 [10] 305.1 ± 40.7 [10]	309.4 ± 48.7 [10] 434. ± 59.9 [10]

Results represent mean ± standard error. Abbreviations and limits of detection/quantification: M, male; F, female; LM, low menthol; HM, high menthol; COHb, carboxyhemoglobin; LOQ, limit of quantification; LOD, limit of detection; HPMA, 3-hydroxypropylmercapturic acid (biomarker for acrolein uptake; LOD 12.6 ng/ml; LOQ 25 ng/ml); NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (total, biomarker for NNK uptake; LOD 1.7 pg/ml; LOQ 5.0 pg/ml); SPMA, S-phenylmercapturic acid (biomarker for benzene uptake; LOD 0.005 ng/ml; LOQ 0.02 ng/ml); CEMA, 2-cyanoethylmercapturic acid (marker for acrylonitrile uptake; LOD 0.08 ng/ml; LOQ 0.25 ng/ml); MI, p-menthane-3,8-diol (LOD 1.7 ng/ml; LOQ 5 ng/ml). Nicotine (LOD 1.0 ng/ml; LOQ 2.0 ng/ml); Cotinine (LOD 0.5 ng/l; LOQ 2.0 ng/l). Menthol (LOD 35.5 ng/l; LOQ 100 ng/l). The sample size is in parentheses. Difference from sham group at 90d: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

**Table 2b**

Statistically significant differences for body weights and bio-monitoring parameters.

Parameter		THS2.2M medium vs.MRC(LM)	THS2.2M high vs. MRC(HM).	MRC (HM) vs. 3R4F
Body weight (day 89)[g]	M	↑*	↑**	=
	F	=	↑*	=
Respiratory frequency [min <sup>-1</sup> ]	M	=	=	=
	F	↑***	=	=
COHb [%]	M	↓***	↓***	=
	F	↓***	↓***	=
Total nicotine metabolites in urine [nmol]	M	↑**	↑***	=
	F	↑***	↑***	=
CEMA in urine [ng/ml]	M	↓***	↓***	=
	F	↓***	=	↓*
SPMA in urine [ng/ml]	M	↓***	↓***	=
	F	↓***	=	=
NNAL in urine [pg/ml]	M	↓***	↓***	=
	F	↓***	=	↓**
HPMA in urine [ng/ml]	M	↓***	↓*	=
	F	↓***	=	=
Nicotine in plasma [ng/ml]	M	=	↑***	=
	F	↑*	↑***	=
Cotinine in plasma [ng/ml]	M	↑***	↑***	=
	F	↑***	↑***	=
Menthol in plasma [ng/ml]	M	↓*	↑**	↑***
	F	↓*	=	↑***
MI in plasma [ng/ml]	M	=	↑*	↑***
	F	=	=	↑***

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; COHb, carboxyhemoglobin; HPMA, 3-hydroxypropylmercapturic acid; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; SPMA, S-phenylmercapturic acid; CEMA, 2-cyanoethylmercapturic acid; MI, p-menthane-3,8-diol.

concentrations, BW of male THS2.2M Medium rats was statistically significantly higher compared with MRC-exposed rats after 90 days. The body weights at the end of the inhalation period (day 89) are shown in Tables 2a and 2b.

BW development in female rats did not show any treatment-related differences from the sham group, which is consistent with results from previous studies (Coggin, 1993; Coggin et al., 1989; Vanscheeuwijck et al., 2002).

After the 42-day post-inhalation period, the progression of BW across all exposure groups was similar to that of sham for both male and female rats, and there was no statistically significant difference between them.

Food consumption was statistically significantly higher in all THS2.2M MA-exposed groups compared with sham.

Clinical chemistry (Tables 3a and 3b) in MRC MS-exposed groups revealed higher activity of the liver enzyme alkaline phosphatase. The concentrations of total cholesterol, triglycerides, and glucose were consistently significantly lower in both sexes compared with sham, as also observed in previous studies (Schramke et al., 2014; Terpstra et al., 2003; Vanscheeuwijck et al., 2002). Similar effects were seen in the THS2.2M-exposed rats, and were dependent on the exposure concentration. These changes seem to be due to nicotine, as demonstrated in a previous study investigating the effects of nebulized nicotine and nicotine pyruvate in a 28-day inhalation experiment (Phillips et al., 2015a).

In all MRC MS- or THS2.2M MA-exposed groups, other sporadic effects were seen in either males or females (Tables 3aa and 3b). After the post-inhalation period, most exposure-induced effects were reversed.

Hematology analysis (Tables 4a and 4b) revealed statistically significant differences of the MA/MS-exposed groups compared with sham, which were consistently higher for both sexes for the number of neutrophils. Lymphocyte counts were also statistically significantly lower in all MRC groups, in THS2.2M female rats, and in THS2.2M High male rats. In addition, female rats exposed to MRC MS showed a statistically significantly higher reticulocyte count

than sham.

Other changes were occasionally statistically significantly different, but in the range of those found in the sham or in the historical range from previous inhalation studies.

Organ weight changes in MS-exposed groups (Tables 5a and 5b) exhibited typical MS exposure-related effects in accordance with previously reported findings (Carmines and Gaworski, 2005; Terpstra et al., 2003; Vanscheeuwijck et al., 2002). There were statistically significant changes in the relative weight (organ weight relative to exsanguinated body weight) of certain organs for all exposure groups compared with sham, including adrenals, thymus, uterus, ovaries, and kidney and lung with trachea and larynx. However, relative weights of lung with trachea and larynx were statistically significantly lower in THS2.2M groups compared with MRC groups. Higher relative organ weight of testis was observed for MRC groups and the THS2.2M High group compared with sham, and for heart in MRC groups only.

An increase in liver weight was observed in the THS2.2M groups, which was statistically significantly higher compared with sham in all THS2.2M groups except for the male THS2.2M Low group, showing an exposure concentration-dependent trend. Furthermore, liver weights of MRC groups were also statistically significantly higher compared with sham, but did not differ from liver weights in THS2.2M Medium groups.

In general, no differences were observed in body weight, clinical chemistry, or organ weight results in MRC groups compared with the 3R4F group. However, erythrocyte counts were statistically significantly higher in the male MRC(HM) group, and reticulocyte counts were statistically significantly higher in the female MRC(HM) group compared with 3R4F. Relative organ weight of the uterus was statistically significantly lower in the female MRC(HM) group compared with 3R4F.

### 3.4. Free lung cell differentiation in BALF

Exposure to MRC caused a statistically significantly higher total

**Table 3a**

Clinical chemistry parameters determined at the end of the 90-day and the post-inhalation periods.

Parameter	90 days							90 + 42d			
	Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Alanine amino - transferase (IU/L)	M 57.1 ± 6.4 [10] F 57.65 ± 11.5 [10]	62.6 ± 3.5 [9] 66.75 ± 7 [8]	53.5 ± 2.8 [10] 80.75 ± 14.9 [10]*	59.9 ± 3.6 [10] 58.4 ± 3.7 [9]	58.4 ± 1.8 [10] 66.7 ± 3.5 [10]*	61.3 ± 4.2 [10] 71.9 ± 5.5 [10]	77.2 ± 5.5 [10]** 62.3 ± 4.3 [10]	50.3 ± 3.7 [8] 48.1 ± 3.8 [7]	48.3 ± 3.7 [8] 42.1 ± 3.6 [8]	45.3 ± 1.2 [8] 44.4 ± 4.6 [8]	44 ± 1.8 [8] 31.9 ± 2 [8]
Alkaline phosphatase (IU/l)	M 170.3 ± 13.5 [10] F 126.5 ± 13.3 [10]	249.8 ± 29.2 [9] 194.6 ± 19.5 [8]	253.4 ± 26.8 [10]* 213.5 ± 19.2 [10]**	203.8 ± 20 [10] 211.7 ± 21.4 [9]**	173.1 ± 13 [10] 152.5 ± 15 [10]	185.5 ± 14.7 [10] 151 ± 14.3 [10]	297.4 ± 24.9 [10]*** 216.7 ± 17.7 [10]***	130.3 ± 7.6 [8] 85 ± 10.8 [7]	151.9 ± 21.3 [8] 72.6 ± 13.2 [8]	127.9 ± 18.8 [8] 88.9 ± 10.5 [8]	155.9 ± 18.3 [8] 69.5 ± 8.7 [8]
Aspartate amino-transferase (IU/l)	M 111.3 ± 16.8 [10] F 147.1 ± 23.8 [10]	127.9 ± 8.2 [9]* 163.2 ± 13.5 [8]	118.7 ± 10.4 [10]* 204.8 ± 60.5 [10]	139.4 ± 5.7 [10] 146.9 ± 17.8 [9]	134.5 ± 8.1 [10] 129.3 ± 6.6 [10]	127.6 ± 10.1 [10] 168 ± 28 [10]	150.1 ± 13.4 [10]*** 135.9 ± 12.7 [10]	114.6 ± 5.7 [8] 88.3 ± 4.3 [7]	118.4 ± 10.9 [8] 95.3 ± 10.3 [8]	83.1 ± 5 [8] 105.2 ± 11.1 [8]	104.3 ± 8.2 [8] 75.7 ± 5.8 [8]
Calcium (mmol/l)	M 2.4 ± 0.1 [10] F 2.4 ± 0.02 [10]	2.4 ± 0.05 [9] 2.4 ± 0.0 [8]**	2.4 ± 0.04 [10] 2.4 ± 0.0 [10]	2.3 ± 0.02 [10] 2.4 ± 0.0 [9]	2.3 ± 0.0 [10] 2.3 ± 0.0 [10]*	2.3 ± 0.1 [10] 2.3 ± 0.0 [10]	2.04 ± 0.1 [10] 2.3 ± 0.0 [10]*	2.4 ± 0.0 [8] 2.6 ± 0.1 [7]	2.5 ± 0.02 [8] 2.6 ± 0.04 [8]	2.5 ± 0.04 [8] 2.6 ± 0.05 [8]	2.6 ± 0.1 [8] 2.6 ± 0.1 [8]
Creatinine kinase (mol/l)	M 36.3 ± 2 [10] F 33.5 ± 2.2 [10]	32.2 ± 1.6 [9] 29.3 ± 2.3 [8]	29.4 ± 2.1 [10]* 28.9 ± 3.3 [10]*	31.9 ± 1.6 [10] 30.1 ± 1.7 [9]*	29.1 ± 1.6 [10]* 26.6 ± 1.5 [10]**	32.1 ± 2.3 [10] 27.8 ± 1.5 [10]	29.6 ± 2.8 [10] 25 ± 1.7 [10]	39.6 ± 1.8 [8] 36.7 ± 1.9 [7]	38.3 ± 1.7 [8] 40 ± 1.0 [8]	40.4 ± 1.1 [8] 36.5 ± 2.2 [8]	43.5 ± 2.3 [8] 37 ± 1.9 [8]
Glucose (mmol/l)	M 13.8 ± 0.8 [10] F 10.9 ± 0.7 [10]	9.5 ± 0.9 [9]** 8.1 ± 0.6 [8]**	9.6 ± 0.8 [10]** 8.7 ± 0.7 [10]*	8.9 ± 0.5 [10]*** 9 ± 1 [9]*	11 ± 0.9 [10]* 8.8 ± 0.4 [10]*	10.1 ± 0.8 [10]** 8.8 ± 0.6 [10]*	10.4 ± 1.4 [10]* 7.9 ± 0.4 [10]**	12.1 ± 0.7 [8] 10.4 ± 0.4 [7]	13.8 ± 1.1 [8] 10.8 ± 0.7 [8]	15.2 ± 0.6 [8] 9.9 ± 0.8 [8]	14.1 ± 1.5 [8] 12.2 ± 1.5 [8]
Total cholesterol (mmol/l)	M 1.22 ± 0.1 [10] F 1.29 ± 0.1 [10]	0.94 ± 0.0 [9]** 0.81 ± 0.1 [8]**	0.99 ± 0.0 [10]* 0.72 ± 0.0 [10]***	0.99 ± 0.1 [10]* 0.8 ± 0.1 [9]***	0.94 ± 0.1 [10]* 0.96 ± 0.1 [10]**	0.91 ± 0.0 [10]** 0.82 ± 0.1 [10]***	1.11 ± 0.1 [10] 1.02 ± 0.1 [10]*	1.35 ± 0.1 [8] 1.28 ± 0.1 [7]	1.39 ± 0.06 [8] 1.48 ± 0.1 [8]	1.25 ± 0.1 [8] 1.17 ± 0.1 [8]	1.47 ± 0.1 [8] 1.27 ± 0.1 [8]
Total protein (g/l)	M 59.6 ± 1.4 [10] F 65.3 ± 1.1 [10]	61.3 ± 0.9 [9] 61.1 ± 1.1 [8]*	60.6 ± 0.8 [10] 61 ± 1.5 [10]*	60 ± 0.6 [10] 60.3 ± 1 [9]**	58.2 ± 1.4 [10] 58 ± 1.2 [10]***	57.5 ± 1.5 [10] 58.6 ± 0.9 [10]***	60.8 ± 1.2 [10] 59.2 ± 0.9 [10]***	61.9 ± 0.6 [8] 67 ± 1.5 [7]	63.1 ± 1.4 [8] 67.6 ± 1 [8]	65.1 ± 1.3 [8] 69.3 ± 1.3 [8]	64.9 ± 1.4 [8] 66.1 ± 1.6 [8]
Triglycerides (mmol/l)	M 0.99 ± 0.1 [10] F 0.77 ± 0.11 [10]	0.39 ± 0.1 [9]*** 0.29 ± 0.1 [8]**	0.48 ± 0.1 [10]*** 0.26 ± 0.0 [10]***	0.55 ± 0.1 [10]*** 0.24 ± 0.0 [9]**	0.86 ± 0.1 [10] 0.58 ± 0.1 [10]	0.75 ± 0.1 [10]** 0.41 ± 0.1 [10]*	0.6 ± 0.11 [10]** 0.4 ± 0.08 [10]*	0.92 ± 0.1 [8] 1.37 ± 0.14 [7]	1.09 ± 0.2 [8] 0.86 ± 0.1 [8]	1.05 ± 0.16 [8] 0.9 ± 0.12 [8]	1.11 ± 0.2 [8] 0.92 ± 0.1 [8]

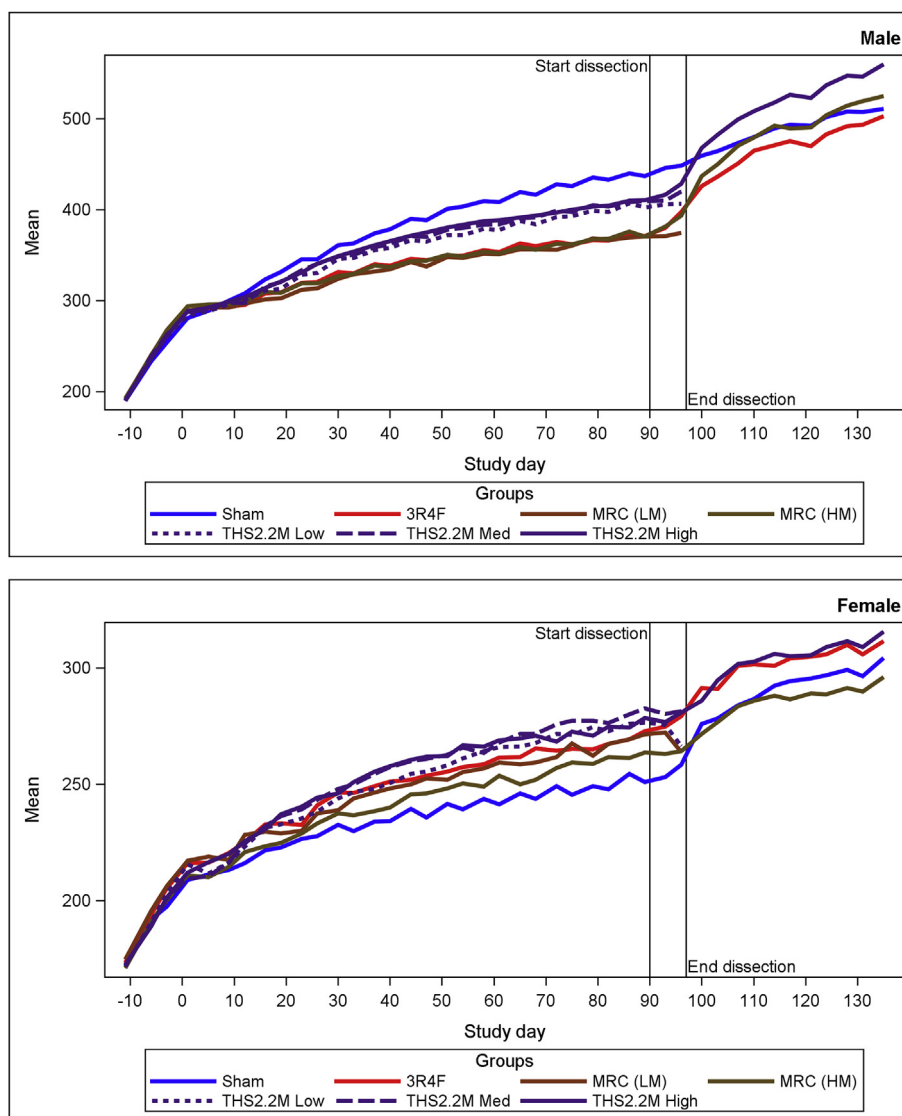
Clinical chemistry parameters measured after 90-day exposure (90 days) and 42-day recovery following a 90-day exposure period (90 + 42d). Results represent mean ± standard error. The sample size is in parentheses. Difference from sham group at 90 days: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 3b**

Statistically significant differences for bio-monitoring parameters at the end of the 90-day and post-inhalation periods.

Parameter	THS2.2M Medium vs.MRC(LM)	THS2.2M High vs. MRC(HM).	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
Alanine amino- - transferase activity (IU/L)	M = F =	↑* =	= =	= =	↓* ↓*	↓** ↓*	↓*** ↓***
Alkaline phosphatase activity (IU/L)	M ↓* F ↓*	↑** =	= =	↓* ↓*	↓* ↓***	↓** ↓***	↓*** ↓***
Aspartate amino-transferase activity (IU/L)	M = F =	= =	= =	= ↓**	= ↓**	↓*** =	↓* ↓***
Calcium (mmol/L)	M = F =	= =	= =	= ↑*	= ↑***	↑*** ↑**	↑*** ↑**
Creatinine kinase (mol/L)	M = F =	= =	= =	= =	↑* ↑**	↑*** ↑*	↑** ↑***
Glucose (mmol/L)	M = F =	= =	= =	= =	↑** ↑*	↑*** =	= ↑***
Total cholesterol (mmol/L)	M = F =	= =	= =	= =	↑*** ↑***	↑** ↑**	↑* ↑*
Total protein (g/l)	M = F =	= =	= =	= =	= ↑***	↑** ↑***	↑* ↑**
Triglycerides (mmol/L)	M ↑** F ↑*	= =	= =	= ↑**	↑** ↑***	↑* ↑***	= ↑**

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.



**Fig. 1.** Body weight development. Mean body weights of male (A) and female (B) rats exposed to fresh air (sham) and aerosols from THS2.2M, 3R4F, and MRC. Animals were weighed twice per week. Day 1 represents the exposure start date. N=6–20. Abbreviations: LM, low menthol; HM, high menthol.

cell count in all groups compared with sham, including statistically significantly higher numbers of neutrophils, eosinophils, macrophages, and lymphocytes, all indicative of the presence of lung inflammation (Tables 6a and 6b). In contrast, there was no inflammation in the lungs of THS2.2M-exposed rats, as evidenced by the absence of statistically significant differences compared with sham, even in the THS2.2M High group where the nicotine exposure concentration was twice as high as in the MRC-exposed groups.

At the end of the 42-day post-inhalation recovery period, lymphocyte counts in MRC groups remained unchanged, and the effects of the MRC MS-exposure on neutrophil counts and eosinophil counts were reduced but still present for both male and female groups.

Similar results were observed in the 3R4F group at the end of the exposure and recovery periods, and are consistent with findings from past studies (Friedrichs et al., 2006; Smith et al., 2002; Werley et al., 2008). Exposure to MRC(HM) caused a statistically significant increase in total cell count and higher numbers of

neutrophils and macrophages in male rats compared with 3R4F after the 90 days of exposure.

### 3.5. Cytokines and chemokines in BALF

Multiple analyte profiling (RodentMAP™) was performed on BALF to quantify 60 analytes; most analytes were below the lower limit of quantification or did not show exposure-related effects. The fold-changes in concentrations of nine analytes in the BALF were statistically significantly elevated in MRC-exposed groups, pointing to the presence of lung inflammation (Fig. 2). Both male and female rats exposed to MRCs had higher levels of the following cytokines and chemokines, which were present at the same magnitude in the BALF of the 3R4F group: Resistin, vascular cell-adhesion molecule-1 (VCAM1), MCP-1 (chemokine (C-C motif) ligand 2 (CCL2)), MCP3 (CCL7), MDC (CCL22), MIP-1beta (CCL4), macrophage colony-stimulating factor (M-CSF-1 (M-CSF or CSF-1)), metalloproteinase 9 (MMP9), and fibrinogen. All nine cytokines were markedly lower or not present in the THS2.2M groups.

**Table 4a**  
Hematological parameters at the end of the 90-day inhalation and post-inhalation periods.

Parameter		90 days							90 + 42d			
		Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Clotting potential	Fibrinogen [s]	M 18.4 ± 2.7 [7]	11.6 ± 1 [9]*	12 ± 0.6 [9]	12.5 ± 0.9 [10]	14.9 ± 0.7 [7]	14.7 ± 2 [10]	12.5 ± 1 [7]	17 ± 0.4 [7]	14.7 ± 1.5 [8]	18.3 ± 1 [8]	18.9 ± 3.7 [8]
		F 22.2 ± 2.1 [8]	19.7 ± 6.8 [7]	14.2 ± 1.7 [9]**	11.4 ± 1.5 [7]**	16.2 ± 1 [10]*	14.8 ± 0.8 [10]**	15.3 ± 2.5 [8]	25.3 ± 1.5 [8]	23.4 ± 1.2 [8]	23.3 ± 2.1 [7]	26.5 ± 1.4 [8]
	Fibrinogen [g/l]	M 3.5 ± 0.9 [7]	4.8 ± 0.5 [9]*	4.4 ± 0.2 [9]	4.4 ± 0.3 [10]*	3.5 ± 0.2 [7]	4 ± 0.4 [10]	4.4 ± 0.4 [7]	3.1 ± 0.1 [7]	3.9 ± 0.6 [8]	2.9 ± 0.1 [8]	2.8 ± 0.4 [8]
		F 2.3 ± 0.3 [8]	3.7 ± 0.7 [7]	4.3 ± 0.4 [9]**	4.9 ± 0.6 [7]**	3.3 ± 0.2 [10]*	3.6 ± 0.2 [10]**	4 ± 0.6 [8]*	1.9 ± 0.2 [8]	2.1 ± 0.2 [8]	1.8 ± 0.2 [7]	2 ± 0.1 [8]
Red blood cells	Prothrombin time [s]	M 15.8 ± 0.4 [7]	17.3 ± 0.4 [9]**	17.2 ± 0.4 [9]**	16.8 ± 0.4 [10]*	16.3 ± 0.5 [7]*	16.4 ± 0.4 [10]	16.2 ± 0.4 [7]	15.1 ± 0.4 [7]	15.1 ± 0.3 [8]	15.4 ± 0.2 [8]	16.4 ± 0.6 [8]
		F 15.3 ± 0.3 [8]	16.9 ± 0.9 [7]	16.7 ± 0.3 [9]**	16.9 ± 0.6 [7]*	15.8 ± 0.3 [10]	16.2 ± 0.2 [10]	15.9 ± 0.4 [8]	15.1 ± 0.3 [8]	15.2 ± 0.3 [8]	15.1 ± 0.2 [7]	14.7 ± 0.2 [8]
	Erythrocyte [10 <sup>6</sup> /μl]	M 8.6 ± 0.3 [10]	8.5 ± 0.2 [9]	8.6 ± 0.2 [10]	9 ± 0.1 [10]	8.7 ± 0.2 [10]	8.4 ± 0.2 [10]	8.3 ± 0.2 [10]	8.4 ± 0.1 [8]	8 ± 0.2 [8]	8.4 ± 0.1 [8]	8.3 ± 0.1 [8]
		F 8 ± 0.1 [10]	8.1 ± 0.1 [8]	7.9 ± 0.2 [10]	7.5 ± 0.2 [9]*	8 ± 0.2 [10]	7.5 ± 0.1 [10]**	7.3 ± 0.2 [10]*	7.7 ± 0.1 [7]	7.4 ± 0.2 [8]	7.3 ± 0.2 [8]	7.6 ± 0.1 [8]
White blood cells	Hematocrit	M 0.4 ± 0 [10]	0.4 ± 0 [9]	0.4 ± 0 [10]	0.5 ± 0 [10]*	0.4 ± 0 [10]	0.4 ± 0 [10]	0.4 ± 0 [10]	0.4 ± 0 [8]	0.4 ± 0 [8]	0.4 ± 0 [8]	0.4 ± 0 [8]
		F 0.4 ± 0 [10]	0.4 ± 0 [8]**	0.4 ± 0 [10]	0.4 ± 0 [9]	0.4 ± 0 [10]	0.4 ± 0 [10]*	0.4 ± 0 [10]*	0.4 ± 0 [8]	0.4 ± 0 [8]	0.4 ± 0 [8]	0.4 ± 0 [8]
	Mean corpuscular hemoglobin [pg]	M 17.5 ± 0.2 [10]	18.5 ± 0.2 [9]**	18.4 ± 0.1 [10]**	18.4 ± 0.1 [10]**	17.8 ± 0.1 [10]	17.7 ± 0.2 [10]	17.6 ± 0.1 [10]	17.5 ± 0.2 [8]	17.7 ± 0.2 [8]	17.8 ± 0.4 [8]	17.8 ± 0.2 [8]
		F 18.4 ± 0.1 [10]	19.3 ± 0.2 [8]**	18.8 ± 0.2 [10]	19 ± 0.2 [9]	18.5 ± 0.2 [10]	18.9 ± 0.2 [10]	18.6 ± 0.2 [10]	18.6 ± 0.1 [7]	18.9 ± 0.1 [8]	18.6 ± 0.2 [8]	18.5 ± 0.1 [8]
White blood cells	Reticulocyte [%]	M 3.2 ± 0.3 [10]	3.3 ± 0.3 [9]	3.9 ± 0.4 [10]	3.2 ± 0.2 [10]	3.3 ± 0.2 [10]	3.6 ± 0.3 [10]	3.8 ± 0.2 [10]	4 ± 0.4 [8]	5.8 ± 1.3 [8]	4.1 ± 0.2 [8]	3.8 ± 0.4 [8]
		F 2.9 ± 0.3 [10]	3.7 ± 0.4 [8]	5 ± 0.5 [10]**	5.3 ± 0.5 [9]**	3.3 ± 0.4 [10]	3.8 ± 0.3 [10]	4.5 ± 0.8 [10]	3.6 ± 0.2 [7]	4 ± 0.2 [8]	4.1 ± 0.5 [8]	3.7 ± 0.4 [8]
	Lymphocyte [10 <sup>3</sup> /μl]	M 5.5 ± 0.6 [10]	4.5 ± 0.5 [9]	4.2 ± 0.2 [10]	4.3 ± 0.3 [10]	5.6 ± 0.5 [10]	5.8 ± 0.6 [10]	4.3 ± 0.5 [10]	7.2 ± 0.6 [8]	6.8 ± 0.3 [8]	6.5 ± 1 [8]	7.6 ± 0.7 [8]
		F 5.3 ± 0.7 [10]	3.7 ± 0.5 [8]	3.5 ± 0.3 [10]*	3.1 ± 0.3 [9]*	4.1 ± 0.3 [10]	4.2 ± 0.5 [10]	3.5 ± 0.3 [10]*	5.3 ± 0.6 [7]	5.1 ± 0.3 [8]	3.8 ± 0.3 [8]	5.8 ± 0.7 [8]
White blood cells	Monocyte [10 <sup>3</sup> /μl]	M 0.4 ± 0 [10]	0.5 ± 0 [9]*	0.6 ± 0 [10]**	0.5 ± 0 [10]	0.4 ± 0 [10]	0.6 ± 0.1 [10]	0.5 ± 0.1 [10]	0.6 ± 0 [8]	0.5 ± 0.1 [8]	0.5 ± 0.1 [8]	0.5 ± 0.1 [8]
		F 0.3 ± 0 [10]	0.5 ± 0.1 [8]*	0.4 ± 0 [10]*	0.5 ± 0.1 [9]	0.3 ± 0.1 [10]	0.4 ± 0 [10]	0.5 ± 0.1 [10]	0.4 ± 0 [7]	0.4 ± 0 [8]	0.3 ± 0.1 [8]	0.4 ± 0 [8]
	Neutrophil [10 <sup>3</sup> /μl]	M 1.4 ± 0.2 [10]	2.8 ± 0.4 [9]*	3 ± 0.5 [10]*	2.3 ± 0.3 [10]*	1.4 ± 0.2 [10]	2.2 ± 0.3 [10]*	2.9 ± 0.3 [10]**	1.6 ± 0.1 [8]	2.3 ± 0.4 [8]	1.8 ± 0.2 [8]	1.5 ± 0.2 [8]
		F 1 ± 0.2 [10]	2.5 ± 0.3 [8]**	2.6 ± 0.5 [10]**	3.6 ± 1 [9]**	1.8 ± 0.4 [10]*	2.4 ± 0.3 [10]**	3.2 ± 0.6 [10]**	1 ± 0.1 [7]	1.1 ± 0.1 [8]	1 ± 0.1 [8]	0.8 ± 0.1 [8]

Results are presented as mean ± standard error. The sample size is in parenthesis. Difference from sham group at 90 d: significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 4b**  
Statistically significant differences for hematological parameters at the end of the 90-day and post-inhalation periods.

Parameter		THS2.2M Medium vs.MRC(LM)	THS2.2M High vs. MRC(HM)	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
Clotting potential	Fibrinogen [s]	M =	=	=	=	=	↑***	=
		F =	=	=	=	=	↑***	↑**
	Fibrinogen [g/l]	M =	=	=	=	=	↓**	↓*
		F =	=	=	=	↓*	↓**	↓*
Red blood cells	Prothrombin time [s]	M =	=	=	=	=	↓*	↓*
		F =	=	=	=	↓**	=	↓**
	Erythrocytes [10 <sup>6</sup> /μl]	M =	↓**	↓**	=	=	=	↓**
		F =	=	↓**	↓*	↓**	=	=
Red blood cells	Hematocrit l/l	M =	↓***	↑*	=	↓**	↓**	=
		F =	=	↑***	=	↓**	↓*	=
	Mean corpuscular hemoglobin [pg]	M ↓**	↓***	=	=	↓*	=	=
		F =	=	=	=	=	=	=
White blood cells	Reticulocytes [%]	M =	=	=	=	=	↑**	=
		F =	=	↑*	↑*	↑**	=	=
	Lymphocytes [10 <sup>3</sup> /μl]	M ↑*	=	=	=	↑**	↑*	↑**
		F =	=	=	=	↑*	=	↑*
White blood cells	Monocytes [10 <sup>3</sup> /μl]	M =	=	=	↑**	=	=	=
		F =	=	=	=	=	=	=
	Neutrophils [10 <sup>3</sup> /μl]	M =	=	=	=	=	=	↓**
		F =	=	=	=	↓**	↓*	↓**

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 5a**

Organ weights relative to body weight determined at the end of the 90-day and post-inhalation periods.

Parameter [mg]		90 days						90 + 42d				
		Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Adrenal gland (right)	M	0.6 ± 0.1 [10]	0.9 ± 0.1 [9]*	0.9 ± 0.1 [10]**	0.8 ± 0.1 [10]	0.8 ± 0 [10]	0.8 ± 0 [10]*	0.8 ± 0.1 [10]	0.4 ± 0 [8]	0.5 ± 0 [8]	0.5 ± 0.1 [8]	0.4 ± 0 [8]
	F	1.3 ± 0.1 [10]	1.6 ± 0.1 [8]**	1.7 ± 0.1 [10]***	1.7 ± 0.1 [9]***	1.6 ± 0.1 [10]**	1.6 ± 0.1 [10]**	1.6 ± 0.1 [10]***	0.9 ± 0.1 [7]	1 ± 0 [8]	1.1 ± 0.1 [8]	1 ± 0 [8]
Adrenal gland (left)	M	0.7 ± 0 [10]	1 ± 0.1 [9]*	1 ± 0.1 [10]**	0.9 ± 0 [10]*	0.8 ± 0 [10]*	0.8 ± 0 [10]**	0.9 ± 0 [10]***	0.4 ± 0 [8]	0.4 ± 0 [8]	0.5 ± 0 [8]	0.4 ± 0 [8]
	F	1.3 ± 0.1 [10]	1.8 ± 0.1 [8]***	1.8 ± 0.1 [10]**	1.8 ± 0.1 [9]***	1.7 ± 0.1 [10]**	1.8 ± 0.2 [10]**	1.7 ± 0.1 [10]***	1 ± 0 [7]	1 ± 0 [8]	1.2 ± 0 [8]	1 ± 0.1 [8]
Brain	M	51.4 ± 1.6 [10]	58.6 ± 1.1 [9]**	58.8 ± 1.6 [10]**	58 ± 1.9 [10]**	55.9 ± 1.3 [10]*	54.3 ± 1.5 [10]	55.2 ± 2.5 [10]	44.6 ± 1.3 [8]	45.9 ± 1.2 [8]	43.9 ± 1.6 [8]	41.3 ± 1.7 [8]
	F	84.4 ± 1.4 [10]	76 ± 0.9 [8]***	76.4 ± 2.2 [10]**	78.4 ± 1.4 [9]*	76.2 ± 1.7 [10]**	73.4 ± 1.9 [10]***	72.8 ± 1.7 [10]***	68.8 ± 3.1 [7]	67.3 ± 1.3 [8]	70.1 ± 1.9 [8]	67.4 ± 1.9 [8]
Heart	M	31.1 ± 0.8 [10]	38.1 ± 1.2 [9]***	38.7 ± 1 [10]***	39.1 ± 1 [10]***	33.4 ± 1.2 [10]	32.2 ± 0.5 [10]	35.4 ± 0.8 [10]***	29.1 ± 0.8 [8]	29.9 ± 0.6 [8]	29.5 ± 0.5 [8]	28.9 ± 1.2 [8]
	F	36.5 ± 1.3 [10]	43.1 ± 1 [8]***	42.8 ± 1.5 [10]**	43.1 ± 1.8 [9]**	36.2 ± 0.9 [10]	40.4 ± 1.7 [10]*	38.3 ± 0.9 [10]	32.1 ± 0.9 [7]	33 ± 0.9 [8]	33.4 ± 0.6 [8]	34.1 ± 1.5 [8]
Kidney (right)	M	28.9 ± 0.9 [10]	33.7 ± 0.5 [9]***	32.8 ± 0.8 [10]**	33.4 ± 0.7 [10]**	32 ± 0.9 [10]*	30.7 ± 0.9 [10]	33 ± 1.1 [10]**	28.2 ± 0.6 [8]	29 ± 1.2 [8]	30.1 ± 1.1 [8]	27.6 ± 0.9 [8]
	F	33.2 ± 0.8 [10]	33.6 ± 0.6 [8]	33.9 ± 0.8 [10]	32.9 ± 0.9 [9]	33.2 ± 0.7 [10]	33.6 ± 0.6 [10]	33.6 ± 1.3 [10]	29.5 ± 1.2 [7]	30.4 ± 0.6 [8]	30.6 ± 1 [8]	30.3 ± 0.8 [8]
Kidney (left)	M	28.8 ± 0.9 [10]	32.8 ± 0.6 [9]**	32 ± 0.8 [10]*	32.3 ± 0.8 [10]**	31.3 ± 0.7 [10]*	30.4 ± 0.8 [10]	32.4 ± 1 [10]*	27.6 ± 0.7 [8]	28.4 ± 1.1 [8]	30.3 ± 0.9 [8]	28.1 ± 1.3 [8]
	F	31.7 ± 0.7 [10]	32.5 ± 0.5 [8]	32.8 ± 0.8 [10]	32.5 ± 0.8 [9]	32.8 ± 0.7 [10]	32.6 ± 0.6 [10]	32.3 ± 1.2 [10]	28.4 ± 1 [7]	28.6 ± 0.7 [8]	29.8 ± 0.5 [8]	29.8 ± 0.9 [8]
Liver	M	330 ± 10.8 [10]	379.7 ± 13.6 [9]*	373.2 ± 13.5 [10]*	379.8 ± 10.9 [10]**	351.3 ± 7.5 [10]	362.3 ± 5.6 [10]*	412 ± 12.6 [10]***	330.3 ± 4.3 [8]	331 ± 4.8 [8]	339.3 ± 8 [8]	342.9 ± 11.7 [8]
	F	357.4 ± 9.1 [10]	416.4 ± 7.6 [8]***	409.5 ± 7 [10]***	415.6 ± 13.5 [9]**	406.5 ± 11.8 [10]**	415.3 ± 7.3 [10]***	425.4 ± 10.9 [10]***	332.6 ± 12.8 [7]	344.7 ± 5.3 [8]	344.4 ± 9.8 [8]	328.1 ± 7.8 [8]
Lung, Larynx, Trachea	M	36.8 ± 0.8 [10]	56.9 ± 1.8 [9]***	57.2 ± 1.8 [10]***	54.5 ± 1.5 [10]***	39.6 ± 1 [10]*	39.5 ± 0.9 [10]*	42.1 ± 1.4 [10]**	33.8 ± 0.7 [8]	39.6 ± 1.2 [8]	38.4 ± 0.9 [8]	33.7 ± 1.1 [8]
	F	52.4 ± 0.9 [10]	64.3 ± 1.5 [8]***	67.3 ± 2.5 [10]***	68.8 ± 3.1 [9]***	49.4 ± 1.3 [10]	48.9 ± 1.1 [10]*	49.8 ± 0.6 [10]*	45.4 ± 2.3 [8]	54.5 ± 2.4 [8]	54.1 ± 2.5 [8]	46.4 ± 0.7 [8]
Ovary (right)	F	1.4 ± 0.1 [10]	2 ± 0.3 [8]*	1.7 ± 0.2 [10]	1.7 ± 0.1 [9]	2 ± 0.1 [10]**	2.1 ± 0.1 [10]**	1.5 ± 0.1 [10]	1.5 ± 0.1 [7]	1.6 ± 0.1 [8]	1.6 ± 0.2 [8]	1.6 ± 0.1 [8]
Ovary (left)	F	1.4 ± 0.1 [10]	2.1 ± 0.4 [8]	1.7 ± 0.1 [10]*	1.5 ± 0.2 [9]	1.8 ± 0.1 [10]**	1.8 ± 0.1 [10]*	1.5 ± 0.1 [10]	1.3 ± 0.1 [7]	1.5 ± 0.1 [8]	1.5 ± 0.2 [8]	1.4 ± 0.1 [8]
Spleen	M	17.5 ± 0.5 [10]	16.2 ± 0.7 [9]	17 ± 0.7 [10]	15.8 ± 0.7 [10]	16.9 ± 0.5 [10]	17.1 ± 0.5 [10]	14.3 ± 0.8 [10]**	17.9 ± 1.1 [8]	18.9 ± 1.1 [8]	17.4 ± 0.7 [8]	16.7 ± 1.1 [8]
	F	20.8 ± 0.9 [10]	16 ± 0.4 [8]***	19.3 ± 0.9 [10]	19.1 ± 1.1 [9]	19.4 ± 0.7 [10]	18.5 ± 0.4 [10]*	18.8 ± 1.2 [10]	21.1 ± 1.1 [7]	21.2 ± 1.1 [8]	21.2 ± 0.5 [8]	20.6 ± 0.6 [8]
Testes (right)	M	35.2 ± 2.9 [10]	48.7 ± 1.4 [9]**	48 ± 1.2 [10]***	49.9 ± 2.3 [10]**	36.2 ± 3 [10]	36.5 ± 2.5 [10]	46.6 ± 1.6 [10]**	33.2 ± 1.8 [8]	37.7 ± 1.1 [8]	39.2 ± 0.9 [8]	35.9 ± 1.1 [8]
Testes (left)	M	34.4 ± 3.3 [10]	48.6 ± 1.5 [9]**	44.2 ± 2.7 [10]*	45.6 ± 2.8 [10]*	35.5 ± 2.5 [10]	35.7 ± 1.7 [10]	46.3 ± 1.4 [10]**	34.1 ± 1.5 [8]	37.2 ± 1 [8]	39.1 ± 1 [8]	34.8 ± 1.2 [8]
Thymus	M	4.9 ± 0.3 [10]	2.6 ± 0.2 [9]***	3 ± 0.4 [10]***	2.7 ± 0.2 [10]***	3.5 ± 0.4 [10]**	3.3 ± 0.3 [10]***	2.7 ± 0.2 [10]***	3.8 ± 0.3 [8]	4.5 ± 0.3 [8]	4.5 ± 0.2 [8]	4.1 ± 0.4 [8]
	F	7.2 ± 0.4 [10]	3.7 ± 0.4 [8]***	3.6 ± 0.3 [10]***	3.9 ± 0.3 [9]***	5.4 ± 0.5 [10]**	3.9 ± 0.5 [10]***	4.2 ± 0.5 [10]***	5.9 ± 0.7 [7]	8.3 ± 0.7 [8]	7.4 ± 0.8 [8]	8.1 ± 0.9 [8]
Uterus	F	29.1 ± 4.2 [10]	18.4 ± 2.2 [8]*	17.4 ± 1.9 [10]***	12.8 ± 1 [9]*	16.7 ± 1.4 [10]**	22.5 ± 5 [10]*	12.8 ± 0.8 [10]***	24 ± 3 [7]	23.2 ± 2.2 [8]	21.5 ± 2.3 [8]	24 ± 4.3 [8]

Results are presented as mean ± standard error. The sample size is in parentheses. Difference from sham group at 90d: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 5b**

Statistically significant differences for organ weights relative to body weight at the end of the 90-day and post-inhalation periods.

Parameter		THS2.2M Medium vs. MRC(LM)	THS2.2M High vs. MRC(HM).	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
Adrenal gland (right)	M	=	=	=	↓**	↓***	↓***	↓***
	F	=	=	=	↓**	↓***	↓***	↓***
Adrenal gland (left)	M	=	=	=	↓***	↓**	↓***	↓***
	F	=	=	=	↓***	↓***	↓***	↓***
Brain	M	=	=	=	↓**	↓***	↓***	↓***
	F	=	↓*	=	↓**	↓***	↓**	=
Heart	M	↓***	↓**	=	=	↓***	↓***	↓***
	F	=	↓*	=	↓*	↓***	↓***	↓*
Kidney (right)	M	=	=	=	=	↓**	↓*	↓**
	F	=	=	=	↓*	↓**	=	↓*
Kidney (left)	M	=	=	=	=	↓**	=	↓*
	F	=	=	=	↓*	↓***	↓*	=
Liver	M	=	=	=	=	↓**	↓**	↓***
	F	=	=	=	=	↓***	↓***	↓***
Lung	M	↓***	↓***	=	↓**	↓***	↓***	↓***
	F	↓***	↓***	=	↓*	↓**	↓**	↓**
Ovary (right)	F	=	=	=	=	=	=	=
Ovary (left)	F	=	=	=	=	=	=	=
Spleen	M	=	=	=	=	=	=	=
	F	=	=	↑*	=	↑**	=	=
Testes (right)	M	↓**	=	=	=	↓***	↓**	↓***
Testes (left)	M	↓*	=	=	=	↓***	=	↓***
Thymus	M	=	=	=	↓*	↑***	↑***	↑**
	F	=	=	=	↑***	↑***	↑**	↑**
Uterus	F	=	=	↓*	=	=	↓**	↓**

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

At the end of the 42-day post-inhalation recovery period, the effects of the MRC and 3R4F exposure on most upregulated analytes at the end of the exposure period were reduced, but still higher than in the sham group (Fig. 2), showing consistency with the BALF cell count results.

### 3.6. Gross pathology

Type, incidence, and severity of the macroscopical observations were not regarded as treatment-related. No significant exposure-related effects were observed (data not shown).

### 3.7. Histopathology of non-respiratory tract organs

The histopathological examination of THS2.2M MA- and MRC MS-exposed animals confirmed exposure-related effects for single endpoints in non-respiratory tract organs, and are regarded as normal within this type of study. These changes included vacuolization of hepatocytes (MRC(HM) and THS2.2M High female groups only) and thymus atrophy, as seen in previous studies (Phillips et al., 2015a; Wong et al., 2016 (in this issue)).

### 3.8. Histopathology of respiratory tract organs

To investigate the morphological effects on respiratory tract organs after exposure to MRC MS and THS2.2M MA, a semi-quantitative histopathological assessment was performed for the nose, larynx, trachea/bifurcation, and left lung.

Histopathological scores and incidences are given in Tables 7a and 7b only for those endpoints that were statistically significantly different from sham-exposed rats.

#### 3.8.1. Nose

At the end of the 90-day inhalation period, histological changes observed in the nasal epithelia for both male and female rats of the MRC groups included goblet-cell hyperplasia or squamous

metaplasia accompanied by the loss of goblet cells along the nasal septum and dorsal meatus, basal cell hyperplasia and/or squamous metaplasia of the respiratory epithelium along the lateral walls, olfactory epithelial cell atrophy and loss of nerve bundles, squamous cell metaplasia of the olfactory epithelium, and degeneration of the glomerular layers in the olfactory bulb. The findings observed in MRC(LM) and MRC(HM) groups were of similar severity and incidence, and had high severity scores in nose level 1 and moderate to high severity scores in nose levels 3 and 4. The incidence and/or mean severity was higher in females compared with males across all groups; however, the overall trend of the findings remained the same. No differences were observed in the nasal passages of MRC-exposed groups in any of the histopathological endpoints compared with the 3R4F group. Furthermore, the presence of menthol in MS did not introduce any novel effect, nor did it alter the severity and/or incidence of histopathological findings observed from 3R4F MS exposure alone.

Changes observed in the THS2.2M High group were moderate at nose level 1 and lower than in MRC-exposed rats, and moderate or absent in nose levels 3 and 4.

With the exception of the changes seen at the nasal septum, all findings diagnosed in the THS2.2 Low and THS2.2 Medium groups were considered incidental. In the THS2.2M exposure groups, there was a concentration-dependent increase in severity and/or incidence of basal cell hyperplasia, and goblet cell loss at the nasal septum.

Following the 42-day post-inhalation recovery period, the number of goblet cells in the anterior nasal septum was statistically significantly higher in THS2.2M High group and MRC MS-exposed groups compared with sham. Proliferation and hypertrophy of goblet cells in the nasal septum were observed in parallel with the loss of goblet cells.

#### 3.8.2. Larynx

In the larynx, severity and incidence of hyperplasia of the squamous epithelium at the base of the epiglottis, lower and upper

**Table 6a**

Statistically significant differences in differential cell counts in BALF at the end of the 90-day and post-inhalation periods.

Parameter	90 days							90 + 42d			
	Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Total cells ( $10^5$ )	M $40 \pm 3.5$ [10]	$227.8 \pm 17.9$ [9]*	$210.7 \pm 30.4$ [10]**	$164.7 \pm 13.1$ [10]	$43.2 \pm 5.3$ [10]	$36.7 \pm 3.7$ [10]*	$48 \pm 5.5$ [10]	$40 \pm 4.6$ [8]	$68.3 \pm 14.7$ [8]	$59.7 \pm 9.4$ [8]	$37.6 \pm 4.9$ [8]
	F $25.2 \pm 3.7$ [10]	$138.1 \pm 12.4$ [8]**	$193.3 \pm 42.1$ [10]***	$147.7 \pm 19.5$ [9]***	$37.3 \pm 4.5$ [10]**	$31.9 \pm 4.2$ [10]**	$32.9 \pm 3.4$ [10]***	$19 \pm 4.7$ [7]	$50.1 \pm 7.4$ [8]	$69.6 \pm 8.1$ [8]	$19.4 \pm 3$ [8]
Eosinophils ( $10^5$ )	M $0 \pm 0$ [10]	$0.3 \pm 0$ [9]*	$0.4 \pm 0.1$ [10]**	$0.3 \pm 0.1$ [10]*	$0.1 \pm 0.1$ [10]*	$0 \pm 0$ [10]**	$0 \pm 0$ [10]***	$0 \pm 0$ [8]	$0.1 \pm 0$ [8]	$0.1 \pm 0$ [8]	$0 \pm 0$ [8]
	F $0 \pm 0$ [10]	$0.3 \pm 0.1$ [8]***	$0.6 \pm 0.2$ [10]**	$0.4 \pm 0.1$ [9]***	$0 \pm 0$ [10]**	$0 \pm 0$ [10]**	$0 \pm 0$ [10]***	$0 \pm 0$ [7]	$0.1 \pm 0$ [8]	$0.2 \pm 0.1$ [8]	$0 \pm 0$ [8]
Lymphocytes ( $10^5$ )	M $0.4 \pm 0.1$ [10]	$4.6 \pm 0.5$ [9]**	$6.6 \pm 1.9$ [10]**	$3.5 \pm 0.5$ [10]*	$0.3 \pm 0.1$ [10]*	$0.3 \pm 0.1$ [10]	$0.3 \pm 0.1$ [10]	$0.5 \pm 0.1$ [8]	$4.7 \pm 1.4$ [8]	$3.6 \pm 0.9$ [8]	$0.7 \pm 0.2$ [8]
	F $0.2 \pm 0$ [10]	$4.1 \pm 0.9$ [8]***	$4.6 \pm 1.3$ [10]**	$3.9 \pm 0.9$ [9]*	$0.1 \pm 0$ [10]**	$0.1 \pm 0$ [10]***	$0.2 \pm 0.1$ [10]***	$0.3 \pm 0.1$ [7]	$4.1 \pm 1.2$ [8]	$4 \pm 0.7$ [8]	$0.2 \pm 0$ [8]
Macrophages ( $10^5$ )	M $39.2 \pm 3.4$ [10]	$135.9 \pm 10.7$ [9]***	$110.9 \pm 15.1$ [10]***	$100.1 \pm 10.2$ [10]***	$39.8 \pm 3$ [10]	$36 \pm 3.7$ [10]	$47.1 \pm 5.5$ [10]***	$39.2 \pm 4.6$ [8]	$50.1 \pm 12.4$ [8]	$42.6 \pm 6.8$ [8]	$36.5 \pm 4.8$ [8]
	F $24.8 \pm 3.7$ [10]	$64.7 \pm 6.4$ [8]***	$104.4 \pm 25.7$ [10]**	$77.7 \pm 10.8$ [9]**	$36.8 \pm 4.4$ [10]	$31.5 \pm 4.2$ [10]*	$32.3 \pm 3.5$ [10]	$18.5 \pm 4.6$ [7]	$35.5 \pm 5.8$ [8]	$48.9 \pm 5.4$ [8]	$19.2 \pm 3$ [8]
Neutrophils ( $10^5$ )	M $0.3 \pm 0.1$ [10]	$87 \pm 9.8$ [9]***	$92.8 \pm 18.7$ [10]**	$60.7 \pm 7.2$ [10]**	$3 \pm 2.4$ [10]*	$0.3 \pm 0.1$ [10]	$0.6 \pm 0.1$ [10]**	$0.2 \pm 0.1$ [8]	$13.5 \pm 3.7$ [8]	$13.4 \pm 2$ [8]	$0.4 \pm 0.1$ [8]
	F $0.1 \pm 0$ [10]	$69 \pm 5.7$ [8]	$83.7 \pm 16.5$ [10]	$65.7 \pm 10$ [9]	$0.3 \pm 0.1$ [10]	$0.3 \pm 0.1$ [10]	$0.5 \pm 0.1$ [10]	$0.1 \pm 0$ [7]	$10.3 \pm 3$ [8]	$16.6 \pm 2.6$ [8]	$0.1 \pm 0$ [8]

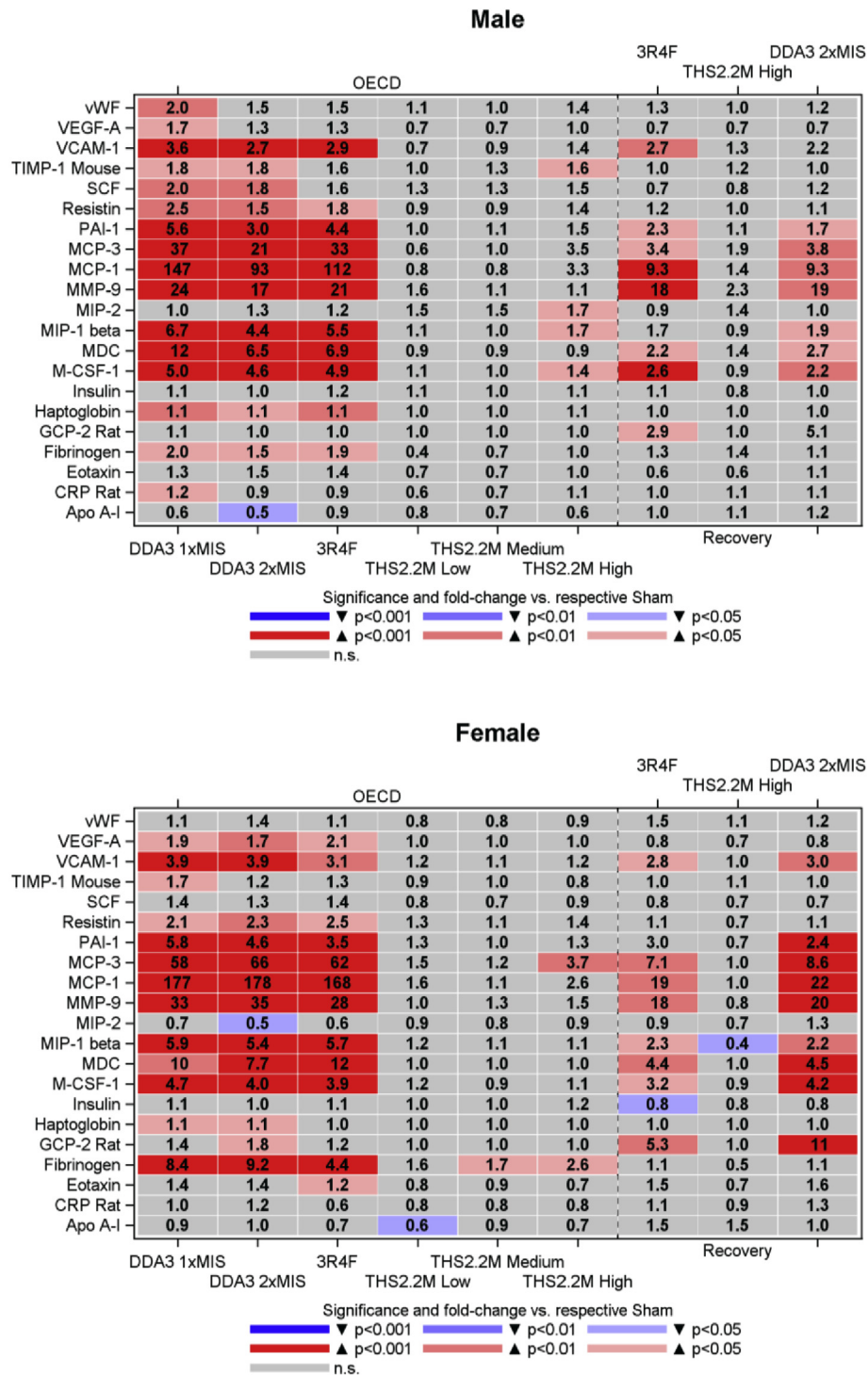
Results are presented as mean  $\pm$  standard error. The sample size is in parentheses. Difference from sham group at 90d: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 6b**

Statistically significant differences in differential cell counts in BALF at the end of the 90-day and post-inhalation periods.

Parameter	THS2.2M Medium vs. MRC(LM)	THS2.2M High vs. MRC(HM)	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
Total cells ( $10^5$ )	M ↓***	↓***	↓*	=	↓***	↓**	↓***
	F ↓**	↓***	=	=	↓***	↓**	↓**
Eosinophils ( $10^5$ )	M ↓***	↓***	=	=	↓***	↓*	=
	F ↓***	↓***	=	=	↓**	=	=
Lymphocytes ( $10^5$ )	M ↓***	↓***	=	=	=	=	=
	F ↓***	↓***	=	=	=	=	=
Macrophages ( $10^5$ )	M ↓***	↓***	↓*	=	↓***	↓***	=
	F ↓*	↓**	=	=	↓**	↓*	↓*
Neutrophils ( $10^5$ )	M ↓***	↓***	↓*	=	↓***	↓***	=
	F ↓***	↓***	=	=	↓***	↓**	↓***

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.



**Fig. 2.** Analysis of selected cytokines and chemokines in bronchoalveolar lavage fluid (BALF). Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol.

arytenoid projections, ventral pouch, and vocal folds in the mid-lateral region were statistically significantly higher in all exposed groups compared with sham. However, severity and incidence were statistically significantly lower in THS2.2M MA-exposed groups compared with MRC MS-exposed groups, which showed a fully developed hyperkeratotic squamous metaplasia.

Larynx morphometry revealed that mean laryngeal epithelial thickness at the base of the epiglottis, lateral floor, and the arytenoid projections was statistically significantly higher in MRC MS-

exposed groups (Tables 8a and 8b). There was also a concentration-dependent increase observed in the THS2.2M MA-exposed groups which was statistically significantly lower compared with MRC groups.

Following the 42-day post-inhalation recovery period, all effects observed in the larynx were completely reversed in THS2.2M MA-exposed groups, and partially reversed in MRC groups.

No differences were observable in the larynx of MRC-exposed groups compared with the 3R4F group. Furthermore, the

**Table 7a**

Statistically significant differences in histopathological findings in the respiratory tract at the end of the 90-day and post-inhalation periods.

Parameter		90d							90 + 42d				
		Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R	
Nose—Level 1													
Nasal Septum - Respiratory Epithelium													
Basal cell	M	0.1 ± 0.1 [1/10]	2.7 ± 0.2 [9/9]***	1.8 ± 0.4 [8/10]**	1.5 ± 0.3 [8/10]**	0.5 ± 0.2 [4/10]	0.7 ± 0.2 [7/10]**	2.1 ± 0.4 [5/10]***	0 ± 0 [0/8]	0.3 ± 0.2 [2/8]	0.3 ± 0.16 [2/8]	0.4 ± 0.18 [3/8]	
hyperplasia	F	0 ± 0 [0/10]	1 ± 0.3 [5/8]**	2.4 ± 0.5 [7/8]***	1.3 ± 0.5 [5/9]**	0.3 ± 0.2 [3/10]	0.6 ± 0.16 [6/10]***	1.7 ± 0.4 [8/10]***	0 ± 0 [0/8]	0.1 ± 0.13 [1/8]	0.3 ± 0.25 [1/8]	0.1 ± 0.13 [1/8]	
Goblet cell	M	0.9 ± 0.28 [6/10]	2.1 ± 0.35 [9/9]*	0.4 ± 0.16 [4/10]	1.1 ± 0.38 [6/10]	0.6 ± 0.31 [3/10]	0.9 ± 0.23 [7/10]	0.5 ± 0.22 [4/10]	0.3 ± 0.16 [2/8]	4.3 ± 0.25 [8/8]	4 ± 0.42 [8/8]	2.3 ± 0.49 [7/8]	
hyperplasia/hypertrophy	F	0 ± 0 [0/10]	0.5 ± 0.27 [6/8]*	1 ± 0.3 [6/10]**	0.7 ± 0.24 [5/9]**	0.6 ± 0.22 [5/10]*	1.3 ± 0.4 [6/10]***	0.1 ± 0.1 [1/10]	0.1 ± 0.13 [1/8]	4.1 ± 0.4 [8/8]	2.9 ± 0.44 [8/8]	2 ± 0.57 [8/8]	
Goblet cell loss	M	0.3 ± 0.15 [3/10]	3.9 ± 0.2 [9/9]***	4.8 ± 0.13 [10/10]***	4.3 ± 0.21 [10/10]***	0.8 ± 0.25 [6/10]	1.2 ± 0.29 [8/10]*	3.9 ± 0.18 [10/10]***	0.3 ± 0.16 [2/8]	1.8 ± 0.41 [8/8]	1.3 ± 0.25 [7/8]**	0.9 ± 0.4 [4/8]	
	F	0.7 ± 0.26 [5/10]	4.3 ± 0.25 [8/8]***	4.4 ± 0.22 [10/10]***	4.6 ± 0.18 [9/9]***	1.3 ± 0.3 [8/10]	1.2 ± 0.33 [7/10]	4.1 ± 0.23 [10/10]***	0.4 ± 0.18 [3/8]	1.9 ± 0.52 [7/8]*	2.1 ± 0.52 [6/8]*	1.3 ± 0.37 [6/8]	
Squamous cell	M	0 ± 0 [0/10]	2.6 ± 0.24 [9/9]***	2 ± 0.3 [9/10]**	2.2 ± 0.39 [9/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.9 ± 0.43 [4/10]*	0 ± 0 [0/8]	0.3 ± 0.25 [1/8]	0.3 ± 0.25 [1/8]	0 ± 0 [0/8]	
metaplasia	F	0 ± 0 [0/10]	3.5 ± 0.19 [8/8]***	3.5 ± 0.27 [10/8]***	3.6 ± 0.34 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1.6 ± 0.4 [7/10]***	0 ± 0 [0/8]	0.4 ± 0.26 [2/8]	1 ± 0.57 [3/8]	0.4 ± 0.38 [1/8]	
Lateral Wall—Respiratory Epithelium													
Basal cell	M	0.1 ± 0.1 [1/10]	1.6 ± 0.24 [8/9]***	0.5 ± 0.22 [4/10]	0.9 ± 0.23 [7/10]***	0.1 ± 0.1 [1/10]	0.5 ± 0.17 [5/10]	1.1 ± 0.23 [8/10]**	0.1 ± 0.13 [1/8]	1 ± 0.27 [6/8]	0.4 ± 0.18 [3/8]	0.4 ± 0.18 [3/8]	
hyperplasia	F	0 ± 0 [0/10]	0.9 ± 0.35 [4/8]*	0.4 ± 0.22 [3/10]	0.6 ± 0.24 [4/9]*	0.1 ± 0.1 [1/10]	0.3 ± 0.15 [3/10]	0.2 ± 0.13 [2/10]	0.1 ± 0.13 [1/8]	0.5 ± 0.27 [5/8]	0 ± 0 [0/8]	0.1 ± 0.13 [1/8]	
Squamous cell	M	0 ± 0 [0/10]	3.1 ± 0.35 [9/9]***	3.6 ± 0.16 [10/10]***	3.7 ± 0.21 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1.9 ± 0.55 [7/10]**	0 ± 0 [0/8]	1.6 ± 0.32 [7/8]	3.3 ± 0.31 [8/8]	1.5 ± 0.57 [5/8]	
metaplasia	F	0 ± 0 [0/10]	3.4 ± 0.32 [8/8]***	3.6 ± 0.31 [10/10]***	4 ± 0.24 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	3.7 ± 0.15 [10/10]***	0 ± 0 [0/8]	2.9 ± 0.48 [7/8]	3.5 ± 0.38 [8/8]	2.9 ± 0.52 [7/8]	
Maxilloturbinate—Respiratory Epithelium													
Basal cell	M	0.2 ± 0.2 [1/10]	0.2 ± 0.22 [1/9]	0.8 ± 0.42 [3/10]	0.5 ± 0.22 [4/10]	0.2 ± 0.13 [2/10]	0.9 ± 0.23 [7/10]*	0.7 ± 0.26 [5/10]	0 ± 0 [0/8]	1.3 ± 0.16 [8/8]	1 ± 0.19 [7/8]	0.5 ± 0.19 [4/8]	
hyperplasia	F	0 ± 0 [0/10]	0 ± 0 [0/8]	0 ± 0 [0/10]	0.2 ± 0.15 [7/9]	0.3 ± 0.15 [3/10]	0.4 ± 0.16 [4/10]*	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	0.8 ± 0.31 [4/8]	0 ± 0 [0/8]	0.4 ± 0.26 [2/8]	
Squamous cell	M	0 ± 0 [0/10]	4 ± 0 [9/9]***	3.6 ± 0.22 [10/10]***	3.5 ± 0.22 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1.4 ± 0.43 [7/10]**	0 ± 0 [0/8]	1.5 ± 0.19 [8/8]	1.5 ± 0.27 [6/8]	0.6 ± 0.32 [3/8]	
metaplasia	F	0 ± 0 [0/10]	4.1 ± 0.13 [8/8]***	4.4 ± 0.16 [10/10]***	3.9 ± 0.2 [10/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	3.1 ± 0.43 [10/10]***	0 ± 0 [0/8]	2 ± 0.33 [7/8]	2.1 ± 0.52 [5/8]	0.6 ± 0.26 [4/8]	
Nasoturbinate—Respiratory Epithelium													
Basal cell	M	0.1 ± 0.1 [1/10]	0.8 ± 0.36 [4/9]	0.7 ± 0.21 [4/10]*	0.4 ± 0.27 [8/10]	0.2 ± 0.13 [2/10]	0.6 ± 0.16 [6/10]*	0.6 ± 0.27 [4/10]	0 ± 0 [0/8]	0.9 ± 0.13 [7/8]	0.5 ± 0.19 [4/8]	0.5 ± 0.19 [4/8]	
hyperplasia	F	0 ± 0 [0/10]	0.3 ± 0.25 [1/8]	0 ± 0 [0/10]	0.1 ± 0.11 [8/9]	0.3 ± 0.15 [3/10]	0.4 ± 0.16 [4/10]*	0.3 ± 0.15 [3/10]	0.4 ± 0.18 [3/8]	0.9 ± 0.35 [5/8]	0.1 ± 0.13 [1/8]	0.4 ± 0.18 [3/8]	
Squamous cell	M	0 ± 0 [0/10]	3 ± 0.29 [9/9]***	2.9 ± 0.31 [10/10]***	3.1 ± 0.23 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1.6 ± 0.45 [7/10]**	0 ± 0 [0/8]	1.9 ± 0.3 [7/8]	2.6 ± 0.32 [8/8]	0.6 ± 0.38 [3/8]	
metaplasia	F	0 ± 0 [0/10]	3.5 ± 0.38 [8/8]***	3.7 ± 0.15 [10/10]***	4.1 ± 0.26 [10/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	2.4 ± 0.34 [10/10]***	0.5 ± 0.5 [1/8]	2.5 ± 0.38 [8/8]	3 ± 0.46 [7/8]	0.4 ± 0.18 [3/8]	
Dorsal Meatus—Respiratory Epithelium													
Squamous cell	M	0 ± 0 [0/10]	3 ± 0.24 [9/9]***	3 ± 0.26 [10/10]***	2 ± 0.54 [7/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.5 ± 0.22 [4/10]*	0 ± 0 [0/8]	0.1 ± 0.13 [1/8]	0 ± 0 [8/8]	0 ± 0 [0/8]	
metaplasia	F	0 ± 0 [0/10]	3.4 ± 0.38 [8/8]**	3.2 ± 0.33 [10/10]***	3.4 ± 0.38 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1 ± 0.42 [5/10]*	0 ± 0 [0/8]	0.4 ± 0.38 [1/8]	0.3 ± 0.25 [7/8]	0 ± 0 [0/8]	
Submucosa/Lamina Propria													
Infiltrate, mixed cells	M	0 ± 0 [0/10]	1 ± 0 [9/9]***	1.0 ± 0 [10]***	1 ± 0.1 [10/10]***	0.1 ± 0 [1/10]	0.1 ± 0.1 [1/10]	0.6 ± 0.2 [4/10] *	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0.9 ± 0.1 [8/8]	
(incidence)	F	0 ± 0 [0/10]	1 ± 0 [8/8]***	1.0 ± 0 [10]***	1 ± 0 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.8 ± 0.1 [2/10] ***	±0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0.8 ± 0.2 [8/8]	
Nasal Cavity—Lumen													
Necrotic cellular debris	M	0 ± 0 [0/10]	1 ± 0 [9/9]***	0.8 ± 0.13 [8/10]***	0.8 ± 0.13 [8/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.5 ± 0.17 [5/10]*	0 ± 0 [0/8]	0.6 ± 0.18 [5/8]	0.5 ± 0.19 [4/8]	0.1 ± 0.13 [1/8]	
	F	0 ± 0 [0/10]	0.8 ± 0.16 [6/8]***	0.7 ± 0.15 [7/10]***	0.9 ± 0.11 [8/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.4 ± 0.16 [6/10]	0 ± 0 [0/8]	0.4 ± 0.18 [3/8]	0.5 ± 0.19 [4/8]	0 ± 0 [0/8]	
Amorphous	M	0 ± 0 [0/10]	0.9 ± 0.11 [8/9]**	1 ± 0 [10/10]***	0.8 ± 0.13 [8/10]***	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0.6 ± 0.16 [6/10]*	0 ± 0 [0/8]	0.5 ± 0.19 [4/8]	0.5 ± 0.19 [4/8]	0.3 ± 0.16 [2/8]	
eosinophilic material	F	0 ± 0 [0/10]	0.9 ± 0.13 [7/8]***	0.8 ± 0.13 [8/10]***	1 ± 0 [9/9]***	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0.5 ± 0.17 [5/10]*	0 ± 0 [0/8]	0.4 ± 0.18 [3/8]	0.4 ± 0.18 [3/8]	0 ± 0 [0/8]	
Nose—Level 2													
Dorsal Meatus—Olfactory Epithelium													
Atrophy/loss	M	0 ± 0 [0/10]	4 ± 0.24 [9/9]***	3.5 ± 0.22 [10/10]***	4 ± 0.15 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.5 ± 0.22 [4/10]*	0 ± 0 [0/8]	3.6 ± 0.26 [8/8]	2.6 ± 0.3 [8/7]	0.4 ± 0.26 [2/8]	
	F	0 ± 0 [0/10]	3.8 ± 0.16 [8/8]***	4 ± 0.21 [10/10]***	3.9 ± 0.35 [8/8]***	0 ± 0 [0/10]	0 ± 0 [0/10]	1.1 ± 0.28 [7/10]**	0 ± 0 [0/8]	3.5 ± 0.27 [8/8]	3.5 ± 0.27 [8/8]	0.5 ± 0.19 [4/8]	
Squamous cell	M	0 ± 0 [0/10]	2.2 ± 0.36 [9/9]***	2.1 ± 0.38 [10/10]***	2 ± 0.3 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	1.3 ± 0.16 [8/8]	1.1 ± 0.14 [8/7]	0 ± 0 [0/8]	
metaplasia	F	0 ± 0 [0/10]	2.4 ± 0.42 [8/8]**	2.7 ± 0.4 [10/10]***	2.1 ± 0.61 [7/8]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	1.1 ± 0.3 [6/8]	1.4 ± 0.26 [7/8]	0 ± 0 [0/8]	
Nasoturbinate—Respiratory Epithelium													
Basal cell	M	0 ± 0 [0/10]	2.3 ± 0.17 [9/9]***	2.4 ± 0.31 [10/10]***	2.7 ± 0.21 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	0.5 ± 0.19 [4/8]	0.9 ± 0.23 [6/8]	0 ± 0 [0/8]	
hyperplasia	F	0 ± 0 [0/10]	2 ± 0.33 [7/8]**	2.1 ± 0.28 [10/10]**	2.6 ± 0.56 [7/8]**	0 ± 0 [0/10]	0 ± 0 [0/10]	0.8 ± 0.2 [7/10]**	0 ± 0 [0/8]	0.4 ± 0.18 [5/8]	0.1 ± 0.13 [7/8]	0 ± 0 [0/8]	
Squamous cell	M	0 ± 0 [0/10]	0.6 ± 0.24 [4/9]*	1.5 ± 0.37 [9/10]**	1 ± 0.3 [6/10]**	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	0.1 ± 0.13 [7/8]	0 ± 0 [0/8]	0 ± 0 [0/8]	
metaplasia	F	0 ± 0 [0/10]	1.3 ± 0.31 [6/8]**	1.1 ± 0.28 [7/10]**	1.1 ± 0.3 [6/8]**	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	0.1 ± 0.13 [7/8]	0 ± 0 [0/8]	0 ± 0 [0/8]	
Submucosa/Lamina Propria													
Loss of nerve bundles—dorsal meatus	M	0 ± 0 [0/10]	4.4 ± 0.18 [9/9]***	4 ± 0.21 [10/10]***	4.3 ± 0.21 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.6 ± 0.27 [4/10]*	0 ± 0 [0/7]	3 ± 0.46 [8/8]	2.7 ± 0.36 [8/7]	0.4 ± 0.2 [4/7]	
	F	0 ± 0 [0/10]	4.4 ± 0.18 [8/8]***	4 ± 0.21 [10/10]***	4.4 ± 0.18 [8/8]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.8 ± 0.22 [6/9]**	0 ± 0 [0/8]	2.9 ± 0.35 [8/8]	3.6 ± 0.26 [8/8]	0.5 ± 0.19 [4/8]	
	M	0 ± 0 [0/10]	1 ± 0 [9/9]***	1 ± 0 [10/10]***	1 ± 0 [10/10]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0 ± 0 [0/8]	

(continued on next page)

Table 7a (continued)

Parameter		90d						90 + 42d				
		Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Infiltrate, mixed cell	F	0 ± 0 [0/10]	1 ± 0 [8/8]***	1 ± 0 [10/10]***	1 ± 0 [8/8]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0 ± 0 [0/8]
Edema	M	—	—	—	—	—	—	—	0 ± 0 [0/8]	4 ± 0.19 [8/8]	3.8 ± 0.16 [8/8]	0.4 ± 0.2 [4/7]
	F	—	—	—	—	—	—	—	0 ± 0 [0/8]	3.8 ± 0.16 [8/8]	3.5 ± 0.33 [8/8]	0.5 ± 0.19 [4/8]
Nose—Level 3												
Ethmoid Turbinate—Olfactory Epithelium												
Atrophy/loss	M	0 ± 0 [0/10]	4 ± 0.17 [9/9]***	3.6 ± 0.34 [9/9]***	4 ± 0.24 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.5 ± 0.22 [4/10]*	0 ± 0 [0/8]	4 ± 0 [8/8]	3.1 ± 0.3 [8/8]	0.3 ± 0.16 [2/8]
	F	0 ± 0 [0/10]	4.3 ± 0.25 [8/8]***	4 ± 0.3 [10/10]***	4 ± 0.24 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.6 ± 0.29 [7/9]	0 ± 0 [0/8]	3.5 ± 0.19 [8/8]	3.5 ± 0.19 [8/8]	0.1 ± 0.13 [1/8]
Squamous cell	M	0 ± 0 [0/10]	1.4 ± 0.18 [9/9]***	1.4 ± 0.24 [9/9]***	1.8 ± 0.28 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.2 ± 0.2 [1/10]	0 ± 0 [0/8]	1.8 ± 0.16 [8/8]	1.5 ± 0.19 [8/8]	0 ± 0 [0/8]
metaplasia	F	0 ± 0 [0/10]	1.1 ± 0.35 [6/8]**	1.6 ± 0.37 [9/10]***	1.7 ± 0.41 [7/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/9]	0 ± 0 [0/8]	1.6 ± 0.18 [8/8]	1.4 ± 0.18 [8/8]	0 ± 0 [0/8]
Submucosa/Lamina Propria												
Loss of nerve	M	0 ± 0 [0/10]	4.3 ± 0.29 [9/9]***	3.8 ± 0.32 [9/9]***	3.9 ± 0.35 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.5 ± 0.22 [4/10]*	0 ± 0 [0/8]	3.1 ± 0.4 [8/8]	2.6 ± 0.32 [8/8]	0.4 ± 0.18 [3/8]
bundles	F	0 ± 0 [0/10]	4.3 ± 0.16 [8/8]***	4.1 ± 0.18 [10/10]***	3.9 ± 0.31 [9/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.3 ± 0.24 [2/9]	0 ± 0 [0/8]	3.1 ± 0.13 [8/8]	3.6 ± 0.26 [8/8]	0.5 ± 0.19 [4/8]*
Infiltrate, mixed cell	M	0 ± 0 [0/10]	1 ± 0 [9/9]***	1 ± 0 [9/9]***	0.9 ± 0.11 [8/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0 ± 0 [0/8]
	F	0 ± 0 [0/10]	0.9 ± 0.13 [7/8]***	0.9 ± 0.1 [9/10]***	0.9 ± 0.11 [8/9]***	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0.1 ± 0.11 [1/9]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0 ± 0 [0/8]
Edema	M	—	—	—	—	—	—	—	0.1 ± 0.13 [1/8]	3.6 ± 0.32 [8/8]	3.4 ± 0.26 [8/8]	0.5 ± 0.19 [4/8]
	F	—	—	—	—	—	—	—	0 ± 0 [0/8]	3.8 ± 0.16 [8/8]	3 ± 0.33 [8/8]	0.6 ± 0.18 [5/8]
Nose—Level 4												
Submucosa/Lamina Propria												
Loss of nerve	M	0 ± 0 [0/10]	1.3 ± 0.44 [6/9]**	1.3 ± 0.37 [6/8]**	0.7 ± 0.26 [4/10]*	0 ± 0 [0/10]	0 ± 0 [0/9]	0 ± 0 [0/10]	0 ± 0 [0/8]	3.1 ± 0.34 [8/7]	1.6 ± 0.26 [8/8]	0.1 ± 0.14 [6/7]
bundles, dorsal meatus/septum	F	0 ± 0 [0/10]	1.1 ± 0.48 [4/8]*	1.6 ± 0.27 [9/10]***	1 ± 0.27 [6/8]**	0 ± 0 [0/10]	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0 ± 0 [0/7]	1.8 ± 0.25 [8/8]	1.8 ± 0.45 [7/8]‡	0 ± 0 [0/8]
Infiltrate, mixed cell	M	0 ± 0 [0/10]	0.8 ± 0.28 [5/9]**	0.6 ± 0.18 [5/9]**	0.5 ± 0.34 [8/10]	0 ± 0 [0/10]	0 ± 0 [0/9]	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	0.9 ± 0.44 [3/8]	0 ± 0 [0/8]	0 ± 0 [0/7]
	F	0 ± 0 [0/10]	0.8 ± 0.16 [6/8]**	0.6 ± 0.22 [5/10]*	0.5 ± 0.19 [4/8]*	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	0.3 ± 0.16 [2/8]	0.5 ± 0.38 [2/8]	0 ± 0 [0/8]
Edema, dorsal meatus/septum	M	0 ± 0 [0/10]	0.6 ± 0.18 [5/9]**	0.3 ± 0.24 [7/9]	0.2 ± 0.2 [9/10]	0 ± 0 [0/10]	0.1 ± 0.1 [1/10]	0.2 ± 0.13 [2/10]	0.1 ± 0.13 [1/8]	0.9 ± 0.46 [4/7]	1.3 ± 0.16 [8/8]	0.2 ± 0.17 [1/6]
	F	0 ± 0 [0/10]	0 ± 0 [0/8]	0.3 ± 0.15 [9/10]	0.4 ± 0.18 [5/8]*	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/7]	1.4 ± 0.26 [7/8]	0.9 ± 0.4 [4/8]‡	0.3 ± 0.16 [2/8]
Larynx												
Base of the Epiglottis												
Squamous cell	M	0.1 ± 0.1 [1/10]	5 ± 0 [1/9]***	4.8 ± 0.13 [10/10]***	4.6 ± 0.18 [9/9]***	1.7 ± 0.26 [9/10]***	2.5 ± 0.17 [10/10]***	3 ± 0.21 [10/10]***	0 ± 0 [0/8]	1.9 ± 0.26 [7/7]	1.3 ± 0.25 [7/8]	0.1 ± 0.13 [1/8]
hyperplasia-metaplasia	F	0 ± 0 [0/10]	4.6 ± 0.18 [0/8]***	4.7 ± 0.15 [10/10]***	4.4 ± 0.18 [9/9]***	2.1 ± 0.23 [10/10]***	2.7 ± 0.21 [10/10]***	3 ± 0.21 [10/10]***	0.1 ± 0.13 [1/8]	1 ± 0.31 [5/7]	1.6 ± 0.18 [8/8]	0.5 ± 0.19 [4/8]
Submucosa/Lamina Propria												
Infiltrate, mononuclear cell	M	0.6 ± 0.22 [5/10]	2.2 ± 0.28 [9/9]**	2.2 ± 0.2 [10/10]***	2.2 ± 0.2 [10/10]***	1.5 ± 0.22 [9/10]*	1.4 ± 0.27 [9/10]*	1.2 ± 0.2 [9/10]	0.3 ± 0.16 [2/8]	1.1 ± 0.34 [5/7]	1.1 ± 0.3 [6/8]	0.1 ± 0.13 [1/8]
	F	0.6 ± 0.24 [4/9]	1.9 ± 0.3 [8/8]**	2.6 ± 0.22 [10/10]***	2.2 ± 0.22 [9/9]**	0.8 ± 0.2 [7/10]	1.4 ± 0.31 [8/10]	2.1 ± 0.18 [10/10]***	0.3 ± 0.25 [1/8]	0.9 ± 0.26 [5/7]	1 ± 0.33 [5/8]	0.4 ± 0.18 [4/8]
Ectasia/congestion, vascular	M	0.2 ± 0.13 [2/10]	0.9 ± 0.13 [7/8]*	0.8 ± 0.15 [7/9]*	0.9 ± 0.1 [9/10]**	0.4 ± 0.16 [4/10]	0.6 ± 0.16 [6/10]	0.7 ± 0.15 [7/10]	0 ± 0 [0/8]	0.7 ± 0.18 [5/7]	0.8 ± 0.16 [6/8]	0.1 ± 0.13 [1/8]
	F	0.1 ± 0.11 [1/9]	0.8 ± 0.16 [6/8]*	0.8 ± 0.13 [8/10]**	0.7 ± 0.17 [6/9]*	0.1 ± 0.1 [1/10]	0.5 ± 0.17 [5/10]	0.5 ± 0.17 [5/10]	0 ± 0 [0/8]	0.1 ± 0.14 [1/7]	0 ± 0 [0/8]	0.1 ± 0.13 [1/8]
Arytenoid Projections												
Lower Medial Region												
Squamous cell	M	0.4 ± 0.16 [4/10]	4.8 ± 0.15 [9/9]***	4.2 ± 0.2 [10/10]***	4.5 ± 0.22 [10/10]***	1 ± 0.15 [9/10]*	1.4 ± 0.16 [10/10]**	2 ± 0.21 [10/10]***	0 ± 0 [0/8]	2 ± 0.27 [8/8]	1.5 ± 0.46 [6/8]	0.3 ± 0.25 [1/8]
hyperplasia-metaplasia	F	0.1 ± 0.1 [1/10]	4.3 ± 0.25 [8/8]***	4.5 ± 0.17 [10/10]***	4.2 ± 0.15 [9/9]***	1.7 ± 0.21 [10/10]***	1.9 ± 0.18 [10/10]***	2.2 ± 0.2 [10/10]***	0.1 ± 0.13 [1/8]	1.9 ± 0.55 [6/8]	2.3 ± 0.47 [7/7]	0 ± 0 [0/8]
Upper Medial Region												
Basal cell	M	0 ± 0 [0/10]	0 ± 0 [0/9]	0.1 ± 0.1 [1/10]	0.1 ± 0.1 [1/10]	0.6 ± 0.16 [6/10]**	1.3 ± 0.15 [10/10]***	0.9 ± 0.23 [7/10]**	0 ± 0 [0/8]	0.5 ± 0.19 [4/8]	0.3 ± 0.16 [2/8]	0.1 ± 0.13 [1/8]
hyperplasia	F	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	0 ± 0 [0/10]	0 ± 0 [0/9]	1.3 ± 0.37 [6/8]**	1.2 ± 0.28 [7/9]**	1.3 ± 0.33 [7/10]**	0.1 ± 0.13 [1/8]	0.8 ± 0.16 [6/8]	0.8 ± 0.17 [7/6]	0 ± 0 [0/8]
Squamous cell	M	0 ± 0 [0/10]	4.6 ± 0.18 [9/9]***	3.9 ± 0.41 [10/10]***	4.3 ± 0.21 [10/10]***	0.3 ± 0.21 [2/10]	0.1 ± 0.1 [1/10]	0.7 ± 0.26 [5/10]*	0 ± 0 [0/8]	0.5 ± 0.27 [3/8]	0.5 ± 0.27 [5/8]	0.3 ± 0.25 [1/8]
metaplasia	F	0 ± 0 [0/10]	4.8 ± 0.16 [8/8]***	4.5 ± 0.17 [10/10]***	4.2 ± 0.22 [9/9]***	0.6 ± 0.26 [4/8]*	1.2 ± 0.32 [7/9]***	1.5 ± 0.37 [8/10]***	0.1 ± 0.13 [1/8]	1.4 ± 0.53 [5/8]	1 ± 0.37 [4/6]	0 ± 0 [0/8]
Ventral Pouch												
Epithelial changes												
Basal cell	M	0.1 ± 0.11 [1/9]	0 ± 0 [0/9]	0.1 ± 0.1 [1/10]	0.3 ± 0.21 [2/10]	0.3 ± 0.15 [3/10]	0.7 ± 0.24 [4/9]*	0.7 ± 0.26 [5/10]	0 ± 0 [0/8]	1.3 ± 0.25 [7/8]	0.6 ± 0.26 [4/8]	0 ± 0 [0/8]
hyperplasia	F	0 ± 0 [0/10]	0.1 ± 0.13 [1/8]	0.3 ± 0.21 [2/10]	0.1 ± 0.11 [1/9]	0.3 ± 0.15 [3/10]	1 ± 0.37 [5/10]*	1.2 ± 0.29 [8/10]***	0 ± 0 [0/8]	0.8 ± 0.25 [5/8]	0.6 ± 0.18 [5/8]	0 ± 0 [0/8]
Squamous cell	M	0 ± 0 [0/10]	4.4 ± 0.44 [9/9]***	3.6 ± 0.62 [9/10]***	3.3 ± 0.62 [9/10]***	0 ± 0 [0/10]	0.2 ± 0.22 [8/9]	0.4 ± 0.27 [2/10]	0 ± 0 [0/8]	0.8 ± 0.37 [3/8]	0.3 ± 0.16 [2/8]	0 ± 0 [0/8]
metaplasia	F	0 ± 0 [0/10]	4.6 ± 0.26 [8/8]***	3.9 ± 0.55 [9/10]***	4.4 ± 0.29 [9/9]***	0 ± 0 [0/10]	0.4 ± 0.31 [8/10]	0.2 ± 0.13 [2/10]	0.1 ± 0.13 [1/8]	0.3 ± 0.16 [2/8]	0.4 ± 0.18 [3/8]	0.1 ± 0.13 [1/8]

Submucosa/lamina propria													
Infiltrate, mononuclear cell	M	0.3 ± 0.15 [3/10]	1.3 ± 0.25 [7/8]**	1 ± 0.21 [8/10]*	1.2 ± 0.25 [8/10]*	0.5 ± 0.22 [4/10]	0.8 ± 0.33 [5/10]	0.6 ± 0.22 [5/10]	0 ± 0 [0/8]	0.5 ± 0.27 [3/8]	0.8 ± 0.25 [5/8]	0.1 ± 0.13 [1/8]	
	F	0 ± 0 [0/9]	1.9 ± 0.35 [7/8]***	1.7 ± 0.3 [9/10]***	2 ± 0.24 [9/9]***	0.2 ± 0.13 [2/10]	1.1 ± 0.31 [7/10]**	0.8 ± 0.25 [6/10]**	0.1 ± 0.13 [1/8]	0.3 ± 0.16 [2/8]	0.5 ± 0.38 [2/8]	0 ± 0 [0/8]	
Ectasia/congestion, vascular	M	0.6 ± 0.16 [6/10]	1 ± 0 [0/8]	1 ± 0 [10/10]	0.9 ± 0.1 [9/10]	0.7 ± 0.15 [3/10]	0.9 ± 0.1 [9/10]	1 ± 0 [10/10]	0.1 ± 0.13 [1/8]	1 ± 0 [8/8]	0.6 ± 0.18 [5/8]	0.3 ± 0.16 [2/8]	
	F	0.2 ± 0.15 [2/9]	1 ± 0 [0/8]**	0.9 ± 0.1 [9/10]**	1 ± 0 [9/9]**	0.5 ± 0.17 [5/10]	0.6 ± 0.16 [6/10]	0.7 ± 0.15 [7/10]	0.1 ± 0.13 [1/8]	0.5 ± 0.19 [4/8]	0.8 ± 0.16 [6/8]	0.4 ± 0.18 [3/8]	
Vocal Folds—Mid-lateral Region													
Squamous cell hyperplasia-metaplasia	M	0.2 ± 0.13 [2/10]	3.7 ± 0.17 [9/9]***	3.8 ± 0.29 [10/10]***	3.8 ± 0.25 [10/10]***	0.8 ± 0.22 [6/9]*	1 ± 0.17 [8/9]**	1.2 ± 0.33 [7/10]*	0.1 ± 0.13 [1/8]	2.3 ± 0.42 [8/7]	1.5 ± 0.33 [7/8]	0.3 ± 0.25 [1/8]	
	F	0 ± 0 [0/10]	4.3 ± 0.25 [8/8]***	4.2 ± 0.13 [10/10]***	3.8 ± 0.22 [9/9]***	0.5 ± 0.17 [5/10]*	1.1 ± 0.23 [8/10]***	1.1 ± 0.23 [8/10]***	0.4 ± 0.38 [1/8]	1.6 ± 0.46 [6/8]*	1.4 ± 0.32 [7/8]*	0.1 ± 0.13 [1/8]	
Tracheal Bifurcation—Carina													
Basal cell hyperplasia	M	0.7 ± 0.24 [5/9]	2.9 ± 0.55 [7/7]**	2.6 ± 0.26 [8/8]***	2 ± 0.33 [9/10]**	0.5 ± 0.22 [4/10]	0.6 ± 0.31 [4/10]	1 ± 0.3 [7/10]	0.6 ± 0.37 [2/7]	1.1 ± 0.26 [6/7]	0.2 ± 0.17 [1/6]	0.6 ± 0.26 [4/8]	
	F	0.1 ± 0.11 [1/9]	2 ± 0.46 [7/8]**	2.1 ± 0.31 [9/10]***	2.6 ± 0.46 [8/8]***	0.4 ± 0.16 [4/10]	0.6 ± 0.24 [4/9]	1.1 ± 0.31 [7/9]**	0 ± 0 [0/7]	0.4 ± 0.2 [3/7]	1 ± 0.33 [5/8]	0.7 ± 0.29 [4/7]	
Squamous cell metaplasia	M	0 ± 0 [0/9]	1.4 ± 0.65 [4/8]*	0.3 ± 0.21 [2/10]	1.1 ± 0.43 [5/10]*	0.1 ± 0.1 [1/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	0 ± 0 [0/7]	0 ± 0 [8/8]	0 ± 0 [0/8]	
	F	0 ± 0 [0/10]	0.8 ± 0.41 [3/8]*	0.2 ± 0.13 [2/10]	0.3 ± 0.16 [6/8]	0 ± 0 [0/10]	0 ± 0 [0/9]	0 ± 0 [0/10]	0 ± 0 [0/7]	0 ± 0 [0/7]	0 ± 0 [8/8]	0 ± 0 [0/7]	
Tracheal Wall													
Goblet cell hyperplasia/hypertrophy	M	0.8 ± 0.16 [2/8]	3.1 ± 0.45 [9/9]**	2.5 ± 0.45 [9/10]**	3.5 ± 0.45 [10/10]***	0.6 ± 0.22 [5/10]	0.9 ± 0.2 [7/9]	0.7 ± 0.21 [6/10]	0.1 ± 0.13 [1/8]	0.6 ± 0.2 [4/7]	1.3 ± 0.25 [7/8]	0.1 ± 0.13 [1/8]	
	F	0.9 ± 0.23 [2/8]	1.3 ± 0.31 [6/8]	1.8 ± 0.29 [10/10]*	2.4 ± 0.41 [0/9]*	0.4 ± 0.16 [4/10]	0.4 ± 0.18 [5/8]	0.2 ± 0.15 [2/9]†	0.4 ± 0.3 [2/7]	0.7 ± 0.29 [4/7]	0.6 ± 0.18 [5/8]	0.1 ± 0.14 [1/7]	
<b>Left Lung—Intra-alveolar Changes</b>													
Unpigmented macrophages	M	0.1 ± 0.1 [1/10]	1 ± 0 [9/9]***	1 ± 0 [10/10]***	1 ± 0 [10/10]***	0.3 ± 0.15 [3/10]	0.2 ± 0.13 [2/10]	0.1 ± 0.1 [1/10]	0.8 ± 0.16 [6/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0.5 ± 0.19 [4/8]	
	F	0.1 ± 0.1 [1/10]	1 ± 0 [8/8]***	1 ± 0 [10/10]***	1 ± 0 [9/9]***	0.2 ± 0.13 [2/10]	0 ± 0 [0/10]	0.4 ± 0.16 [4/10]	0.6 ± 0.18 [5/8]	1 ± 0 [8/8]	1 ± 0 [7/7]	0.6 ± 0.18 [3/8]	
Pigmented macrophages	M	0.1 ± 0.1 [1/10]	1 ± 0 [9/9]***	1 ± 0 [10/10]***	1 ± 0 [10/10]***	0 ± 0 [0/10]	0.4 ± 0.16 [6/10]	0.2 ± 0.13 [2/10]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [8/8]	0 ± 0 [0/8]	
	F	0 ± 0 [0/10]	1 ± 0 [8/8]***	1 ± 0 [10/10]***	1 ± 0 [9/9]***	0 ± 0 [0/10]	0.1 ± 0.1 [9/10]	0.1 ± 0.1 [1/10]	0 ± 0 [0/8]	1 ± 0 [8/8]	1 ± 0 [7/7]	0 ± 0 [0/8]	
Macrophage aggregates	M	0 ± 0 [0/10]	3.6 ± 0.58 [9/9]***	3.9 ± 0.35 [10/10]***	3.1 ± 0.53 [10/10]***	0.1 ± 0.1 [1/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	5 ± 0 [8/8]	4.5 ± 0.19 [8/8]	0.1 ± 0.13 [1/8]	
	F	0 ± 0 [0/10]	3 ± 0.46 [8/8]***	3.3 ± 0.54 [9/10]***	3.6 ± 0.47 [8/9]***	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/10]	0 ± 0 [0/8]	4.3 ± 0.25 [8/8]	3.6 ± 0.69 [7/7]	0 ± 0 [0/8]	

Results are presented as mean ± standard error. The sample size is in parentheses. Difference from sham group at 90d: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

presence of menthol in MS did not introduce any novel effects, nor did it alter the severity and/or incidence of histopathological findings observed from 3R4F MS exposure alone.

### 3.8.3. Trachea

Alterations in the trachea which were statistically significantly higher in the MRC groups compared with sham and THS2.2M included basal cell hyperplasia, squamous cell metaplasia, and goblet cell hyperplasia. There were no differences in severity and incidence of the findings between the MRC groups and the 3R4F group, indicating that the presence of menthol in MS had no effect on the alterations in the trachea.

In THS2.2M-exposed groups, all observed findings were considered incidental.

There was complete reversal of adaptive changes and near-complete reversal of inflammatory infiltrates following recovery from MRC MS exposure.

### 3.8.4. Lung

In the left lung, MS-induced histologic findings included basal cell hyperplasia, goblet cell hyperplasia, squamous metaplasia of the main-stem bronchus, basal cell hyperplasia of the lower bronchiolar epithelium, unpigmented and pigmented macrophages scattered within alveoli, dense macrophage aggregates in alveolar spaces, and alveolar type 2 pneumocyte hyperplasia. In the THS2.2M MA-exposed groups, all observed findings were of similar incidence and/or severity compared with sham, and slightly deviating findings were considered incidental.

Overall, findings in the lung following 3R4F and MRC MS exposure were similar. Basal cell hyperplasia and goblet cell hyperplasia along the bronchus, along with squamous cell metaplasia and basal cell hyperplasia of the lower bronchioles, were statistically significantly higher compared with sham. Following the 42-day post-inhalation recovery period, there was complete reversal of basal cell and goblet cell hyperplasia.

In the lower airways and within the alveoli, pigmented and unpigmented macrophages were observed at 100% incidence in MRC MS-exposed groups. The severity of macrophage aggregates within the alveoli was comparable between the MRC groups. These findings were all statistically significantly higher than in the sham and THS2.2M exposure groups. Aggregates of pigment-laden alveolar macrophages were scattered randomly throughout the lower airways adjacent to the alveolar ducts, and were often associated with slight local hyperplasia of alveolar epithelial cells. Incidence of type 2 pneumocyte hyperplasia was associated with the severity of macrophage aggregates. Following a 42-day recovery period, there was a notable increase in macrophage aggregates within alveoli in MRC MS-exposed groups. This was accompanied by an increased incidence of type 2 pneumocyte hyperplasia.

No differences were observable in any of the histopathological endpoints in MRC groups compared with the 3R4F group. Furthermore, the presence of menthol in MS did not introduce any novel effects, nor did it alter the severity and/or incidence of histopathological findings observed from 3R4F MS exposure alone.

## 3.9. Transcriptome and proteome responses (“OECD plus”)

As part of our overall systems toxicology assessment framework (Sturla et al., 2014), the standard toxicological endpoints were complemented by measurements of global transcriptome and proteome changes in RNE and lung tissue from animals that were exposed alongside those from the main study. Effects on the transcriptome were measured by microarray expression profiling, and a robust isobaric tag-based mass spectrometry method was employed to quantify changes in the proteome (Phillips et al.,

2015b; Titz et al., 2014). Here, we report on the global systems response patterns that summarize the molecular perturbations for the different exposure conditions. More details and mechanistic insights are provided in our accompanying manuscript (Kogel et al., 2016 (in this issue)).

### 3.9.1. Systems response profiles of RNE

To assess the response of the RNE transcriptome, differentially expressed genes (DEGs) were first identified for each exposure group in comparison with their respective sham groups as all genes with FDR-adjusted *p*-values below 0.05. Fig. 3A and B summarize the exposure response of these differentially expressed genes for all smoke and aerosol exposure groups. Note that the overall response profiles are discussed here, and a more detailed biological analysis of the molecular exposure response is presented in the accompanying manuscript (Kogel et al., 2016 (in this issue)). For both male and female rats, THS2.2M MA exposure induced fewer changes in the RNE transcriptome compared with MRC MS exposures. For male rats, the highest number of DEGs was observed in the MRC(HM) group (7729 DEGs). For female rats, the highest number of DEGs was observed in the MRC(LM) group (8054 DEGs). In contrast, only 1 DEG was found in male rats and no DEGs in female rats after exposure to THS2.2M Medium MA at the same nicotine concentration (23 µg nicotine/l). Exposure to higher nicotine concentrations led to 9 DEGs in the respiratory epithelium in male rats and 2118 DEGs in female rats of the THS2.2M High groups. After the recovery period, only 5% of all DEGs were still statistically significantly differentially expressed in the THS2.2M High group relative to sham.

Quantitative proteomics revealed up to 1300 significantly differentially expressed proteins (DEPs) in the RNE of MRC MS-exposed rats in comparison with sham (FDR adjusted *p*-value <0.05) (Fig. 4A) – with a higher number of DEPs in female than in male rats. In contrast, THS2.2M MA-exposed rats showed much lower differential protein expression in the RNE (Fig. 4A). In the THS2.2M Low and THS2.2M Medium groups, a maximum of 7 DEPs was found; the differential expression response to THS2.2 High was more pronounced, with 266 (males) and 478 (females) DEPs. The 25 proteins with the overall highest fold-change included several epithelial cell markers (Krt6a, Krt13, Sprr1a, Clu, Sfn), and were indicative of gross changes in the cellular architecture of the epithelium upon MS exposure (see Discussion section and Kogel et al. (Kogel et al., 2016 (in this issue))). These proteins exemplified the overall trends, and were regulated in the same direction upon THS2.2M MA exposure (up- or downregulated) as after MRC MS exposure (Fig. 4C). However, consistently, the fold-changes were much lower upon MA exposure, and statistical significance was generally only reached after exposure to the highest concentration of THS2.2M MA. Whereas female rats exposed to MRC(HM) showed only a partial recovery after 42 days post-exposure, all differentially expressed proteins did recover for the THS2.2M High exposure condition (Fig. 4C).

Taken together, both the transcriptome and proteome response profiles of RNE supported an overall reduction of the THS2.2M aerosol-induced molecular effects compared with reference smoke exposure. In addition, the molecular changes induced by THS2.2M aerosol appeared largely to revert after the 42-day recovery period.

### 3.9.2. Systems response profiles of lung tissue

The molecular response of rat lung tissue to the tested exposure conditions was analyzed by both transcriptomics and proteomics. Here, the overall response profiles are discussed; a more detailed analysis of the affected transcripts and proteins is presented in the accompanying manuscript (Kogel et al., 2016 (in this issue)). Of note, the number of DEGs identified in the lung tissue of rats

**Table 7b**

Statistically significant differences in histopathological findings in the respiratory tract at the end of the 90-day and post-inhalation periods.

Parameter		THS2.2M Medium vs. MRC(LM)	THS2.2M High vs. MRC(HM).	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
<b>Nose—Level 1</b>								
<i>Nasal Septum—Respiratory Epithelium</i>								
Basal cell hyperplasia	M	↓*	=	=	=	↓***	↓**	↓*
	F	↓***	=	=	=	↓*	↓***	=
Goblet cell hyperplasia/hypertrophy	M	=	=	=	=	↑**	↑**	↑**
	F	=	↓*	=	=	↑***	↑***	↑**
Goblet cell loss	M	↓***	=	=	=	↓**	↓**	↓***
	F	↓***	=	=	=	↓***	↓***	↓***
Squamous cell metaplasia	M	↓***	↓*	=	=	↓**	=	↓**
	F	↓***	↓**	=	=	↓***	↓*	↓**
<i>Lateral Wall—Respiratory Epithelium</i>								
Basal cell hyperplasia	M	=	=	=	=	=	↓*	=
	F	=	=	=	=	=	=	↓*
Squamous cell metaplasia	M	↓***	↓*	=	=	↓*	=	=
	F	↓***	=	=	=	=	=	=
<i>Maxilloturbinate - Respiratory Epithelium</i>								
Basal cell hyperplasia	M	=	=	=	=	↑**	=	=
	F	↑*	=	=	=	↑*	=	=
Squamous cell metaplasia	M	↓***	↓**	=	=	↓***	=	↓***
	F	↓***	=	=	=	↓***	↓**	↓**
<i>Nasoturbinate—Respiratory Epithelium</i>								
Basal cell hyperplasia	M	=	=	=	=	=	=	=
	F	↑*	=	=	↑*	=	=	=
Squamous cell metaplasia	M	↓***	↓*	=	=	↓*	=	=
	F	↓***	↓**	=	=	=	↓***	↓*
<i>Dorsal Meatus—Respiratory Epithelium</i>								
Squamous cell metaplasia	M	↓***	↓*	↓*	=	↓***	=	↓**
	F	↓***	=	=	=	↓***	↓*	↓**
<i>Submucosa/Lamina Propria</i>								
Infiltrate, mixed cells (incidence)	M	↓***	=	=	=	=	=	=
	F	↓***	=	=	=	=	=	=
<i>Nasal Cavity—Lumen</i>								
Necrotic cellular debris	M	↓***	=	=	=	=	=	=
	F	=	=	=	=	=	=	=
Amorphous eosinophilic material	M	↓***	=	=	=	=	=	=
	F	↓**	↓*	=	=	=	↓*	↓**
<b>Nose—Level 2</b>								
<i>Dorsal Meatus—Olfactory Epithelium</i>								
Atrophy/loss	M	↓***	↓***	=	=	=	↓**	=
	F	↓***	↓***	=	=	=	=	=
Squamous cell metaplasia	M	↓***	↓***	=	=	↓*	↓*	=
	F	↓***	↓***	=	=	↓*	=	=
<i>Nasoturbinate—Respiratory Epithelium</i>								
Basal cell hyperplasia	M	↓***	↓***	=	=	↓***	↓***	=
	F	↓***	↓*	=	=	↓**	↓**	↓**
Squamous cell metaplasia	M	↓***	↓**	=	=	↓*	↓*	=
	F	↓**	↓**	=	=	↓**	=	↓**
<i>Submucosa/Lamina Propria</i>								
Loss of nerve bundles—dorsal meatus	M	↓***	↓***	=	=	↓*	↓**	=
	F	↓***	↓***	=	=	↓**	=	↓*
Infiltrate, mixed cell	M	↓***	↓***	=	=	=	=	=
	F	↓***	↓***	=	=	=	=	=
Edema	M	=	=	=	=	=	=	=
	F	=	=	=	=	=	=	=

(continued on next page)

Table 7b (continued)

Parameter		THS2.2M Medium vs. MRC(LM)	THS2.2M High vs. MRC(HM).	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
<b>Nose—Level 3</b>								
<i>Ethmoid Turbinate—Olfactory Epithelium</i>								
Atrophy/loss	M	↓***	↓***	=	=	=	↓*	=
	F	↓***	↓***	=	=	↓*	=	=
Squamous cell metaplasia	M	↓***	↓***	=	=	=	=	=
	F	↓***	↓***	=	=	=	=	=
<i>Submucosa/Lamina Propria</i>								
Loss of nerve bundles	M	↓***	↓***	=	=	↓*	↓*	=
	F	↓***	↓***	=	=	↓***	=	=
Infiltrate, mixed cell	M	↓***	↓**	=	=	=	=	=
	F	↓**	↓**	=	=	=	=	=
Edema	M	=	↓***	=	=	=	=	=
	F	=	↓***	=	=	=	=	=
<b>Nose—Level 4</b>								
<i>Submucosa/Lamina Propria</i>								
Loss of nerve bundles, dorsal meatus/septum	M	↓**	↓*	=	=	↑*	=	↑*
	F	↓***	↓**	=	=	=	=	=
Infiltrate, mixed cell	M	↓*	=	=	=	=	=	=
	F	↓*	↓*	=	=	=	=	=
Edema, dorsal meatus/septum	M	=	=	=	=	=	=	↑**
	F	=	↓*	=	=	↑**	=	=
<b>Larynx</b>								
<i>Base of the Epiglottis</i>								
Squamous cell hyperplasia-metaplasia	M	↓***	↓***	=	=	↓***	↓***	↓***
	F	↓***	↓***	=	=	↓***	↓***	↓***
<i>Submucosa/Lamina Propria</i>								
Infiltrate, mononuclear cell	M	↓*	↓**	=	=	↓*	↓**	↓*
	F	↓**	=	=	=	↓*	↓***	↓*
Ectasia/congestion, vascular	M	=	=	=	=	=	↓*	=
	F	=	=	=	=	↓*	=	↓**
<b>Arytenoid Projections</b>								
<i>Lower Medial Region</i>								
Squamous cell hyperplasia-metaplasia	M	↓***	↓***	=	↓*	↓***	↓***	↓***
	F	↓***	↓***	=	=	↓**	↓***	↓**
<i>Upper Medial Region</i>								
Basal cell hyperplasia	M	↑***	↑**	=	=	↑*	↓*	=
	F	↑***	↑**	=	=	↑**	↓**	↑**
Squamous cell metaplasia	M	↓***	↓***	=	=	↓***	=	↓***
	F	↓***	↓***	=	=	↓***	↓**	↓**
<i>Ventral Pouch</i>								
<i>Epithelial Changes</i>								
Basal cell hyperplasia	M	↑*	=	=	=	↑***	↓*	=
	F	=	↑**	=	=	↑*	↓**	↑*
Squamous cell metaplasia	M	↓***	↓**	=	=	↓***	=	↓**
	F	↓***	↓***	=	=	↓***	=	↓***
<i>Submucosa/Lamina Propria</i>								
Infiltrate, mononuclear cell	M	=	=	=	=	=	=	=
	F	=	↓**	=	=	↓**	↓*	↓**
Ectasia/congestion, vascular	M	=	=	=	=	=	↓**	=
	F	=	=	=	=	=	=	=

<i>Vocal Folds—Mid-lateral Region</i>									
Squamous cell hyperplasia-metaplasia	M	↓***	↓***	=	=	↓*	↓*	↓***	
	F	↓***	↓***	=	=	↓**	↓**	↓***	
<i>Tracheal Bifurcation—Carina</i>									
Basal cell hyperplasia	M	↓**	↓*	=	=	↓*	=	↓**	
	F	↓**	↓*	=	=	↓*	=	↓*	
Squamous cell metaplasia	M	=	↓*	=	=	↓*	=	↓*	
	F	=	=	=	=	=	=	=	
<i>Tracheal Wall</i>									
Goblet cell hyperplasia/hypertrophy	M	↓**	↓***	=	↓*	↓**	↓*	↓**	
	F	↓**	↓***	=	=	=	=	↓**	
<b>Left Lung—Intra-alveolar Changes</b>									
Unpigmented macrophages	M	↓***	↓***	=	↑*	=	=	=	
	F	↓***	↓*	=	↑*	=	=	=	
Pigmented macrophages	M	↓*	↓***	=	=	=	=	=	
	F	↓***	↓***	=	=	=	=	=	
Macrophage aggregates	M	↓***	↓***	=	=	↑*	=	=	
	F	↓***	↓***	=	=	↑*	=	=	

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 8a**  
Laryngeal epithelial thickness at the end of the 90-day and post-inhalation periods.

Localization	OECD							90 + 42d			
	Sham	3R4F	MRC(LM)	MRC(HM)	THS2.2M Low	THS2.2M Medium	THS2.2M High	Sham R	3R4F R	MRC(HM) R	THS2.2M High R
Base of epiglottis [μm]	M 22 ± 1.2 [10]	61.7 ± 2.1 [9]***	70 ± 3.2 [10]***	63 ± 3.5 [10]***	39.3 ± 2.4 [10]***	48 ± 2.4 [10]***	51.2 ± 2.9 [10]***	21.9 ± 1 [7]	34.9 ± 3.2 [8]	32.3 ± 2.2 [7]	23.9 ± 1.4 [8]
	F 18.8 ± 0.9 [10]	69.6 ± 4.2 [8]***	67.8 ± 3.5 [10]***	64.2 ± 4 [9]***	37.7 ± 2.2 [10]***	40.6 ± 1.6 [10]***	50.5 ± 3.3 [10]***	21.5 ± 1.6 [8]	29.8 ± 1.7 [8]	29.2 ± 1.9 [8]	22.8 ± 1.1 [8]
Lateral floor [μm]	M 13 ± 0.8 [9]	37.7 ± 1.9 [8]***	37.2 ± 1.6 [10]***	36.8 ± 2.3 [10]***	16.1 ± 0.8 [10]***	21.4 ± 1.3 [10]***	27.4 ± 2.6 [8]***	12 ± 0.8 [8]	25.1 ± 2.2 [8]	25.4 ± 1.3 [7]	11.7 ± 0.3 [8]
	F 10.5 ± 0.4 [10]	41.3 ± 3.4 [6]***	38.8 ± 2.7 [9]***	37.4 ± 1.7 [9]***	16.3 ± 0.7 [10]***	18.4 ± 1 [9]***	25.4 ± 1.8 [8]***	10.8 ± 1.3 [8]	22.1 ± 1.8 [8]	22.6 ± 1.1 [6]	11.6 ± 0.5 [8]
Arytenoid projections [μm]	M 19.8 ± 0.6 [10]	49 ± 3.1 [9]***	45.3 ± 2.8 [10]***	46 ± 1.5 [10]***	24.1 ± 0.8 [10]***	30.1 ± 1.2 [10]***	31 ± 1.3 [10]***	17.2 ± 0.5 [8]	31.9 ± 1.2 [8]	27.9 ± 1.6 [8]	18.2 ± 0.6 [8]
	F 18.2 ± 0.5 [10]	48.6 ± 2.6 [8]***	48.8 ± 1.3 [10]***	45.9 ± 1.7 [9]***	24.1 ± 0.8 [9]***	27.1 ± 0.6 [10]***	30.1 ± 1.2 [10]***	17.3 ± 1 [8]	27.9 ± 2 [8]	27.5 ± 1.1 [8]	18 ± 1 [8]

Results are presented as mean ± standard error. The sample size is in parentheses. Difference from sham group at 90d: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

**Table 8b**  
Statistically significant differences in larynx epithelial thickness at the end of the 90-day and post-inhalation periods.

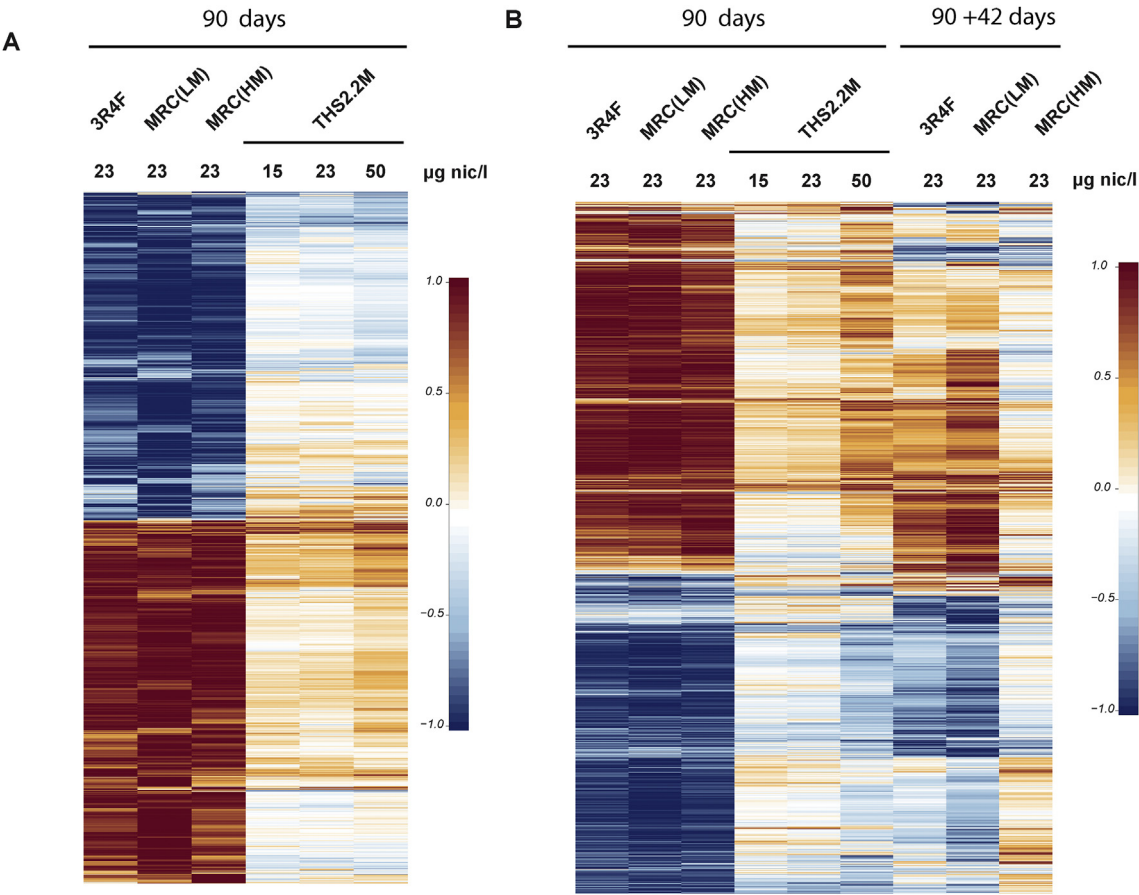
Localization	90d			90 + 42d			
	THS2.2M Medium vs. MRC(LM)	THS2.2M High vs. MRC(HM).	MRC (HM) vs. 3R4F	Sham R vs. Sham	3R4F R vs. 3R4F	MRC(HM) R vs. MRC(HM)	THS2.2M High R vs. THS2.2M High
Base of epiglottis	M ↓***	↓*	=	=	↓***	↓***	↓***
	F ↓***	↓**	=	=	↓***	↓***	↓***
Lateral floor	M ↓***	↓***	=	=	↓***	↓***	↓***
	F ↓***	↓***	=	=	↓***	↓***	↓***
Arytenoid projections	M ↓***	↓***	=	=	↓***	↓***	↓***
	F ↓***	↓***	=	=	↓***	↓***	↓***

Difference between groups: Significance: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Symbols, ↓ indicates lower response in group 1 than in 2; ↑ indicates higher response in group 1 than in 2; = indicates no significance. Abbreviations: M, male; F, female; LM, low menthol; HM, high menthol; R, recovery groups.

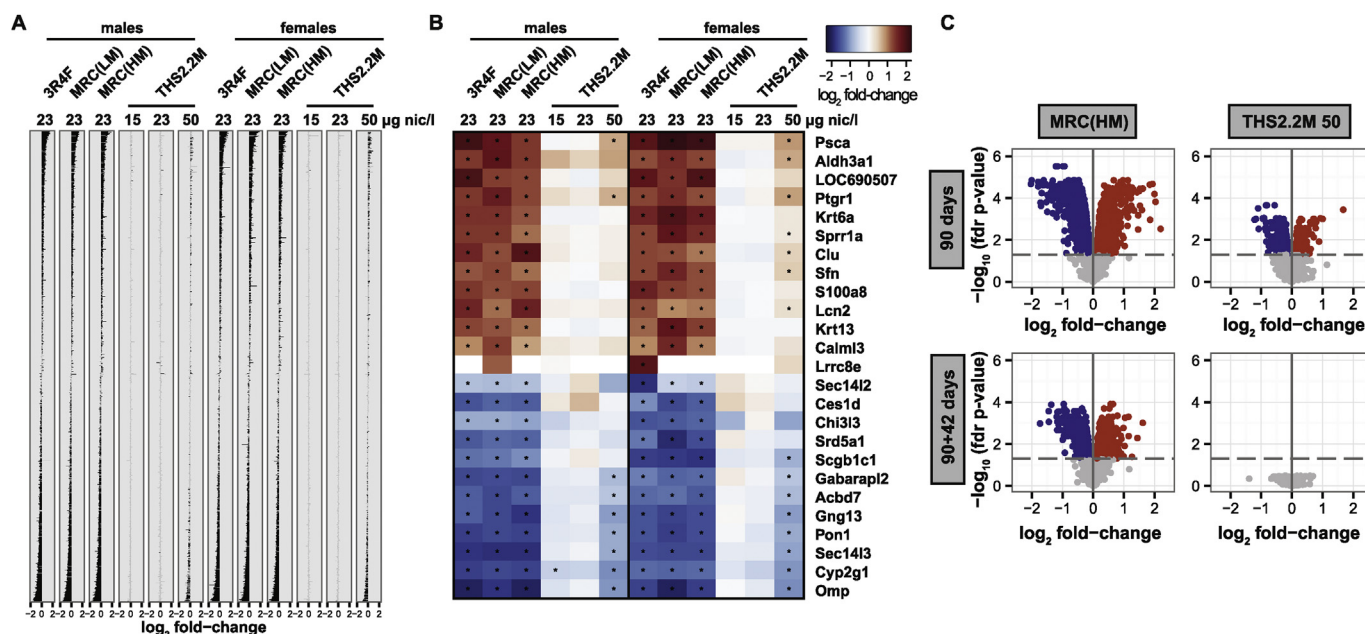
exposed to the reference cigarette controls was approximately one third of the number of DEGs found in the RNE. THS2.2M exposure resulted in fewer DEGs than reference cigarette exposure – even at the high exposure concentration of 50 µg/l nicotine for THS2.2M vs. 23 µg/l nicotine for the MRC. For example, in female rats, THS2.2M High induced 45 DEGs, whereas 1245, 2865, and 1972 DEGs were induced by 3R4F, MRC(LM), and MRC(HM), respectively. In addition, the gene expression fold-changes compared with sham-exposed rats were less pronounced in THS2.2M-exposed lung tissue than in reference cigarette-exposed lung tissue (Fig. 5). Forty-two days after THS2.2M High MA exposure, all genes except one reverted to sham-level expression.

The lung proteome was also clearly affected by exposure to MRCs (Fig. 6). The expression levels of only a few proteins in the

lung of female rats were statistically significantly changed in the THS2.2M High group, i.e. a maximum of 16 DEPs for THS2.2M High exposure vs. 168 DEPs for MRC(HM) exposure (numbers for iTRAQ® set 2, see Materials and Methods section). This global response pattern was further exemplified by the 25 proteins with the greatest overall response, which included upregulated immune response proteins (e.g. Lcn2), oxidative stress and xenobiotic response proteins (e.g. Nqo1, Sod2, Aldh3a1, and Akr1b8), and lipid metabolism proteins (e.g. Lipa and Fabp5) (see Discussion section and Kogel et al. (Kogel et al., 2016 (in this issue))). These proteins demonstrated much lower fold-changes for THS2.2M exposure than after exposure to the reference cigarettes (Fig. 6B). In addition, all proteins with changed expressions after THS2.2M High exposure recovered after 42 days post-exposure (Fig. 6C).



**Fig. 3.** Effects of MS and THS2.2M exposure on the nasal epithelium transcriptome. Heatmap representing the  $\log_2(\text{fold-change})/\max(\text{abs}(\log_2(\text{fold-change})))$  for all the genes for which at least one comparison induced a significant change. The color scaling is done based on the absolute values. A) male rats, B) female rats; LM, low menthol, HM, high menthol.



**Fig. 4.** Effects of MS and THS2.2M exposure on the nasal epithelium proteome. (A) Differential protein expression profiles after 90 days of exposure. Each vertical bar chart shows the protein fold-changes (length of bar) and their significance (black FDR-adjusted  $p < 0.05$ , grey FDR-adjusted  $p > 0.05$ , not significant) for one exposure group compared with sham exposed animals. Only proteins significantly differentially expressed in at least one group are shown. The proteins are ranked from top to bottom by the sum of their fold-changes, i.e. strongly and consistently upregulated proteins are at the top. (B) Differential expression heatmap for the top 25 (by absolute fold-change) differentially expressed proteins after 90 days of exposure. Each column represents an exposure group, each row a differentially expressed protein (FDR-adjusted  $p$ -value  $< 0.05$  for any group). The color of the matrix indicates the fold-change compared with sham exposed animals (see color key); \*\*\*\* indicates significant differential expression (FDR-adjusted  $p$ -value  $< 0.05$ ). (C) Evaluation of recovery effects. The volcano plots show the significance (y-axis,  $-\log_{10}$  FDR-adjusted  $p$ -value) and the effect size (x-axis,  $\log_2$  fold-change) compared with the respective sham groups. Significantly up- and downregulated proteins are indicated with red and blue dots, respectively. Note that two different isobaric-tag sets were defined for the 90-day exposure effect and the 90 + 42d recovery effect (see methods). With this, data for the same exposure group in panels A and B and panel C are not necessarily identical. LM, low menthol, HM, high menthol.

### 3.10. Discussion and conclusion

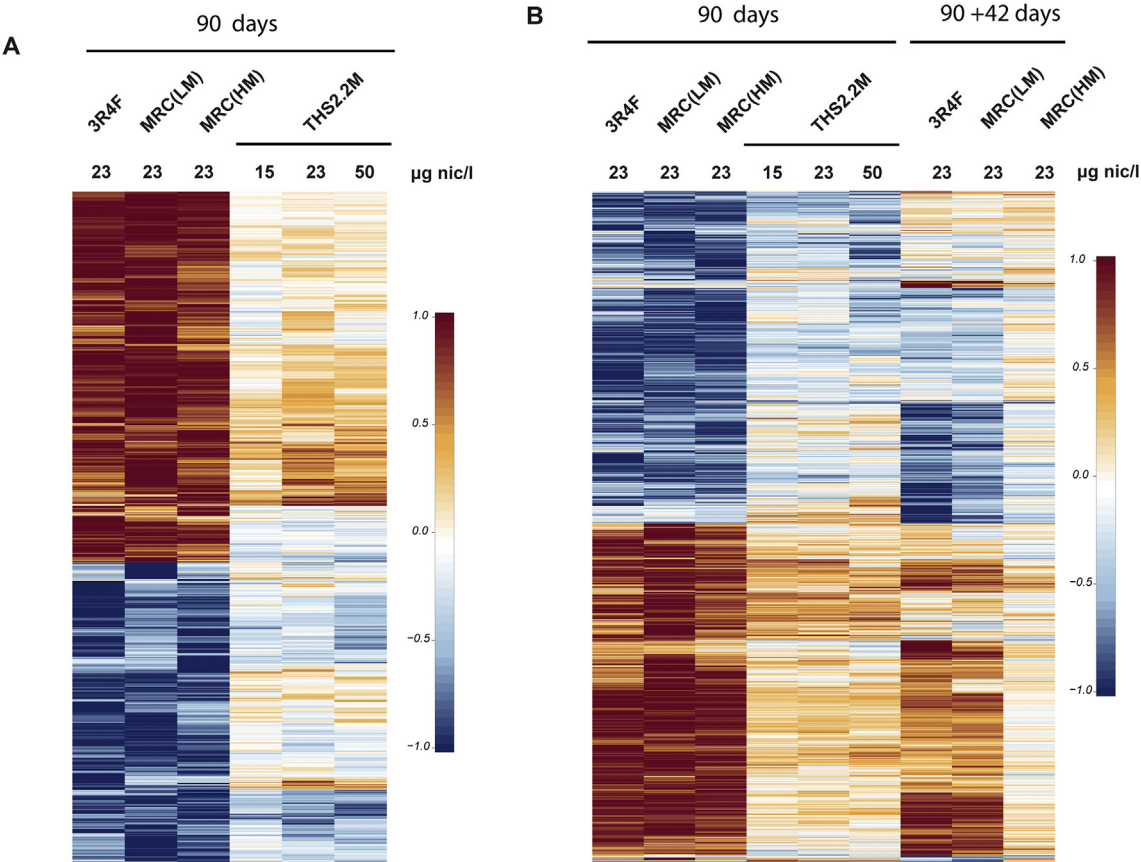
The main objective of the current study was to characterize toxicological effects of MA from THS2.2M, including pulmonary inflammation, and to compare them with those elicited by MS from MRC in a 90-day sub-chronic rat inhalation study. The current report provides a comprehensive assessment of all endpoints required by the OECD Test Guideline 413, with the additional strength of providing supplementary endpoints from molecular BALF analysis, gene expression and proteomic analysis from nasal epithelium and lung tissue, shedding light on the molecular basis for the observed changes.

Further molecular endpoints, are described in full detail in Kogel et al. (Kogel et al., 2016 (in this issue)). The used study design is however not suited for assessing behavioral aspects of the use of mentholated cigarettes.

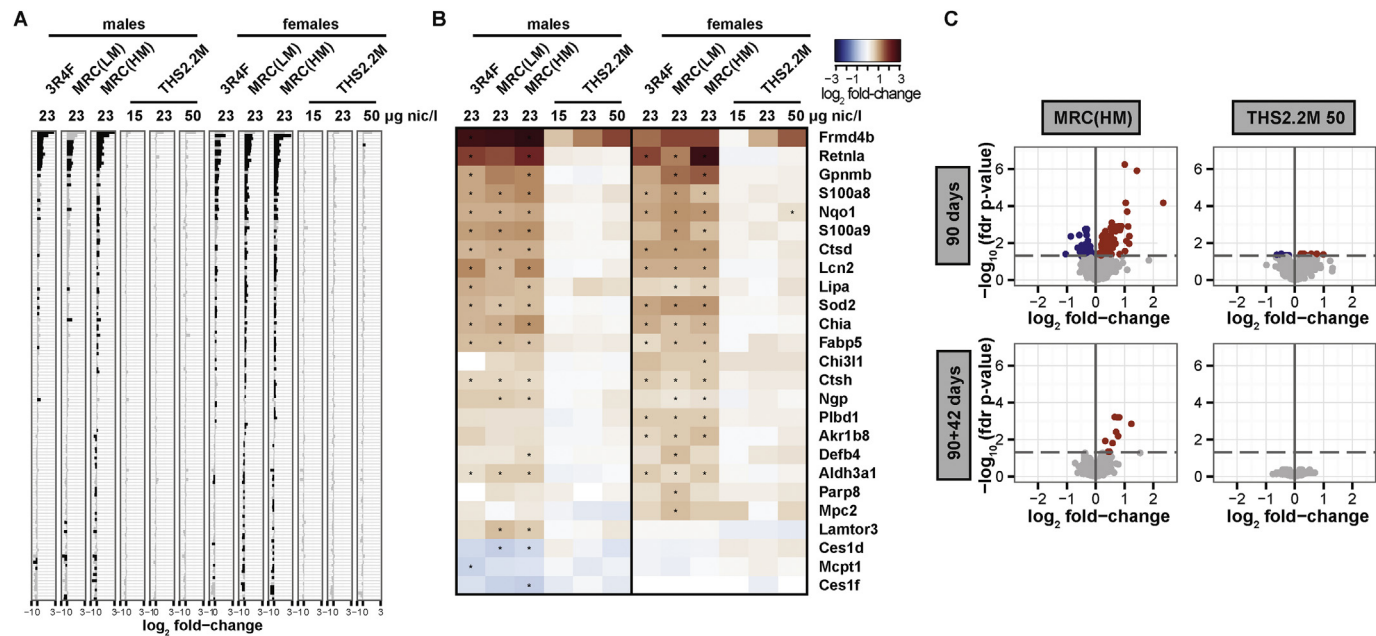
Test atmospheres were generated and diluted to target nicotine concentrations in MA and MS according to ISO 3308 (3308:2012, 2012). In contrast to other cigarette smoke inhalation studies where TPM was used as a dosing parameter (Fujimoto et al., 2015; Terpstra et al., 2003), nicotine concentration in the test atmosphere was used in this study for two reasons. First, TPM from cigarettes and aerosols from heat-not-burn tobacco products have completely different compositions. TPM from 3R4F consists of approximately 32% water, whereas TPM from THS2.2M consists of 68% water; nicotine levels in THS2.2M are 30% lower than those in 3R4F cigarettes (Roemer et al., 2004). As nicotine is present in the aerosols of both cigarettes and THS2.2M, it is used as the dosing parameter here. Second, exposure based on nicotine levels better reflects human smoking behavior. It has been shown that smokers adapt their smoking behavior, and to a lesser extent cigarette

consumption, to achieve a certain nicotine uptake when smoking cigarettes that contain less nicotine (Scherer and Lee, 2014).

Exposure concentrations were chosen according to OECD recommendations, i.e. that the exposure should be conducted at concentrations low enough to avoid causing marked pain and distress, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. The concentrations selected were based on the results of a concentration range-finding study, according to OECD guideline 413 (data not shown). For MRCs and 3R4F, the maximum exposure concentration was limited to a 23  $\mu\text{g}$  nicotine/l test atmosphere because of the high CO level which leads to COHb proportions of up to 26.5%. In contrast, the maximum THS2.2M exposure concentration was driven by tolerance to nicotine, because of the lower levels of CO and other constituent chemicals in the diluted aerosol. Based on the results of a range-finding study, the maximum nicotine concentration in the test atmosphere for THS2.2M was set at 50  $\mu\text{g/l}$ . For a rat of 300 g, with a theoretical respiratory minute volume of 200 ml and a daily exposure of 6 h, the calculated daily nicotine dose is  $(50 \mu\text{g/l} \times 0.2 \text{ l/min} \times 360 \text{ min})/0.3 \text{ kg} = 12 \text{ mg/kg}$ . This corresponds to a daily nicotine uptake of 720 mg for a human being of 60 kg, or the nicotine content of approximately 700 cigarettes per day, if 100% retention is considered. Using a more conservative approach based on body surface area as described in an FDA guidance for industry document (FDA, 2005), the Human Equivalent Doses (HED) for a rat exposed to 12 mg/kg corresponds to a human daily exposure of 12 mg/kg/6.2 or 1.94 mg/kg nicotine. For a human being of 60 kg, this corresponds to the uptake of 116 mg nicotine, or slightly more than 100 cigarettes per day. Nicotine serum analysis showed that exposure to 50  $\mu\text{g/l}$  nicotine for 6 h led to nicotine concentrations in blood of 762 ng/ml in male and



**Fig. 5.** Effects of MS and THS2.2M exposure on the lung transcriptome. Heatmap representing the  $\log_2(\text{fold-change})/\max(\text{abs}(\log_2(\text{fold-change})))$  for all the genes for which at least one comparison induced a significant change. The color scaling is done based on the absolute values. A) male rats, B) female rats.



**Fig. 6.** Effects of MS and THS2.2M exposure on the lung proteome. As in Fig. 4, but showing the exposure effects on the lung proteome.

1207 ng/ml in female rats, which is up to 40-fold and 60-fold higher, respectively, than nicotine levels found in humans (Benowitz et al., 1988; Hukkanen et al., 2005; Rose et al., 1999).

Target nicotine concentrations were confirmed in MA from THS2.2 and MS from MRCs and 3R4F, and particle size distribution measurements indicated that particles were equally respirable in

all groups.

In line with the analytical chemistry results, toxicologically-relevant smoke constituents were dramatically reduced in the THS2.2M MA compared with 3R4F (Schaller et al., 2016a (in this issue)). The concentrations of aldehydes measured in the diluted MA from THS2.2M were approximately only 10%–25% of the concentrations present in MS from MRCs; the concentrations of CO were reduced by up to 98%.

Exposure efficiency was confirmed by monitoring the levels of nicotine, cotinine, menthol, and M-I in blood plasma. A dose-dependent increase was observed, in correspondence with test atmosphere in THS2.2M MA- and MRC MS-exposed groups.

Female rats had approximately 50% higher nicotine levels in blood compared with male rats, while cotinine levels in blood and nicotine metabolites in urine were similar in the two sexes. This may indicate differences in metabolic rates in female rats compared with male rats, as suggested by Harrod et al. (2007). It is noteworthy that the quantities of recovered nicotine metabolites in the urine of the THS2.2M Medium group were higher than the quantities of nicotine metabolites in rats exposed to MS at the same test atmosphere nicotine exposure concentration, i.e. 23 µg/l. This is most probably due to the fact that MS is a more potent respiratory irritant than MA from THS2.2M due to the test atmosphere concentrations of carbonyls, and supported by the data reported here. The respiratory frequency, which is the main driver of the respiratory minute volume (but not measured in the current study), was lower in the MS-exposed groups than in sham and THS2.2M Medium-exposed groups. Therefore, it can be anticipated that the uptake of aerosol would be higher in the THS2.2M-exposed groups than in the MS groups. This is consistent with finding from previous studies (such as Terpstra et al., 2003), and has been described for a similar product (Wong et al., 2016 (in this issue)).

CO concentrations in the test atmospheres were consistent with the nicotine exposure levels when cigarettes were used, and there was a good correlation between the proportions of COHb and CO concentrations in the test atmospheres. The concentrations of CO in the test atmospheres of THS2.2M, however, were so low that COHb levels were only marginally higher than those observed in sham-exposed rats.

In line with reduced quantities of aldehydes and CO in MA from THS2.2M, the concentrations of representative urinary metabolites of toxicologically-relevant smoke constituents (acrolein, NNK, benzene, acrylonitrile) were much lower than those in MRC MS-exposed groups, confirming that rats inhaling MA from THS2.2M were exposed to lower quantities of these constituents.

The decrease in BW gain of male THS2.2M MA-exposed rats was statistically significantly less pronounced than in rats exposed to MRC after 90 days of exposure. This can be attributed to the low levels of toxicologically-relevant smoke constituents such as acrolein in the MA which contribute to the decreased BW gain observed in smoke-exposed rats (Feron et al., 1978). On the other hand, the BW of male rats exposed to MA was statistically significantly lower than that of sham-exposed male rats. This effect is most likely caused by the exposure to high concentrations of nicotine, and was also observed in a previously published nicotine inhalation study (Phillips et al., 2015a), as well as in other studies where nicotine was administered (Chowdhury, 1990; Ijomone et al., 2014; Seoane-Collazo et al., 2014). It has been shown that nicotine treatment increases brown adipose tissue thermogenesis and lower blood glucose levels, leading to an overall negative energy balance (Seoane-Collazo et al., 2014) which could explain blood glucose levels and lower BW in THS2.2M groups compared with sham. In addition, food consumption in THS 2.2M rats was higher than in sham-exposed rats, supporting this hypothesis.

No differences in BW development were observed in female

rats; that seems to be a typical observation in 90-day smoke exposure studies (Ayres et al., 2001; Renne et al., 2006).

The respiratory tract is the main target organ system for inhalation exposure. Therefore, the most significant differences between MS from MRC and the MA from THS2.2M were expected in the respiratory tract. Histopathological alterations induced by smoke from cigarettes are well described (Baker et al., 2004; Coggins et al., 1980, 1989; Fujimoto et al., 2015; Kogel et al., 2014; Roemer et al., 2004; Schramke et al., 2014; Terpstra et al., 2003; Vanscheeuwijck et al., 2002), and similar changes have been observed with MS exposure in the present study. The alterations caused by the MA of THS2.2M in respiratory tract organs were much less pronounced than in the rats exposed to MS from all reference cigarettes, and similar to those observed in rats exposed to THS2.2 (Wong et al., 2016 (in this issue)). To better understand the observed changes, nasal epithelium, lung, and BALF were investigated on the molecular level to complement standard toxicological endpoints.

Rats are obligate nose breathers, and therefore the nasal epithelia showed the most pronounced responses. Typical smoke-related phenotypic alterations were observed in the nose of MRC MS-exposed rats, including hyperplasia or squamous metaplasia of goblet cells, respiratory epithelium, or olfactory epithelium, cell atrophy, and degeneration of the glomerular layers in the olfactory bulb. In line with the significant changes in tissue architecture, gene expression analysis of the RNE showed that up to 8000 genes and 1300 proteins were differentially altered in MRC MS-exposed rats. As part of this response, gene set analysis identified several affected biological processes that could be linked to the adaptive changes in the epithelium, such as epithelial cell differentiation, extracellular structure organization and tissue remodeling, and cell proliferation and migration processes. In addition, the analysis of epithelial cell marker panels clearly corroborated the histopathological findings on the molecular level (for more details, see accompanying manuscript by Kogel et al. (2016 (in this issue))). In contrast, in the THS2.2M MA-exposed rats, the histopathological alterations described above were present only in the high THS2.2M group at nose level 1, and absent from the more distal nasal section levels. This difference was further confirmed on the molecular level, as the nasal epithelium of THS2.2M-exposed rats showed a much lower number of genes or proteins with significantly differential expression compared with MRC-exposed rats at the same nicotine concentration. More DEGs and DEPs were found in female than in male rats; this is consistent with the slightly higher severity of the histopathological findings in the female rats. A more detailed description of the molecular changes associated with the MS/MA exposure is provided in the accompanying article (Kogel et al., 2016 (in this issue)).

Following the 42-day post-inhalation recovery period, most of the observed nasal alterations were reversed or reduced, compared with the end of the inhalation period. One exception to this was the increase in goblet cell proliferation after a significant loss of goblet cells observed immediately following the 90-day exposure to MRC MS and THS2.2M High MA. This effect can be considered a positive physiological response to regenerate the cells which were lost previously, and is in line with observations previously described (Hotchkiss et al., 1995).

Changes observed in the larynx in response to MRC MS exposure consisted mainly of basal cell hyperplasia and squamous metaplasia with or without keratosis in the ventral epithelium overlying the submucosal gland, arytenoids, ventral pouch, and the vocal folds, with a high incidence and maximum severity scores. In contrast, a concentration-dependent increase in basal cell hyperplasia was noted in THS2.2M MA-exposed rats, with low incidences and severities only. Unlike histopathological changes in the nose,

which are mainly related to irritant volatile gas-phase constituents, alterations in the larynx are generally particle-driven. The fact that the histopathological findings in THS2.2M High-exposed rats were much less pronounced compared with MRC-exposed rats at the same TPM concentration indicates that the TPM compositions of THS2.2M and MRC are significantly different, and further supports our study design, whereby target nicotine concentrations, not TPM, are the dosing parameter.

Previous studies have shown that the typical changes, such as cell hyperplasia, result from a local inflammatory response (Lam, 1980; Vidic et al., 1974). Exposure to high concentrations of MS as used in this study resulted in the loss or necrosis of larynx epithelial cells. Epithelial regeneration was rapid, and followed by development of hyperplasia within days. Basal cell hyperplasia and squamous metaplasia can therefore be considered protective during repeated test atmosphere exposures (Lam, 1980).

In the lung, the morphological alterations of MRC MS-exposed rats were analogous to those reported in the literature for sub-chronic MS inhalation studies using reference cigarettes such as 1R4F or 2R4F (Coggins et al., 1980, 1989; Gaworski et al., 1997; Vanscheeuwijck et al., 2002). These changes include accumulation of macrophages in the alveolar lumen and macrophage nests in the alveolar epithelium, all indicative of inflammation. In addition to the reported higher number of free lung cells in the BALF from MS-exposed rats, cytokines and chemokines that play an important role in the recruitment and activation of inflammatory cells, such as macrophages, neutrophils, and lymphocytes, were also upregulated. Gene expression and proteomic analysis of the lung tissue showed that up to 2800 genes and 100 proteins were differentially expressed in the lung of MRC MS-exposed rats compared with sham-exposed rats. Analysis of this response at the level of biological networks, gene sets, and immune cell marker panels clearly supported the activation of a lung immune response involving macrophages and neutrophils by MS exposure (Kogel et al., 2016 (in this issue)). Some highly expressed proteins were identified, including S100A8, which forms a heterocomplex with S100A9, termed S100A8/A9 or calprotectin. Functions of S100A8/A9 includes pro-inflammatory activities (Miyasaki et al., 1993).

In contrast to MS exposure, no MA exposure-related inflammatory changes were detectable in the lung. Gene and protein profiling confirmed the overall much lower impact of MA on the molecular level – including the aforementioned immune response (Kogel et al., 2016 (in this issue)).

Finally, the absence of morphologic and inflammatory alterations was also in line with a lower relative and absolute lung weight compared with MS-exposed rats. The absence of changes in the lung of the rats exposed to THS2.2M is consistent with what has been reported for THS2.2 (Wong et al., 2016 (in this issue)).

In contrast to findings in the respiratory tract organs, systemic effects of test atmosphere exposure, such as higher activities of the enzymes alkaline phosphatase, lower cholesterol, triglycerides, and glucose concentrations, higher numbers of neutrophils in blood, and changes in the relative weight of certain organs were of the same magnitude in MRC- and THS2.2M-exposed rats.

These findings have been reported as MS inhalation findings (Boelsterli et al., 1984; Everds et al., 2013; Piade et al., 2014; Terpstra et al., 2003; Vanscheeuwijck et al., 2002), and considered to be most likely nicotine- and stress-related (Everds et al., 2013). However, more recent work has demonstrated that when rats are exposed to nicotine-containing aerosols, similar changes are observed (Phillips et al., 2015a), indicating that the mild toxicological changes observed upon THS2.2M exposure are most likely caused by nicotine.

A second MRC with higher menthol level in MS was included in the study to match the menthol level in THS2.2M High. This

provided the opportunity to investigate menthol-related effects in MS-exposed rats, analyzing the results from MRC(LM), MRC(HM), and 3R4F.

Only a few endpoints showed a statistically significant effect of menthol exposure in the MRC(HM) groups compared with 3R4F, including a lower total BALF cell count, lower number of neutrophils and macrophages, higher numbers of erythrocytes in male rat peripheral blood but lower in female rats, higher hematocrit in male rats but lower in female rats, and lower relative uterus weight in female rats. These findings are, however, not supported by the limited published data, or are not consistent with other results obtained in this study.

Gaworski et al. exposed rats to cigarettes spiked with 5000 ppm menthol in a 13-week nose-only smoke inhalation toxicity study (Gaworski et al., 1997). Analysis of the MS produced by the mentholated cigarettes indicated that approximately 2% of the TPM was menthol, whereas the highest menthol concentration in our study was about 0.5% in TPM. In another 13-week nose-only smoke inhalation toxicity study, in which cigarettes were spiked with 18,000 ppm menthol (Vanscheeuwijck et al., 2002), no menthol-related changes were reported that concerned numbers of erythrocytes and reticulocytes or uterus weight.

Exposure to MRC(HM) led in male rats to lower total BALF cell counts, as well as lower neutrophil and macrophage numbers compared with 3R4F, indicating a slightly lower inflammatory response. However, these results are not supported by other study endpoints, such as cytokine expression in BALF, pathological findings in the lung, or differential gene and protein expression in the lung.

Willis et al. reported that menthol attenuated respiratory irritation responses in mice to multiple cigarette smoke irritants, such as acrolein and acetic acid, concluding that menthol may facilitate smoke inhalation (Willis et al., 2011). Likewise, Ha et al., reported that L-menthol, through Transient Receptor Potential Melastatin 8 (TRPM8), is a strong suppressor of respiratory irritation responses in smoke-exposed mice, and increases blood cotinine (Ha et al., 2015). The potential reduction of the irritant by menthol is not confirmed by the data collected from exposure to MRC as compared to 3R4F, as there was no difference in the respiratory frequency or in the levels of COHb, nicotine, or cotinine in blood between 3R4F- and MRC-exposed groups, regardless of the menthol level in smoke. Similarly, Gaworski (Gaworski et al., 1997) found similar blood levels of nicotine and cotinine in rats exposed for 1 h/day for 90 days to smoke from mentholated and nonmentholated cigarettes, but lower COHb levels in rats exposed to smoke from mentholated cigarettes.

In conclusion, heating tobacco rather than burning it leads to a remarkable reduction in toxicologically-relevant constituents in the produced aerosols and the test atmospheres, resulting in notable and significantly smaller biological effects. As reported in previous studies for heated tobacco products (Ayres et al., 2001; Kogel et al., 2014; Roemer et al., 2004; Terpstra et al., 2003; Werley et al., 2008), exposure to MA from THS2.2M induced lower BW gain suppression, subdued histopathological changes in the respiratory tract organs, and decreased pulmonary inflammation compared with MRC MS. Furthermore, gene and protein expression investigations demonstrated that no new adverse effects of THS2.2M MA were present. Addition of menthol to heat-not-burn products results in effects that do not appear to differ from effects of non-mentholated similar products (Wong et al., 2016 (in this issue)). Study result meta-analysis may shed some additional light on the effects of menthol in THS products; for which, to achieve the best possible outcome for such a comparison, a single pathologist should read both studies, and in a group designation-blinded manner.

In addition, and as evidenced by the similarity of the results between 3R4F-exposed animals and those exposed to MS from cigarettes with two added concentrations of menthol, menthol displays no apparent effects when added to cigarettes.

### Conflict of interest statement

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

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### Transparency document

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