

Chapter 3

Regional variability in cigarette smoke-induced lung emphysema in mice

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in preparation

Abstract

To validate a mouse model of cigarette smoke-induced emphysema it was studied whether the superior regions of the lung, like in humans, are predisposed to the disorder. Female C57BL/6J mice were exposed nose-only to the diluted smoke of 3 simultaneously burning cigarettes, two times every weekday, for 6 months. Airway responsiveness to increasing concentrations of inhaled nebulised methacholine was determined monthly, 24 hours after exposure. Lungs were sampled, fixed and embedded in acrylic copolymer 24 hours after the last exposure. Sections were cut from the left lung in the frontal plane along the dorsoventral axis at 400 μm intervals and stained with haematoxylin & eosin. Per section two digitised images were captured each in the caudal, medial and cranial region to assess mean linear intercept (Lm) as a measure of smoke-induced lung damage. Smokers showed an average increase in Lm of 10% compared to control animals ($p < 0.05$). Plotting Lm on the dorsoventral axis showed the most damage at the most ventral section depth of 400 μm , whereas damage on the caudocranial axis was most pronounced in the cranial region of the lung. When ΔLm was plotted simultaneously on the caudocranial and dorsoventral axes, striking regional differences were observed. Generally, values were highest in the cranial region of the lungs, where they followed a bell-shaped curve in the frontal plane, with differences of up to 20% between smokers and controls. Both in the medial and caudal regions, the distribution almost mirrored that seen in the cranial region. The caudal region showed a nadir of -1% in the middle of the frontal plane and differences of about 7% ventrally and dorsally of it. Lung lavage fluid of smokers and controls contained > 97% macrophages and no neutrophils, while total cell numbers were not increased in smokers. Also interstitial inflammation was minimal. Smoking did not cause significant cholinergic airway hyperreactivity at any of the time points measured. Since damage was preferentially seen in the upper part of the lung, we believe this mouse model to be a good approximation of smoke-induced emphysema in humans.

Introduction

Chronic inhalation of cigarette smoke is generally considered to be the main etiological factor in the development of lung emphysema. The disease is characterised by dilatation and destruction of the alveolar walls and respiratory bronchioles, and progressive airflow limitation [93]. Emphysema can be divided in to pure centrilobular, pure panacinar, or mixed type. In centrilobular emphysema, the respiratory bronchioles are predominantly involved, while in panacinar the whole acinus is uniformly damaged. As the disease progresses, a mixture of centrilobular and panacinar patterns can be observed in the lung [22]. Centrilobular emphysema, generally associated with cigarette smoking, is the most common form [94]. One hallmark of centrilobular emphysema in man is a predisposition of the upper parts of the lungs towards the disease [22, 23]. So far, there are several theories to explain the pathology of emphysema, of which the protease/antiprotease [8] and oxidant/antioxidant [80] theories have received the most attention. Because most patients are only seen by a doctor when the disease has become symptomatic, most studies in humans so far concern relatively advanced emphysema.

To study the etiology of this debilitating condition, rodent models of cigarette smoke-induced emphysema are being increasingly employed, since they offer the ability to track the progress of the disease in time. However, rodent airways differ considerably from those of humans. This difference has been cited as a reason why models employing rodents may not be able to reproduce the effects seen in humans [95]. Since most studies in rodents that use the mean linear intercept (Lm) as a measure of extent of emphysema either do not report on the spatial location where lung samples are taken, or sample at random locations throughout the lung [81, 96-99], we wanted to document regional differences in smoke-induced lung damage in the mouse. To this end, smoke-induced damage was assessed throughout the left lung, by measuring Lm at a range of depths and heights.

Materials and methods

Animals

Seven-week-old female C57BL/6J mice were purchased from Charles River (Maastricht, The Netherlands) and housed under controlled conditions, in macrolon cages under filter-tops, in the local animal facility. Animals received water and feed (Hope Farms, Woerden, The Netherlands) *ad libitum*. All experiments were conducted in accordance with the Animal Care Committee of Utrecht University.

Study outline

At the start of the experiment, mice were randomly divided into two groups: smoke ($n=12$) and control ($n=12$). Of each group, 6 mice were marked on the tail with water-resistant marker bi-weekly for identification in the airway reactivity measurements. Animal weights were monitored weekly throughout the experiment.

Smoke exposure

Cigarette smoke was generated by the burning of commercially available Lucky Strike™ cigarettes without filter (British-American Tobacco, Groningen, The Netherlands), using the TE-10z smoking machine (Teague Enterprises, Davis, CA) which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35 ml puff volume drawn for 2 seconds once per minute). A set number of cigarettes are automatically loaded, ignited, smoked and the butts ejected and extinguished during 1 run of 9 minutes. Before starting smoke exposure, mice were accustomed to the exposure tubes by gradually prolonging their stay in the tubes over the course of two weeks. Then, smoke exposure was started with 1×1 cigarette, increasing the dosage to 2×3 cigarettes within two weeks. Mice were exposed nose-only to the diluted mainstream and sidestream smoke of 3 simultaneously burning cigarettes, twice every weekday for 6 months using the In-Tox 24-port nose-only exposure chamber (In-Tox Products Inc., Albuquerque, NM). Control mice underwent the same procedures, but were allowed to breathe room air throughout the whole exposure period.

Determination of smoke parameters

Carbon monoxide content of smoke inside the exposure chamber was measured by sampling with the Monoxor II CO analyzer (Bacharach Inc., New Kensington, PA, USA) at 15 seconds intervals during 2 runs. Total Suspended Particulates (TSP) concentration of smoke inside the exposure chamber was determined gravimetrically. The weight of Emfab™ filters (Pall Corporation, East Hills, NY, USA) was determined before and after smoke had been drawn through during 1 run. Dividing weight increase by smoke volume yielded TSP concentration.

Determination of smoke exposure parameters

Plasma nicotine and cotinine levels were determined in a separate group of mice (n=5). Animals went through the training and first two weeks of the smoke exposure regimen (see below). Immediately following the last exposure, mice were sacrificed. Following injection of an overdose of sodium pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands, 1 µl/g body weight, i.p.) blood was obtained by cardiac puncture, mixed with heparin and centrifuged to yield plasma. Plasma levels of nicotine and cotinine were measured as described before [100], at the Laboratory of Analytical Chemistry of the National Institute for Public Health and the Environment.

Lung preparation and volume measurement

Twenty-four hours after the last smoke exposure, animals were sacrificed. Following injection of an overdose of sodium pentobarbital (Nembutal™, Ceva Santé Animale, Naaldwijk, The Netherlands, 1 µl/g body weight, i.p.) a cannula was inserted into the trachea and fixed with a ligature. Lungs and heart were removed *en bloc* and the lungs were inflated via the cannula with Carnoy's fixative (ethanol:chlorophorm:acetic acid 60:30:10) (Sigma-Aldrich, Zwijndrecht, The Netherlands) at a fluid pressure of 25 cm for 5 min. Fixation was continued for at least 4 hours before rinsing the lungs in 3 changes of 100% ethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands). After removal of heart, trachea and oesophagus, the volume of the fixed lungs was measured by fluid displacement. Then, the left lung was embedded in acrylic copolymer consisting of 75 parts of butyl methacrylate and 25 parts of methyl methacrylate (Sigma-Aldrich, Zwijndrecht, The Netherlands). After polymerisation, sections of 3 µm were cut at 400, 800, 1200, 1600 and 2000 µm depth in the dorsal-ventral plane, mounted on cover slips, de-plasticised using acetone and stained with haematoxylin & eosin.

Morphometry

The extent of smoke-induced alveolar wall damage was determined morphometrically, using digitised images of representative fields not containing vessels or bronchi. Grids of 8 horizontal and 10 vertical lines were superimposed on the images, and the number of intersections with alveolar walls was counted using the Image Pro™ 4.0 software package and a custom-written macro.

Per section two digitised images were captured each in the cranial, medial and caudal region, at 5× magnification. By dividing total grid length by the number of intersections, the mean linear intercept (Lm) was calculated. In total, 30 fields were used per lung to calculate the average Lm. For each combination of section-depth and height, a regional ΔLm was calculated by subtracting Lm of controls from that of smokers. ΔLm was expressed as the relative difference from the control Lm (set at 100%).

Measurement of intraluminal inflammation

Twenty-four hours after the last smoke exposure, bronchoalveolar lavage was performed. After sodium pentobarbital anaesthesia, lungs were lavaged with 1 ml of PBS containing 5% BSA and 4 times with 1 ml saline. Lung lavage cells of each mouse were collected after centrifugation, pooled and resuspended in 150 µl saline. For differential cell counts, cytopsin preparations were made and stained with Diff-Quick™ (Dade A.G., Düringen, Switzerland). Total number of cells was determined and cells were differentiated by standard morphology.

Histopathological assessment

Interstitial inflammation and morphological changes in lung structures were assessed by light microscopy using the same sections of lung tissue that had been used for morphometry.

Determination of airway responsiveness

Airway responsiveness to increasing concentrations of inhaled nebulised methacholine was determined 24 hours after smoke exposure in weeks 3, 7, 11, 16, 20 and 24 following the start of exposure. Measurements were performed in conscious, free-moving mice using whole body plethysmography (Buxco, EMKA, Paris, France) as described before [101]. Airway responsiveness was expressed as enhanced pause (Penh).

Statistics

Values were compared between groups using an unpaired, two-tailed Student's *t*-test for equal variance in Excel 2000 for Windows or a two-way ANOVA followed by Bonferroni's correction for multiple comparisons in GraphPad Prism 4.03 for Windows. A confidence level of $p < 0.05$ was considered significant.

Results

Smoke and smoke exposure parameters

TSP concentration of smoke inside the exposure chamber was 798 mg/m^3 (average over 2 runs). Carbon monoxide concentration averaged $> 1639 \pm 571 \text{ ppm}$, with levels above the upper limit of detection (2000 ppm) during half of the exposure period. Animals that had been exposed at these levels for 2 weeks had plasma levels of $378 \pm 117 \text{ ng/ml}$ nicotine and $552 \pm 225 \text{ ng/ml}$ cotinine, immediately following exposure.

Effects of smoke exposure on body weights

Body weights did not differ between room air breathing controls and smoke-exposed animals in the first week. But from then on, until the end of the experiment, smokers weighed significantly less than room air breathing control mice. The difference in average body weights increased with time, but did not exceed 10% (Figure 1).

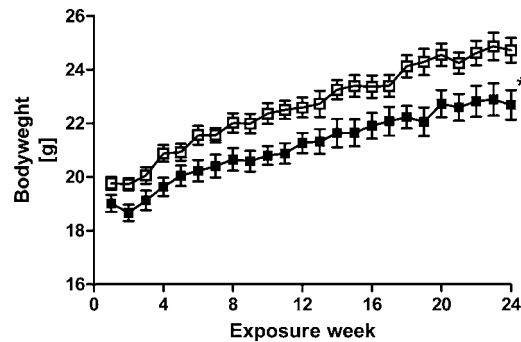


Figure 1: Body weights during 6 months of smoke exposure. Mice were exposed to cigarette smoke (■) or room air (□) for 5 days a week during 6 months. Every Monday morning before smoke exposure body weight was recorded. Values are means \pm SEM of 12 animals per group. *: $p < 0.05$ compared to room-air breathing controls.

Effects of smoke exposure on Lm

Compared to room-air breathing controls, average Lm throughout the lungs was significantly increased (+10%) in smoke-exposed mice (Figure 2). When Lm was plotted as a function of depth, along the dorsoventral axis (Figure 3), or of height, along the caudocranial axis (Figure 4), values for room air breathing control animals were similar throughout the lung. In smokers however, Lm increased in the cranial direction reaching significance in the cranial region. Along the dorsoventral axis no clear trend could be discerned, but at the most ventral section depth of $400 \mu\text{m}$, the Lm of smokers is significantly higher compared to the Lm of room air breathing controls.

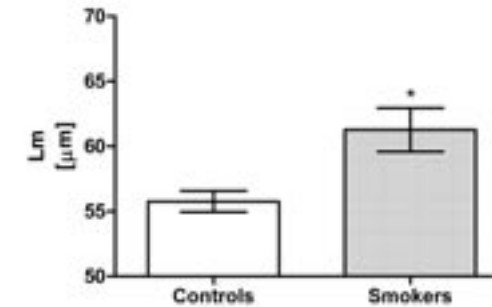


Figure 2: Lung damage, as judged by Lm, after 6 months of smoke exposure. Mice were exposed to cigarette smoke or room air, for 5 days a week during 6 months. Lungs were processed 24 h after last exposure, to prepare sections of the left lobe in the frontal plane at 5 different depths, for measurement of the Lm at 2 sites each in the caudal, medial and cranial region of each section. Bars represent means \pm SEM of 30 fields per animal and 3 animals per group. *: $p < 0.05$ compared to room-air breathing controls.

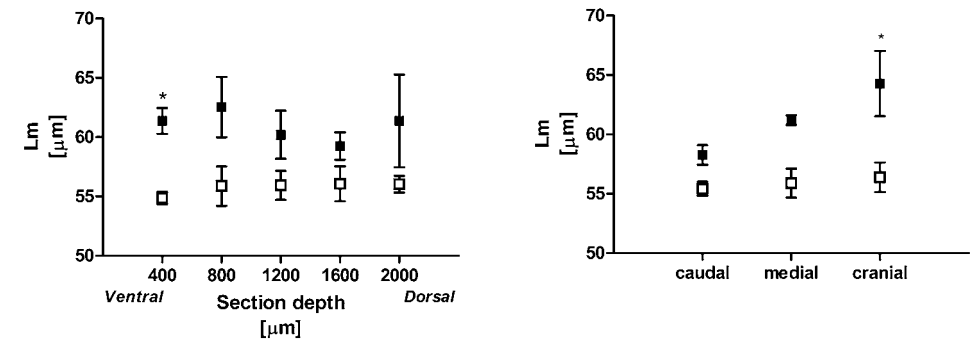


Figure 3: Lung damage at varying depths in the lung, as judged by Lm, after 6 months of cigarette smoke exposure. Mice were exposed to cigarette smoke (■) or room air (□) for 5 days a week during 6 months. Lungs were processed 24 h after last exposure, to prepare sections of the left lobe in the frontal plane at 5 different depths, for measurement of the Lm at 2 sites each in the caudal, medial and cranial region of each section. Values are means \pm SEM of 6 fields per animal and 3 animals per group. *: $p < 0.05$ compared to room-air breathing controls at same depth.

Figure 4: Lung damage at varying heights in the lung, as judged by Lm, after 6 months of cigarette smoke exposure. Mice were exposed to cigarette smoke (■) or room air (□) for 5 days a week during 6 months. Lungs were processed 24 h after last exposure, to prepare sections of the left lobe in the frontal plane at 5 different depths, for measurement of the Lm at 2 sites each in the caudal, medial and cranial region of each section. Values are means \pm SEM of 5 fields per animal and 3 animals per group. *: $p < 0.05$ compared to room air breathing controls at same height.

Plotting ΔLm (i.e. the relative increase in Lm caused by smoke exposure) simultaneously along the caudocranial and dorsoventral axes, revealed striking regional differences (Figure 5). Generally, values were highest in the cranial region of the lungs, where they followed a bell-shaped curve along the dorsoventral axis, with differences of up to +20% between smokers and controls. Both in the medial and caudal regions, ΔLm values formed almost a mirror image of those seen in the cranial region. The ΔLm in the caudal region showed a nadir of -1% in the middle of the dorsoventral axis and increases of about 7% ventrally and dorsally of it.

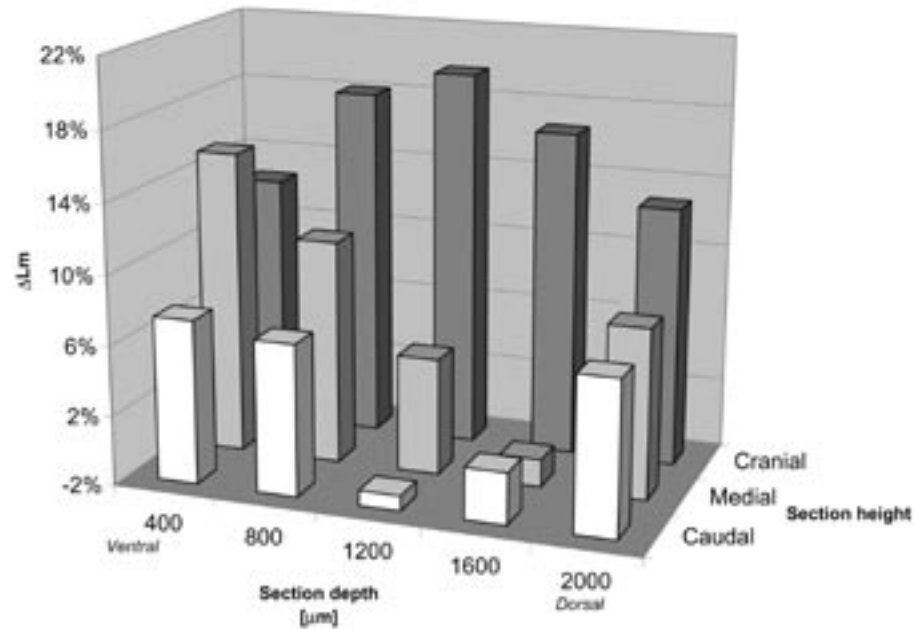


Figure 5: Lung damage at varying heights and depths in the lung, as judged by ΔLm , after 6 months of cigarette smoke exposure. Mice were exposed to cigarette smoke or room air, for 5 days a week during 6 months. Lungs were processed 24 h after last exposure, to prepare sections of the left lobe in the frontal plane at 5 different depths, for measurement of the Lm at 2 sites each in the caudal, medial and cranial region of each section. Bars represent ΔLm , which was calculated by subtracting average Lm of smokers from that of controls (3 animals per group) for each spatial location. ΔLm is expressed as percentage change from control Lm.

Effects of smoke exposure on lung volume and bronchoalveolar lavage cell numbers

Lung volume, corrected for body weight, was significantly increased following smoke exposure (Figure 6). Total cell numbers in bronchoalveolar lavage fluid were similar in smokers and room air breathing controls (Figure 7). The lavaged cells consisted almost exclusively of macrophages and 2-3% lymphocytes, while neutrophils were absent, both in smokers and room air breathing controls (data not shown).

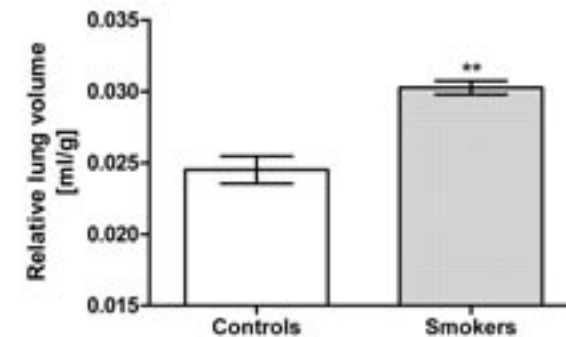


Figure 6: Organ volume of mouse lungs, after 6 months of cigarette smoke exposure. Mice were exposed to cigarette smoke or room air, for 5 days a week during 6 months. Lungs were fixed 24 h after the last exposure, and organ volume was determined by fluid displacement. To correct for differences in animal size, organ volumes were divided by body weight. Bars represent means \pm SEM of 3-4 animals per group. **: $p < 0.005$ compared to room-air breathing controls.

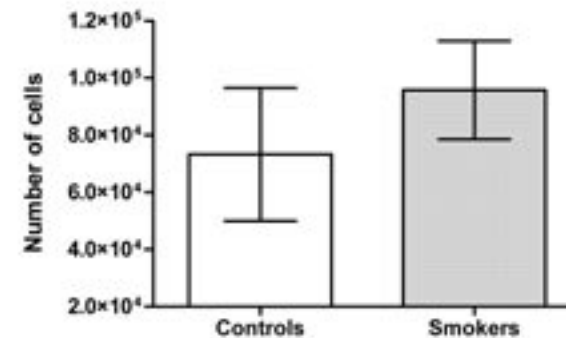


Figure 7: Total number of cells in bronchoalveolar lavage fluid, after 6 months of cigarette smoke exposure. Mice were exposed to cigarette smoke or room air for 5 days a week during 6 months. Lungs were lavaged with PBS containing 0.5% BSA and protease inhibitor cocktail, 24 hours after the last smoke exposure. Cells were counted using a Bürker-Türk chamber, after staining with Türk solution. Bars represent means \pm SEM of 3-4 animals per group.

Effects of smoke exposure on lung histopathology

Interstitial inflammation was minimal, if any, in smokers and absent in room air breathing controls. In smokers, the distal part of the terminal bronchiole and the alveolar ducts were distended compared to room-air breathing controls. Combined with the lack of destruction of alveolar septa this indicates a predominantly centrilobular emphysema.

Effects of smoke exposure on airway reactivity

Airway reactivity to inhaled aerosols of increasing concentrations of methacholine did not differ between smoke-exposed mice and room air breathing controls at all points of time measured (data not shown).

Discussion

In this study we exposed female C57BL/6J mice to cigarette smoke to induce lung emphysema, as seen in 10-20% of chronic smokers. The smoke to which the mice were exposed contained on average more suspended particulate matter compared with previously published rodent smoke exposure studies [102-104] namely 897 mg/m³ versus 75 to 657 mg/m³. Moreover, the CO level was at least 2.5 times higher than reported in a study exposing C57BL/6J mice to smoke with a concentration of 600 mg/m³ of suspended particulate matter, giving rise to a CO/TSP ratio of 1.82 vs. 1.06. The relatively high smoke exposure was also apparent from the nicotine and cotinine plasma levels directly after smoke exposure, since these were about 1.5 to 2 times higher, respectively, compared to that same study [102]. At the given exposure conditions, the mice developed emphysematous lesions after 6 months of smoke exposure, as judged by the average Lm assessed at 30 sites in the left lung, when compared to room air-exposed controls. The smoke-induced damage was not equally distributed over the lung. Whereas the Lm values in the lungs of room air-exposed mice were similar at all sites measured and showed no trends along the craniocaudal or dorsoventral axes, the Lm of the smoke-exposed mice revealed that damage was clearly most severe in the cranial region and decreased in the caudal direction. In this respect, the smoke-induced lung emphysema in our mice resembles that of human smoking-related lung emphysema, reportedly being most extensive in the apical region [105-107] although a homogeneous distribution was recently reported [108]. Along the dorsoventral axis, the most extensive damage was seen most ventrally, decreasing in the dorsal direction, to increase again at the most dorsal level. Regional patterns in damage became more clear when Δ Lm was plotted simultaneously on the dorsoventral and caudocranial axis. The damage in the cranial region of the mouse lungs appeared maximal in the middle when plotted along the dorsoventral axis. In the medial and caudal region, however, lung damage appeared lowest in the central portion of the lungs and to increase in the dorsal, and particularly, the ventral direction. These results correlate well with the few studies that looked at regional deposition patterns of cigarette smoke particles in lungs. A study in rats showed an increase in deposition when moving cranially along the caudocranial axis [103], and in humans cranial deposition was greater than expected from the distribution of resting ventilation [109]. Leukocyte counts in lung lavage fluid were similar for smoke-exposed and control animals, in contrast to the generally reported increase in smoking humans and rodents [110-113]. Although a cigarette smoke-induced inflammatory process is generally considered to be at the root of the majority of pathologic lesions associated with chronic obstructive pulmonary disease (COPD) [114], a lack of correlation between the intensity of severity of inflammation, presence of intraluminal macrophages and emphysema score in upper and lower lobes of human smokers has been reported as well [115]. Moreover, there are data that point to an anti-inflammatory effect of cigarette smoke [116], and more specifically of the nicotine contained in the smoke, as reviewed in [117].

Also for CO anti-inflammatory effects have been observed [31, 32]. Since our smoke-exposed mice were exposed to smoke with a high level of CO, and had high plasma levels of nicotine, these could have caused a suppression of the inflammatory response. The lack of hyperreactivity we observed at all time points measured is not surprising, considering that Cosio et al. previously showed that airway reactivity in patients with centrilobular emphysema is dependent on the degree of cellular inflammation [21], and we observed centrilobular emphysema in our smoke-exposed C57BL/6J mice, as did Takubo et al. [118], but no evidence of inflammation. An additional explanation for this last finding is the fact that, in the naïve state, C57BL/6J mice are hyporeactive to smooth muscle constrictors, including methacholine, compared to other mouse strains [92]. Consequently, it seems likely that this baseline hyporeactivity will also translate in a smaller likelihood of developing hyperreactivity following exposure to cigarette smoke. Summarising, after exposing female C57BL/6J mice to cigarette smoke for 6 months, we found a significant increase in Lm, indicative of smoke-induced lung damage. This increase is highest in the ventral and cranial regions. The observed predisposition of damage to the upper parts of the lung is similar to that seen in emphysema in smoking humans. In view of the observed spatial differences it is recommended to always sample at similar 3D-coordinates within the lungs of cigarette smoke-treated animals, when performing morphometric studies.

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