Respiratory mechanics and lung structure in rodent models of emphysema

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List of papers included in this thesis:

I. Z. Hantos, Á. Adamicza, T. Jánosi, **M.V. Szabari**, J. Tolnai and B. Suki: **Lung volumes and respiratory mechanics in elastase-induced emphysema in mice.** *Journal of Applied Physiology*, 105: 1864–1872 (2008), impact factor: 3.658

II. J. Tolnai, **M.V. Szabari**, G. Albu, B.A. Maár, H. Parameswaran, E. Bartolák-Suki, B. Suki and Z. Hantos: **Functional and morphological assessment of early impairment of airway function in a rat model of emphysema.** *Journal of Applied Physiology*, 112: 1932–1939 (2012), impact factor: 3.484

III. **M.V. Szabari**, J. Tolnai, B.A. Maár, H. Parameswaran, E. Bartolák-Suki, B. Suki and Z. Hantos: **Lung structure and function relation in elastase-treated rats: a long-term follow up study.** *Respiratory Physiology & Neurobiology*, 215: 13–19 (2015), impact factor: 1.971

IV. **M.V. Szabari**, H. Parameswaran, S. Sato, Z. Hantos, E. Bartolák-Suki and B. Suki: **Acute mechanical forces cause deterioration in lung structure and function in elastase-induced emphysema.** *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 303: L567–L574 (2012), impact factor: 3.523

List of papers related to the subject of this thesis:

V. B. Suki, S. Sato, H. Parameswaran, **M.V. Szabari**, A. Takahashi and E. Bartolák-Suki: **Emphysema and mechanical stress-induced lung remodeling.** *Physiology*, 28: 404–413 (2013), impact factor: 5.645

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third major cause of death worldwide after ischemic heart disease and stroke. COPD is a type of obstructive lung disease characterized by airway narrowing, dyspnea, cough and sputum production. It is an umbrella term for two disorders: chronic bronchitis and emphysema.

Emphysema is a long-term disease, with progressive structural changes in the lung in which the alveolar walls are destroyed. This disorder is characterized clinically by a loss of elastic recoil and significant hyperexpansion of the lungs, as a result of permanent destruction of the parenchymal tissue structure. A variety of mechanisms, including a protease–antiprotease imbalance, inflammation, abnormal extracellular matrix remodeling and mechanical forces, have been proposed to be involved in its pathogenesis. From the point of view of respiratory mechanics, the decrease in the retraction capacity due to the parenchymal destruction modifies the elastic balance between the chest wall and the lungs, and this leads to an elevation of the end-expiratory lung volume, without the involvement of dynamic hyperinflation resulting from the expiratory flow limitation characteristic of COPD. Additionally, parenchymal destruction weakens the tethering forces transmitted to the airway wall by the alveolar attachments and the rest of the parenchymal structure from the pleural space. While it appears plausible that this change in the elastic forces leads to lower transbronchial pressures and a consequent decrease in the airway lumen, it has remained unclear whether or not the weaker tethering *per se* can lead to the obstructive conditions in COPD. The limited clinical knowledge available on the early phase of COPD can be explained by the symptomless period of the disease, when there is no airway involvement and hence no decline in lung function: FEV1 (forced expiratory volume in the first second, the standard measure of the COPD severity) and the airway conductance are not affected. This early phase of the disease is therefore one of the most challenging aspects in the understanding of the pathomechanisms of COPD, highlighting both the importance of a sensitive and detailed assessment of the respiratory mechanics and the need for appropriate animal model studies on the pathomechanisms.

These aspects are closely linked. While the alterations in lung volumes are a solid landmark of emphysema, the assessment of the airway function is overwhelmingly based on forced spirometry. The most detailed measurement of respiratory mechanics, the low-

frequency forced oscillation technique (LFOT)[1], permits a separation of the airway and tissue mechanics[2]. However, LFOT measurements assume apneic conditions and hence have been employed in merely a few studies on ventilated COPD patients. Also, while a few investigations on rodent emphysema models have used LFOT, changes in lung volumes have been assessed with rudimentary methods at most, and the relationships between lung volume and mechanics have been left unaddressed.

Rodent models of PPE (porcine pancreatic elastase)-induced emphysema are commonly used for the rapid generation of an emphysema-like airspace enlargement that can be studied easily within a month; the investigation period is usually limited to 2-4 weeks. In contrast, the human disease is a slowly progressing pathological condition with long-term effects on the patient, which usually starts around midlife. To promote the understanding of the chronic aspects of human emphysema, follow-up measurements may be made in rodents in order to reveal how changes in the tissue structure impact on the lung function on a long-term basis. Since repeated intubations during the anesthesia of small animals pose technical challenges, most rodent studies have been limited to cross-sectional analyses of the disease progression. Additionally, while elastolytic, parenchymal destruction has been investigated widely in PPE-induced rodent animal models, less attention has been paid to the possible link between the decreased tethering forces and the airway involvement, a potential limitation of the PPE-induced emphysema model.

COPD patients are usually characterized by increased lung volumes and expiratory resistance, resulting in dynamic hyperinflation and increased overall lung strain and stretch. From time to time, the acute exacerbation of COPD (AECOPD) can occur, with periods of severe productive cough and shortness of breath; these flare-ups increase the mechanical stress and its destructive effect on the lung. AECOPD worsens the patient's respiratory symptoms, which exceed the normal day-to-day variations and can necessitate to changes in medication. Moreover, exacerbations are becoming increasingly more frequent as the disease progresses. However, a detailed understanding of how exacerbation augments the irreversible functional changes is lacking. Since mechanical forces have been shown to be the governing mechanism of tissue destruction in emphysema, the combination of mechanical and chemical effects may constitute a clinically more relevant model of the human disease.

AIMS

The overall goal of this PhD thesis was to study the structural and functional changes during the development and the progression of PPE-induced emphysema in rat and mice, using the LFOT for the detailed characterization of lung mechanics, body plethysmography for the measurement of lung volumes, and various visualization techniques to investigate the lung structure. A further aim was also to create a partial model of COPD exacerbation involving only mechanical stress to the lungs. The specific aims were as follows.

Study I: The purpose of this study was to map the mechanical properties of the airways and respiratory tissues as functions of the lung volume and transrespiratory pressure in mice treated with PPE. We tracked the resistive and elastic parameters of the respiratory system during slow inspiratory-expiratory maneuvers.

Study II: The aim of this study was to examine in further detail whether PPE-treatment induces pure parenchymal destruction or whether it also involves airway abnormalities.

Study III: Since emphysema is a slowly progressing, chronic pathological condition, we studied the structural and functional alterations in a PPE-induced rat model of emphysema in follow-up experiments starting at 3 days after the PPE treatment and lasting for 105 days.

Study IV: The goal here was to create an "emphysema exacerbation" model involving a combination of elastolytic and mechanical interventions. We hypothesized that, while infections are the triggers in the AECOPD, the mechanical forces during the exacerbation phase play a key role in the tissue rupture. We tested this hypothesis by superimposing deep inspirations (DIs) during mechanical ventilation on the remodeled parenchyma at different stages of PPE-induced emphysema in mice.

METHODS

ANIMAL PREPARATION: 1. Relationship between lung volumes and respiratory mechanics (Study I)

CBA/Ca mice were anesthetized and intubated. The elastase-treated animals (*group E, N=14*) received PPE (0.3-0.6 international unit (IU) via intratracheal instillation. The control animals (*group C, N=14*) were treated with saline. Three weeks thereafter, the mice were anesthetized, tracheotomized and placed in a body plethysmograph and ventilated with a small-animal respirator (rate (f)=160 min^{-1}, tidal volume (V_T)=0.25 ml).

2. Functional and morphological assessment of early impairment of the airway function (Study II)

Sprague-Dawley rats were anesthetized and intubated. Eight of them were treated with an intratracheal instillation of 50 IU PPE and 6 rats were treated with saline. The animals were anesthetized again, tracheotomized 6 weeks after the treatment. The rats were then placed in a plethysmograph and ventilated (f=80 min^{-1}, V_T=8 $ml.kg^{-1}$). At the end of the study, the lungs were removed for histological evaluation.

3. Long-term changes in lung structure and function in emphysema (Study III)

Sprague-Dawley rats were anesthetized, intubated and placed into a plethysmograph and ventilated (f=75.min^{-}1, V_T =7-8 $ml.kg^{-1}$). After the lung function measurements, the rats were treated with an intratracheal instillation of PPE (50 IU) (PPE-treated group: T, N=21) or saline (control group: C, N=19), and were allowed to recover. The same animals were intubated and measured again at 3, 10, 21 and 105 days after treatment.

4. The effects of mechanical forces on lung structure and function (Study IV)

C57BL/6 mice were anesthetized and treated via oropharyngeal aspiration with PPE (6 IU). Fifteen animals served as intact controls. At 2 days, 1 week and 3 weeks after the treament, animals were anesthetized, tracheotomized and placed into a plethysmograph and ventilated with the FlexiVent (V_T=8 $ml.kg^{-1}$, f=240 min^{-1}) for 1 hour with or without deep inspiration (DI). DI is defined as an inflation up to an airway pressure of 35 cmH_2O 2 times per minute. The following groups were formed: Control DI (0 DI, N=7), Control no-DI (0 no-DI, N=8), 2 days treated DI (2d DI, N=8), 2 days treated no-DI (2d no-DI, N=8), 1 week treated DI (1w DI, N=7), 1 week treated no-DI (1w no-DI, N=8), 3 weeks treated DI (3w DI, N=7), and 3 weeks treated no-DI (3w no-DI, N=6).

MEASUREMENT OF LUNG VOLUMES

In *Study I*, the thoracic gas volume (TGV) at end-expiration at zero transrespiratory pressure (TGV0), i.e. at functional residual capacity (FRC), was measured with the plethysmographic technique, modified for the measurement of TGV in anesthetized mice that have a weak or no respiratory effort. Briefly, 2-3 s after the respirator was stopped, the tracheal cannula was occluded and the intercostal muscles were stimulated with electrodes. The plethysmograph pressure (Pbox) and tracheal pressure (Ptr) were measured. TGV0 was estimated from the Pbox vs. Ptr relationship on the basis of Boyle's principle. Following the

measurement of TGV0, the tracheal cannula was connected to a loudspeaker through a wave tube. After a 5-s pause at end-expiration, Pbox was lowered until -20 cmH_2O was reached, and Pbox was then allowed to return to 0 cmH_2O. P_1 and P_2, the inlet and outlet lateral pressures of the wave tube were measured. Inflation volume V(t) was obtained by integration of the flow (V') determined as $V' = P_2/R_{wt}$, where R_{wt} is the direct current resistance of the wave tube. TGV as a function of time was obtained as TGV(t) =TGV0+V(t). Transrespiratory pressure, P_{rs} was calculated as Ptr - Pbox. FRC was measured with a similar arrangement of the plethysmographic technique in *Study II-IV* during spontaneous breathing efforts.

In *Study II* and *Study III*, the inspiratory capacity (IC) and expiratory reserve volume (ERV), respectively, were defined as the volume changes between -35 and 20 cmH_2O Pbox. The total lung capacity (TLC) and residual volume (RV) were calculated as TLC = FRC + IC and RV = FRC – ERV.

MEASUREMENT OF LUNG MECHANICS

Tracking of lung mechanics: Total respiratory system impedance (Zrs) was measured as the load impedance of the wave tube by using a pseudorandom signal (4-38 Hz) during slow inflation-deflation maneuvers in *Study I*. The mean Zrs was computed for the first 5 s of oscillation before the inflation started (to estimate Zrs at TGV0) and for every successive 0.5-s interval during the maneuver. Each Zrs spectrum was fitted by a model containing a Newtonian (airway) resistance (R_N), an inertance (I), and a constant-phase tissue unit characterized by the coefficients of damping (G) and elastance (H). Hysteresivity (η) was calculated as $\eta = G/H$. In the rats in *Study II* and *Study III*, the same method was used to measure lung mechanics with a pseudorandom signal (0.5–16 Hz).

Lung mechanics measurements with the FlexiVent in Study IV: The FlexiVent is a computer-controlled precision piston pump that can perform mechanical ventilation and measurements of respiratory mechanics. The low-frequency respiratory impedance (2-19 Hz) was measured at 3 cmH_2O PEEP (positive end-expiratory pressure). Respiratory compliance ($C=1/H$), R_N and G were calculated by fitting the constant-phase model to input impedance.

LUNG MORPHOMETRY

Epifluorescence microscopy: In *Study I* the collapsed lungs were slowly inflated with low-melt 4% agarose. Epifluorescence microscopy was performed to investigate the alveolar morphometry. Following thresholding, the alveolar or terminal airspace cross-sectional areas

(A_{alv}) were measured by using image-processing software, and an equivalent alveolar diameter (D_{alv}) was calculated as $D_{alv}=(4A_{alv}/\pi)^{1/2}$. From the values of A_{alv}, an area-weighted mean equivalent diameter (D_2) was computed[3]. The D_2 is very useful in that it is not sensitive to shape and it can characterize the alveolar structure better than other morphological indices in the case of heterogeneity.

Traditional histology: Formaldehyde fixation and hematoxylin-eosin (H&E) staining were common methods in *Studies II, III and IV*. *Study II*: The digitized images were then segmented into individual airspaces. D_{alv} was then calculated as in *Study I*. From the readings of bronchial perimeter (Pb), the diameter of an equivalent circular cross-section (D_b) was computed. The mean airway wall thickness (T_w) and the number of septal attachments (N_S) were determined. Septal attachment density was calculated as N_S/Pb. *Study III:* Samples were stained with H&E, and modified Movat's and Masson's methods were used to visualize elastin and collagen, respectively. D_{alv}, D_2, the alveolar wall thickness (Wt) were calculated. *Study IV:* D_{alv}, D_2 were calculated. Wt was measured, and the attachment density (Ad) was determined by dividing the number of septal walls attached to a small airway by the outer perimeter of the airway wall. Collagen and elastin fibers were visualized with modified Masson's trichrome and Verhoeff's methods, respectively. The total number of end tips, as a measure of the number of septal ruptures (N_{sr}) per image per tissue fraction was measured.

Orthogonal polarization spectral (OPS) imaging: The lungs and heart were removed *en bloc* in *Study III*. The lung surface was scanned, while the transpulmonary pressure was 25 cmH_2O. Custom-made software was used to identify the septal borders from pictures of the video recording, and D_{alv} was calculated.

RESULTS

1. Relationship between lung volumes and respiratory mechanics (Study I.)

Morphometric evaluation of the lung slices revealed a significant enlargement of the alveolar airspace sizes in the PPE-treated animals: the D_{alv} increased from 46.5±13.8 μm (control mice) to 70.3±34.2 μm. Additionally, the variance of the D_{alv} was also significantly higher in the treated mice. There was a significant increase in D_2, from 55 μm to 107 μm. The distribution of the D_{alv} in the treated mice exhibited a significantly longer tail.

PPE treatment resulted in changes in the *lung volumes*: relative to the control, TGV0 and TGV at a P_{rs} of 20 cmH_2O (TGV20) increased by 52% and 45%, respectively. The inspiratory

volume (TGV20-TGV0) increased by 37% in the treated animals, which was accompanied by a 27% decrease in the chord tissue elastance between 0 and 20 cmH_2O.

The mechanical parameters in the PPE-treated mice exhibited marked changes: as compared with the control, the decreases in H were 57% and 27%, respectively, at P_{rs} values of 0 and 20 cmH_2O. The values of η were also altered by the PPE treatment, with increases more marked at 0 cmH_2O (55%) than at 20 cmH_2O (12%). The mean R_N data did not differ between the groups at any value of P_{rs}.

2. Functional and morphological assessment of early impairment of the airway function (Study II)

Lung volumes: FRC and RV were higher in the PPE group as compared with the controls (by 38% and 53%, respectively).

Respiratory mechanics: G and H were smaller in the PPE-treated rats than in the controls (76% and 62%). Because of the larger decrease in H than in G in the treated rats, η was elevated (129%). There was no difference in R_N between the groups.

Alveolar and bronchial morphometry: In the PPE group, the distribution of D_{alv} was shifted to higher values and the median of D_{alv} was higher in the treated group (68.4 μm vs. 61.8 μm). The distributions of D_b were not different between the groups. The attachment density N_S/Pb was mildly lower in the PPE group (median 0.0175 vs. 0.0189 μm^{-1}). The mean value of T_w was higher in the PPE group than in the controls (12.4 vs. 10.8 μm).

Elastin and collagen density in the bronchial wall: There was no difference between the D_b of the airways of the control and PPE-treated animals for which the elastin and collagen density was evaluated. The mean and SD (standard deviation) of the elastin grayscale representing the average and the spatial variability, respectively of the elastin density within the bronchial wall, were not different in the groups. Interestingly, the collagen density and its intra-bronchial SD were increased by 12% and 17% in the treated group. Furthermore, the inter-airway variance of the collagen density was substantially higher (67%) in the treated animals.

3. Long-term changes in lung structure and function in emphysema (Study III)

Lung volumes: During the 105 days of the study, FRC, TLC and RV increased with time, and also as a result of growth, but the increase was faster in the treated animals: while group C showed an increase of 75% in FRC, and 56% in RV from the beginning to the end of the

study, the corresponding changes in group T were 194% and 244%, respectively. There were differences between the control and treated rats throughout the study: for FRC and RV, a difference already appeared on day 3, whereas there was no difference in TLC until day 21. The increases in all lung volumes reflected the combined effects of PPE and time.

Respiratory mechanics: There was no difference between R_N in the control and the treated animals at any time point. Differences between the groups in H and η developed from day 3.

Morphological evaluations: The H&E-stained samples from the 105 days after the PPE treatment-group exhibited large areas of tissue destruction, with enlarged airspaces and septal wall breaks, and the distribution of the D_{alv} values included diameters larger than 300 μm. The D_{alv} difference between the control and the treated animals became and remained significant from day 21 up to day 105. D_2 already revealed a difference in alveolar size between the two groups by day 3. The highest values of Wt were found on day 3 in both groups, which was followed by decreases on day 21 and a reversal on day 105. The treated groups exhibited significantly higher values of Wt than the controls at all time points. The *ex vivo* images obtained with the OPS technique revealed enlargements of the subpleural airspaces in the PPE group at 105 day as compared with those in the control animals, which resulted in a median increasing from 66.3 to 78.4 μm.

Inverse relationship between H and D_{alv} were found, which reveals a strong structure-function correlation in the control and the treated rats.

4. The effects of mechanical forces on lung structure and function (Study IV)

Lung volumes: PPE increased FRC before ventilation, with mean values of 0.25 ml in the control, 0d group, and 0.39 ml in the PPE-treated 21d group. Independently of time, the DIs increased FRC. Following a 1-h ventilation, FRC was 0.32 ml and 0.19 ml in the 0d DI group and the 0d no-DI group, respectively. FRC increased from 0.37 ml in the 21d no-DI group to 0.45 ml in the 21d DI group.

Lung mechanics: PPE also increased C with time before ventilation, with values of 0.031 ml/cmH_2O and 0.055 ml/cmH_2O in the 0d and 21d groups, respectively. Similarly to FRC, a strong effect of the DIs was seen on C at each time point. R_N gradually decreased with time, the difference reaching significance at 21d. η significantly differed between the DI and no-DI groups and increased with time, except from 0d to 2d and from 7d to 21d.

Morphological evaluations: The PPE treatment increased D_{alv} from 29.6 μm in the 0d group to 34.9 μm at 21 days after treatment. D_2, revealed much more pronounced differences between the groups in time. However, neither of these lumped structural indices indicated a difference between the DI and no-DI groups at any time point. To explore the possibility that the DIs caused structural change in some preferential airspace size, the alveoli were pooled from all animals and grouped into small, medium and large airspaces according to their D_{alv} values. For the smallest airspaces, differences were found between the DI and no-DI groups at 0d and 21d and at 7d. For the medium-sized airspaces, there were differences at 21d and 0d, whereas for the largest airspaces, D_{alv} was different only at 0d. Even though the percentage differences are small, it is interesting that, in the control animals, the no-DI group had a larger D_{alv}, whereas in the treated animals, the DI groups always had a larger D_{alv}.

Wt increased by 47%, from 2.5 μm at 0d to 3.4 μm at 21d. Moreover, at 21d, the DIs induced a 13% elevation in Wt relative to the no-DI group. N_{sr} was significantly larger in the DI group than in the no-DI group, with values of 165 and 134, respectively. Ad was 11% lower in the DI group than in the no-DI group on 21d.

DISCUSSION

We studied the early and long-term effects of elastolytic intervention on the pulmonary structure and function in rodents. We observed emphysematous changes in lung function: increased lung volumes (FRC, TLC and RV) and altered lung tissue mechanics due to parenchymal destruction with no airway function impairment. Traditional histology, epifluorescence microscopy and OPS imaging confirmed alveolar enlargement, increased alveolar and airway wall thickness, decreased alveolar attachment density around the airways and increased heterogeneity in the PPE-treated lungs. Elastin and collagen contents, visualized with specific staining methods, and confirmed ECM (extracellular matrix) remodeling in the follow-up after the elastolytic insult. We also investigated the effects of acute mechanical stress (DIs) on the structure and mechanics of emphysematous lungs at different stages of emphysema, thereby creating a partial model of COPD exacerbation.

1. Lung volumes and respiratory mechanics in PPE-induced emphysema

In accordance with the other emphysema model studies that we performed, the results of the most comprehensive functional investigation (Study I) indicated that 1) the lung volumes (FRC, RV and TLC) were increased in the PPE-induced animals 21 days after the treatment,

similarly as seen in patients with emphysema; 2) the tissue elastance was lower in the emphysematous animals at all lung volumes and P_{rs} levels, except in a narrow medium range of P_{rs}; and 3) the airway resistance (R_N) did not differ between the control and the PPE-treated groups at any P_{rs} level.

Longitudinal functional assessment: The data from our follow-up animal study (*Study III*) reflected the progression of emphysema in the PPE-treated rats and the somatic growth in all animals up to day 105. These changes affected the lung volumes and the tissue mechanics. The lung volume increases in the PPE-treated groups resembles the typical feature of the human disease. Our finding that the differences between the two groups increased further after day 21 of the study (the recommended investigation time for the PPE model) indicates the need for the research of late-stage emphysema. The changes in the mechanical properties of the respiratory tissues with time paralleled those in the lung volume. While natural growth leads to a decrease in elastance, PPE induced an accelerated decrease in H. Interestingly, the difference in G between the control and the treated group did not develop as quickly and to such an extent as that in H; as a result of this, η increased abruptly in the treated group.

Airway function: Uniformly in *Studies I-II-III*, LFOT did not reveal any difference in R_N between the control and treated groups. The lack of difference in R_N indicates that the airway function is not affected by the loss of parenchymal tethering forces, as expected from the fall in elastance, and some observations on human emphysema also indicate that an airspace enlargement and an airflow limitation do not necessarily combine. In this context, the changes following PPE treatment may characterize an initial or mild degree of emphysema, where the loss of alveolar attachments (which must have accompanied the increase in alveolar size and lung volumes) is compensated by new elastic equilibria within the lung parenchyma and between the lung and chest wall, so that the patency of the airways is retained. There were mild decreases in R_N, which were similar in the control and the PPE-treated groups, and can be attributed to body (and lung) growth with age. It is important to point out that there was no difference in R_N between the control and treated animals at the actual operating lung volumes.

2. Lung morphometry in PPE-induced emphysema

The results of histology from *Studies I-III* revealed that 1) the measures of alveolar size were significantly higher and the distribution of the alveolar diameters was shifted toward higher values in the PPE group; 2) the alveolar attachment density was lower, and the alveolar

and bronchial wall thicknesses were higher in emphysematous animals; 3) the collagen content per unit wall thickness of the bronchial wall was higher and more heterogeneous in the treated than in the control animals; and 4) the elastin component of the ECM increased, while the collagen component decreased in PPE-induced emphysema.

Alveolar structure: The increases in D_{alv} and D_2 in the treated animals are consistent with the morphological features of emphysema, i.e. the destructured wall of the airspaces and the enlarged alveoli. The wall thickness of the alveoli in the histological images was higher in the PPE-treated animals due to tissue remodeling. Previous studies[4] also indicated an elevated alveolar septal wall thickness in PPE-treated mice, although there was no difference between the control and the PPE-treated animals. In rats, the same results were found[5], where the mean thickness was 2.1 and 2.9 μm in the control and the emphysematous animals, respectively, 38 days after the PPE treatment. Increased septal wall thickness was likewise observed in human emphysema within moderate lesions[6], though, the wall thickness was twice that in the healthy controls (5.2 vs. 2.2 μm), indicating that the PPE model may mimic early emphysema.

Subpleural alveoli: The OPS technique was designed to image the human microcirculation [7], using reflected light from the surface of a solid organ such as the brain, skin or liver. Although this imaging technique can be used *in vivo*, we chose the *ex vivo* condition so as to avoid the confounding effects of the beating heart, which allowed us to investigate the subpleural alveoli in their physiological condition. The distribution of the subpleural alveoli exhibited a slight shift in the treated group due to septal wall ruptures, which lead to enlarged airspaces. Thus, this technique is capable of detecting structural differences between the normal and the emphysematous lung. The distribution of the alveoli from the traditional histological samples revealed a similar scenario, but the histogram demonstrated a longer tail, which may indicate that the subpleural alveoli are smaller in the OPS images in both the control and the PPE-treated animals than in the histological samples, where the entire plane of the section was investigated. This difference may be explained by the cohesive effect of the pleura, which can mitigate any increase in alveolar size in both groups. While we acknowledge the possible size differences between the subpleural and interior alveolar populations, we argue that the OPS images display an intact structure free from irregular airspaces (such as ductal structures resulting in a long tail in the size distributions toward

large values in the histological samples). The OPS alveolar size distribution in the control rats is normal, while it becomes asymmetrical in the treated ones.

Airway structure: In *Study II* we quantified the parenchymal tethering by measuring the number of attachments per unit airway wall perimeter, and found that the treatment reduced the average attachment density by 7%. The elastin density of the walls measured from the histological images in a band of fixed width around the bronchi was similar in the two groups. Since the wall thickness in the treated animals was larger by 15%, it is likely that the total elastin content was also higher in the bronchial walls of the treated rats, implying cellular remodeling of the airway walls. However, collagen also contributes to stiffness, especially at higher transmural pressures. The increased collagen density of the wall and the elevated intra-wall heterogeneity suggest disordered remodeling following PPE treatment.

Structure–function relationship: An important finding of *Study III* is a strong structure–function relation that links the lung elasticity (parameter H) to the mean alveolar diameter (D_{alv}). The mechanism behind this relation is probably involves rupture of the septal walls. There are two consequences of a septal wall rupture: 1) a coalescence of two neighboring alveoli and increase of the alveolar diameters; and 2) a decrease in the number of walls carrying the load in the parenchyma, which in turn decreases the lung stiffness. Moreover, a single rupture transmits the tension in the wall to its neighbors, which increases the likelihood of a subsequent rupture. A sufficient number of ruptures results in a macroscopically measurable decrease in H. Thus, through a common mechanism, the rupture of the septal walls, the alveolar structure and the parenchymal stiffness become intimately linked.

3. Mechanical effects on chemically weakened lung tissue

The acute increases in mild mechanical forces achieved by the frequent DIs in *Study IV* resulted in 1) large changes in the functional properties of the respiratory system, such as FRC and compliance, and 2) structural alterations in the parenchyma and airways, seen on day 21 after treatment. The latter suggests that, when the ECM of the lung is sufficiently weakened, mechanical forces are able to induce irreversible changes in the lung structure.

Lung volumes and respiratory mechanics: FRC, C and η increased with time as a result of the development of emphysema after PPE treatment. The DIs increased FRC and C in the control and treated mice. To interpret these results, we first note that anesthetized mice in the supine position easily derecruit large regions of the lung. A single DI recruits some regions,

which tend to collapse again with time. Further DI-activated mechanisms that may contribute to the elevation in C in this experimental setting are the ruptures of fibers in the septal wall and the large stretches triggering surfactant release. Thus, the changes in C in the control mice are due to recruitment and surfactant secretion, whereas the contribution to the increase in C in the treated lung made by the mechanical failure.

Alveolar structure: The progression of emphysema was accompanied by increases in D_{alv} and D_2. In a more refined analysis, we pooled D_{alv} from all animals and grouped them into small, medium and large size categories. Differences were detected between the no-DI and DI groups. In the control animals, the DIs decreased the sizes of the airspaces in all 3 airspace size categories. This may be related to the finding by Namati et al.[8] that the alveoli became smaller at end-inspiration, due to recruitment in the lung. They suggested that at higher inflation pressures (similar to those in our DI protocol), secondary (daughter) alveoli pop open and inflate via the primary (mother) alveoli through the pores of Kohn. In contrast, in our treated animals, the DIs generated larger D_{alv} in the two smallest size categories, suggesting that at the same fixation pressure the DIs definitely increased the airspace diameters. These results imply that the septal walls of some small and medium-sized airspaces became softer or the septal walls actually ruptured during ventilation with the DIs. The microstructural changes caused by the DIs were most pronounced on day 21. Indeed, while the septal wall thickness Wt steadily increased with time, the DIs caused a significant increase only on day 21. We note that the increase in Wt is associated with an increase in C in the DI group, most probably as a consequence of capillary rupture, leakage and edema.

Changes in the ECM: On day 21, analysis revealed that N_{sr} was larger in the DI group than in the no-DI group. Ad around the airways increased due to the DIs. In the control animals, we did not see any evidence of ruptures, and hence the increase in FRC was caused by pure recruitment. Additionally, on day 21, the DI-induced increases in N_{sr} and Ad resulted in larger D_{alv} and a proportion of the increases in FRC and C must therefore have resulted from ruptures. These results prove that our model reproduces some features of AECOPD, with irreversible changes in structure and function. We also found wall thickening, fiber and septal wall rupture only on day 21; a threshold in ECM remodeling may therefore exist below which acute increases in mechanical forces do not seem to induce irreversible changes.

SUMMARY AND CONCLUSIONS

- We have demonstrated (*Studies I-III*) that PPE-induced destruction of the lung parenchyma is accompanied by elevated respiratory compliance and lung volumes, and hence modeling the changes in human emphysema; however, the decrease in the tethering forces, together with the observed subtle remodeling of the bronchial wall, does not result in increased R_N. This model may correspond to an early symptomless phase of COPD and suggests that, in addition to the altered elastic equilibrium between the lungs and the chest wall, the involvement of other factors is necessary for airway obstruction.
- We followed the progression of emphysema induced by a single dose of PPE (*Study III*). During the 105-day follow-up, there were increases in lung volumes and pulmonary compliance, without any impairment in the airway function, further raising the necessity of additional pathomechanisms that target the bronchi. This study is the first to employ OPS imaging to visualize the subpleural alveoli *ex vivo*, and its results complement those of conventional morphometry by avoiding the usual artifacts of the latter. At the level of the ECM, we observed that elastin remodeling was delayed as compared with collagen remodeling. Overall, a *strong structure–function relationship* has been identified between alveolar size and lung compliance, which is apparently driven by septal wall ruptures.
- We have demonstrated that exacerbation-like irreversible changes in structure and function can be induced by acute increases in mechanical forces in the absence of bacterial or viral infections, as long as the lung ECM is sufficiently weak (*Study IV*). Thus, it is possible that, the increases in mechanical stresses on the emphysematous tissue associated with cough and hyperinflation are also important contributors to the irreversible changes in AECOPD in humans. While we acknowledge that infection is a likely cause of the generation of mechanical stresses and is ultimately needed for impairment of the airway function, our study also suggests that the clinical treatment of AECOPD should minimize mechanical stresses, for instance by e.g. attenuating coughing or carefully choosing ventilation parameters if the patient needs to be ventilated.

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