

# Methacholine and Ovalbumin Challenges Assessed by Forced Oscillations and Synchrotron Lung Imaging

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**Rationale:** Methacholine (Mch) is routinely used to assess bronchial hyperreactivity; however, little is known about the differences in the lung response pattern between this provocation and that observed with ovalbumin (Ova) after allergic sensitization.

**Objectives:** To compare (1) the central versus peripheral effects of Mch and Ova within the lung by combining measurements of airway and tissue mechanics with synchrotron radiation (SR) imaging, and (2) to assess the extent to which mechanical and imaging parameters are correlated.

**Methods:** We used the low-frequency forced oscillation technique and SR imaging in control ( $n = 12$ ) and ovalbumin-sensitized ( $n = 13$ ) rabbits, at baseline, during intravenous Mch infusion ( $2.5 \mu\text{g/kg/min}$ ,  $5.0 \mu\text{g/kg/min}$ , or  $10.0 \mu\text{g/kg/min}$ ), after recovery from Mch, and after intravenous Ova injection ( $2.0 \text{ mg}$ ). We compared intravenous Mch challenge with inhaled Mch ( $125 \text{ mg/ml}$ ,  $90 \text{ s}$ ) in a separate group of control animals ( $n = 5$ ).

**Measurements and Main Results:** Airway conductance and tissue elastance were measured by low-frequency forced oscillation technique. The central airway cross-sectional area, the ventilated alveolar area, and the heterogeneity of specific ventilation were quantified by SR imaging. Mch infusion induced constriction predominantly in the central airways, whereas Ova provocation affected mainly the peripheral airways, leading to severe ventilation heterogeneities in sensitized animals. Mch inhalation affected both conducting and peripheral airways. The correlations between airway conductance and central airway cross-sectional area ( $R = 0.71$ ) and between tissue elastance and ventilated alveolar area ( $R = -0.72$ ) were strong.

**Conclusions:** The pattern of lung response caused by intravenous Mch and Ova are fundamentally different. Although inhaled Mch induces a heterogeneous lung response similar to that observed with intravenous allergen, these similar patterns are due to different mechanisms.

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Methacholine (Mch) is routinely used to assess bronchial hyperreactivity; however, little is known about the differences in the lung response pattern between this provocation and that observed with ovalbumin (Ova) after allergic sensitization.

### What This Study Adds to the Field

Intravenous Mch acts primarily on the central airways, whereas intravenous ovalbumin induces a predominantly peripheral and heterogeneous lung response. The latter pattern is also produced by inhaled Mch, despite a different underlying mechanism.

**Keywords:** asthma; airways; tomography, X-ray computed; synchrotrons; respiratory mechanics

The site of airway constriction primarily involved after exposure to various constrictor agonists is frequently a matter of debate. The pattern of response depends on many factors, including the type and route of provocation (1) and the presence of inflammation (2, 3). Methacholine (Mch) for instance, has been shown to act mainly on the central airways (4, 5), whereas other studies reported its effects on the pulmonary tissue resistance (6, 7). On the other hand, measurement of the airway and lung tissue responses separately via the respiratory impedance spectra suggests that provocation with antigens, such as ovalbumin (Ova), in sensitized animals may produce a fundamentally different lung response, with more pronounced tissue contributions than with Mch (8). The precise quantification of the contributions of the central and peripheral lung compartments to the overall lung response is hampered by the fact that the partitioning of airway and tissue contributions is extremely sensitive to inhomogeneous constriction (9–11). Some of the discrepancies in previous results may therefore be due to the methodological limitations of lung mechanical measurements, because it has been shown that both Mch (1) and Ova (12) provocation increase ventilation heterogeneities.

Whereas the assessment of detrimental changes in the lungs by means of various imaging techniques is gaining interest, the currently available imaging techniques have their limitations regarding the structural and functional details. For instance, standard high-resolution computed tomography does not allow measurement of regional lung ventilation (13). Although other imaging techniques, such as magnetic resonance with hyperpolarized helium (14), and positron emission tomography (PET) with  $^{13}\text{N}$  (15), permit a qualitative assessment of ventilation heterogeneity, their ability to demonstrate the structural details of the lungs, and thus their suitability for the study of structure–function correla-

tions, is limited. Contrast-enhanced computed tomography (CT) has recently been used for regional measurements of lung structure and function in large animal models (16).

We earlier introduced a novel CT imaging method that uses synchrotron radiation for the simultaneous quantitative imaging of airway structure and regional lung ventilation with high spatial resolution, allowing the *in vivo* study of small animal models (17–19). The combination of this imaging technique with the estimations of airway and tissue parameters obtained with the low-frequency forced oscillation technique (LFOT) is promising for the elucidation of the mechanisms responsible for a compromised lung function, following two different pathways (the cholinergic and acute-phase allergic responses) encountered in pathological situations. The aims of this study were (1) to compare the main sites of action of intravenous Mch and Ova challenges in the lungs by combining measurements of airway and tissue mechanics with synchrotron radiation CT imaging in control and sensitized rabbits, (2) to determine the role of the inhaled route on the pattern of Mch response, and (3) to assess the extent to which mechanical and imaging parameters are correlated.

## METHODS

A more detailed description of the experimental set-up and methods can be found in the online supplement.

### Animal Preparation

New Zealand rabbits were sensitized by intraperitoneal ovalbumin (Ova) 30 and 17 days before the experiments, followed by daily exposures to aerosolized Ova starting 5 days preceding the study (20). Experiments were performed in 17 control and 13 sensitized, anesthetized, tracheostomized, and paralyzed animals. The left carotid artery and jugular vein were catheterized. The animals were immobilized in a vertical position in a plastic holder for imaging.

### Synchrotron Radiation CT Imaging

The rationale for the use of synchrotron radiation can be found in the online supplement and in previous studies (17, 21). Dynamic imaging during xenon wash-in with the synchrotron lung CT technique known as K-edge subtraction imaging allows quantitative measurements of regional specific ventilation ( $\dot{V}$ ) (i.e., ventilation normalized to voxel volume). Two CT image slices were selected, at the sixth (middle) and eighth (lower) thoracic vertebral levels. The cross-sectional areas (CAaw) of the two largest central airways in each lung slice were calculated by fitting an ellipse to the lumen in monoenergetic CT images. The local  $\dot{V}$  was calculated from the time constant ( $\tau$ ) of the xenon wash-in by using a monoexponential fit (17). The heterogeneity of ventilation was calculated as the coefficient of variation (CV) of  $\dot{V}$ . To determine well-ventilated lung zones, a histogram of  $\dot{V}$  was calculated and fit with a weighted log-normal function. The ventilated alveolar area (VA) was defined as the area of the ventilation image where  $\dot{V}$  was greater than the median  $\mu$  of distribution minus two standard deviations:  $\mu - 2\sigma$ .

### Measurement of Respiratory Mechanics

The input impedance of the respiratory system ( $Z_{rs}$ ) was measured at end-expiration by LFOT using the wave-tube technique (10). To separately estimate airway and tissue mechanics, a distributed-parameter model including parallel pathways with airway resistances following a uniform probability distribution (22) most important to constant-phase tissue compartments (23) was fitted (24) to the  $Z_{rs}$  spectra. The mean airway resistance (Raw), the standard deviation of the resistances of the parallel pathways (SDRaw), the common inertance (Iaw), and the tissue damping (G) and elastance (H) were estimated from these fits.

### Study Protocol

After baseline acquisitions of  $Z_{rs}$  and imaging data, Mch was administered by continuous intravenous infusion through the jugular vein,

according to protocols A and B (Figure 1); protocol A: 2.5 followed by 5.0  $\mu\text{g/kg/min}$  in six control and six sensitized animals; protocol B: 10  $\mu\text{g/kg/min}$  in six control and seven sensitized animals. After a 30-minute recovery from the effect of Mch, a new set of data was collected before Ova provocation (2.0 mg) in both groups, and 2 minutes later data acquisition was repeated. We compared intravenous Mch challenge with inhaled Mch (125 mg/ml, 90 s) in a group of control animals (protocol C,  $n = 5$ ), based on  $Z_{rs}$  and SR imaging data. The stability of the experimental preparation and the mechanical and imaging parameters were studied in two sham-treated animals.

### Statistical Analysis

We used repeated-measures analysis of variance with the Student-Newman-Keuls multiple comparison to test significances (25). The scatter in the data was expressed as SEM. Each test was performed with a significance level of  $P < 0.05$ .

## RESULTS

### Stability of the Preparation

None of the mechanical and imaging parameters measured in the rabbits undergoing the sham protocol exhibited a significant change during the study period.

### Airway Responses

Monoenergetic synchrotron CT images showing the lung morphology in representative rabbits at the middle and lower thoracic levels under the different experimental conditions are displayed in Figure 2. The highest dose of intravenous Mch induced a marked reduction in CAaw at both the middle and lower levels of the chest, which was fully reversible in the recovery period. Similarly, inhaled Mch induced a strong decrease in CAaw. However, Ova had much smaller effects on the central airway calibers. Airway mechanical parameters obtained from the inhomogeneous mechanical model and images related to the central airways are shown in Figure 3. Raw increased during both intravenous and inhaled Mch challenges, whereas Ova induced marked and statistically significant elevations in Raw and SDRaw only in the sensitized animals. The ratio SDRaw/Raw increased markedly during infusion of the highest dose of Mch in both groups of rabbits, after inhaled Mch and after Ova in the sensitized animals, indicating the appearance of airway mechanical heterogeneities under these conditions. No significant changes in Iaw were detected during the intravenous Mch or Ova provocations. To assess the changes in CAaw at both image levels, the two largest airways in opposite lung fields were chosen for detailed analysis. In the baseline images, the airway diameter ranged from 0.75 to 3.13 mm, corresponding to CAaw values of from 0.44 to 7.68 mm<sup>2</sup>. These airway dimensions approximately correspond to airway generations four to eight in rabbit (26). In the sensitized animals, CAaw at the middle level decreased earlier than the increase in Raw, which was significant even at the lowest Mch infusion rate ( $P < 0.001$ ). The two highest Mch infusion rates generated significant decreases in CAaw ( $P < 0.001$ ) independently of the image slice level. Similarly, inhaled Mch caused a strong decrease in CAaw. In the sensitized animals, Ova induced significant decreases in CAaw only at the middle level ( $P < 0.001$ ), whereas CAaw at the lower level was unaffected.

### Lung Peripheral Responses

Figure 4 demonstrates images of regional ventilation in a representative rabbit at baseline and after Mch or Ova provocation. The infusion of Mch exerted a moderate effect on the regional ventilation. In contrast, both inhaled Mch and intravenous Ova induced marked ventilation heterogeneities in the lung periph-

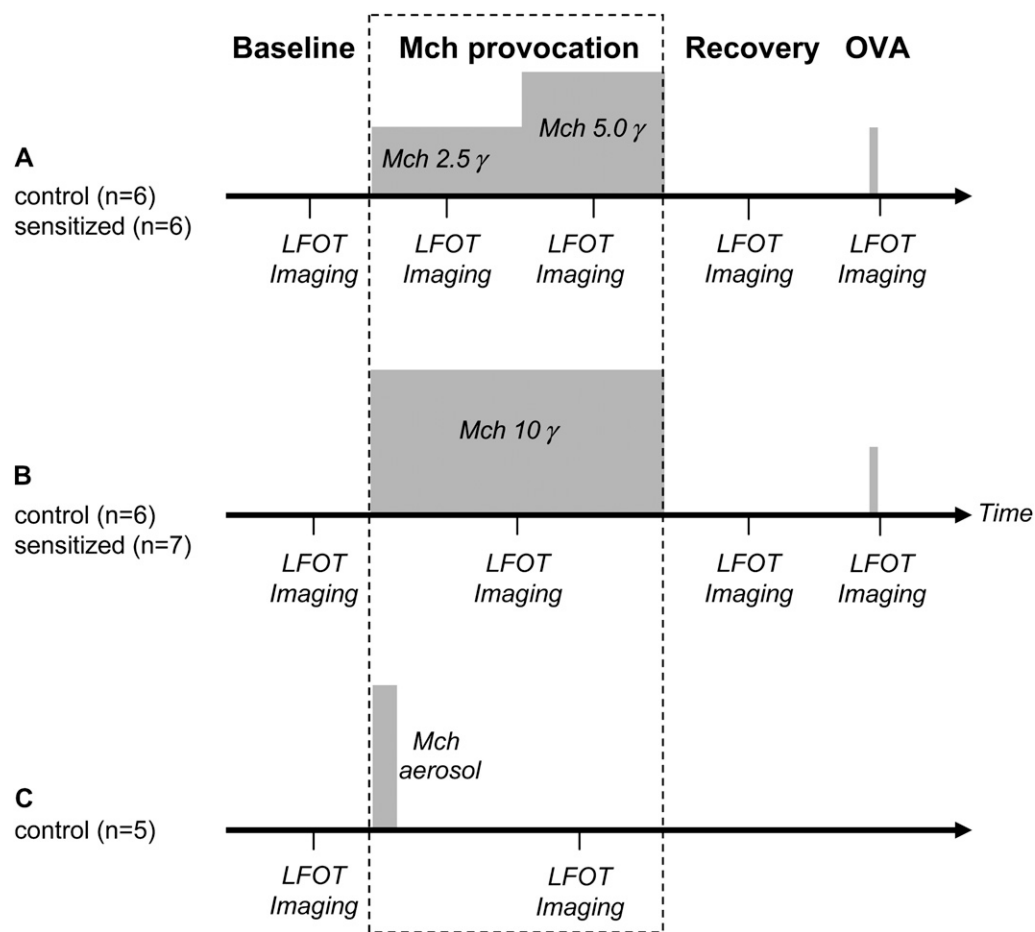


**Figure 1.** Experimental protocol. LFOT = low-frequency forced oscillation technique; Mch = methacholine; Ova = ovalbumin provocation;  $\gamma$  =  $\mu\text{g/kg/min}$ .

ery, with a highly scattered distribution, and areas of closure or near-closure. On the other hand, the challenges with intravenous Mch did not generate statistically significant changes in G, whereas H decreased slightly, but systematically, in both the control and the sensitized rabbits (Figure 5). These changes during Mch infusion were associated with mild but significant decreases in VA and increases in CV in the sensitized rabbits, which were consistently present at both the middle and the

basal lung levels ( $P < 0.05$  at both levels). Ova injection in the sensitized animals led to marked increases in both tissue mechanical parameters ( $P < 0.001$ ), whereas no change in either parameter was detected after Ova administration in the control animals. Similar increases in G and H were observed after inhaled Mch. Furthermore, VA decreased and CV increased markedly in the sensitized animals after intravenous Ova and in the control animals after inhaled Mch, indepen-

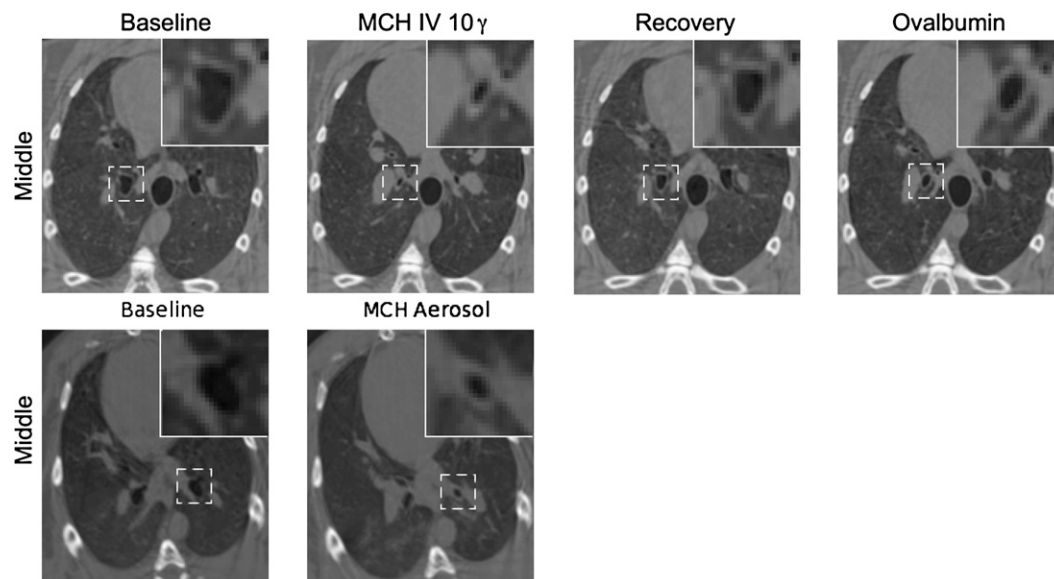


**Figure 2.** Monoenergetic computed tomography images showing changes in the central airway cross-sectional area in the middle image slices at the different experimental stages in one representative animal in protocols A and B (upper panel), and C (lower panel). Magnifications of the indicated square areas are shown in the right upper corners.

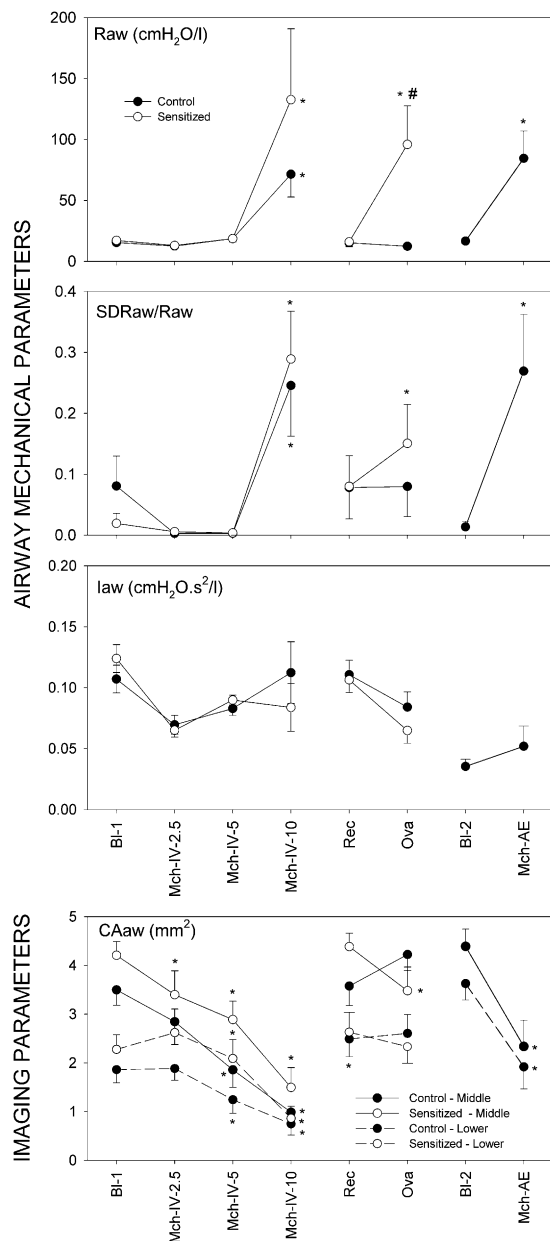


**Figure 3.** Values of the airway mechanical parameters: mean airway resistances (Raw), the coefficient of variation of the parallel pathway resistances (SDRaw/Raw), common inertance (law); and imaging parameters: central airway cross-sectional area (CAaw) in control and sensitized rabbits in middle and lower image slices at baseline (BI-1 and BI-2), after methacholine (Mch) aerosol inhalation (AE), or during infusion (IV), on recovery (Rec), and after ovalbumin (Ova) provocation. Data are means  $\pm$  SEM. \* $P < 0.05$  versus baseline; # $P < 0.05$  versus control.

dently of the slice level studied ( $P < 0.01$  at both levels). These imaging parameters remained at their baseline levels in the control rabbits given intravenous Ova.

#### Relationships between Changes in Mechanical and Imaging Parameters

The associations between the alterations in the mechanical and imaging parameters are depicted in Figure 6. For a better visualization, the changes in the conductance parameters ( $1/\text{Raw}$ ) were related to the changes in the square of CAaw. There

was a very strong association between the Mch-induced changes in the airway conductance assessed from the Zrs spectra, and CAaw measured from the images ( $R = 0.71$ ,  $P < 0.0001$ ). In contrast with Mch, the correlations between the airway mechanical and imaging parameters were extremely weak after Ova injection in sensitized animals ( $R = 0.22$ ,  $P = 0.4$ ). Conversely, the inhaled Mch- and Ova-induced changes in H and VA correlated very strongly ( $R = -0.72$ ,  $P < 0.001$ ; Figure 6, *bottom*), whereas intravenous Mch caused no changes in these parameters.

#### DISCUSSION

This is the first study to combine separate estimates of airway and parenchymal contributions to the overall lung mechanics with direct quantitative measurements of the central airway cross-section and regional lung ventilation. We demonstrated that the patterns of changes induced in the various mechanical and imaging parameters by Mch and Ova in sensitized animals are fundamentally different through the intravenous route. Although Mch exerted constriction mainly in the central airways, Ova induced severe heterogeneities in the lung periphery, with minor central effects in the sensitized animals. Because Mch challenge is performed through the inhaled route in the clinical setting, we compared the effect of this route with Mch infusion. Our results show that inhaled Mch provocation reproduces a response pattern resembling that of intravenous antigen challenge in the lung periphery. A possible mechanism could be a heterogeneous peripheral pattern of Mch deposition through the inhaled versus the intravenous route. There were concordances between the changes in the mechanical parameters and the indices obtained from K-edge subtraction images both during Mch provocation and after the administration of Ova. The significant correlations between the airway and tissue mechanical responses and those in the airway and regional ventilation imaging parameters suggest that the model-based evaluation of the low-frequency Zrs spectra can give separate estimates of changes in the airway and parenchymal mechanics after constrictor stimuli.

#### Methodological Considerations

The technical features of analyzing structural and functional changes by using K-edge subtraction imaging methodology have been extensively discussed (17). Xenon is a dense gas, which may have an effect on the xenon wash-in time constants and therefore on the value of  $s\dot{V}$  (27). However, this effect was similar in all experimental conditions and it is therefore unlikely that the density influenced our main findings. Due to the time necessary to obtain images of regional ventilation, the number of lung levels assessed was limited to two. Sampling of these levels was appropriate to follow the changes in CAaw and also to provide a representative picture of the alterations in the lung periphery.

Although ideally a uniform definition of the well- and poorly ventilated lung zones should be applied in all animals in the ventilation images, the appropriate determination of this definition would need (1) a complete three-dimensional assessment of regional ventilation, which was not feasible in the present study protocol; and (2) that the specific ventilation in the image slice remain the same between subjects and among experimental conditions within a subject. However, because the overall specific ventilation in the imaged slice is subject to change, taking a unique cutoff for poorly ventilated zones could lead to erroneous estimations of the extent of regional ventilation defects. To minimize this error, we chose to use a definition

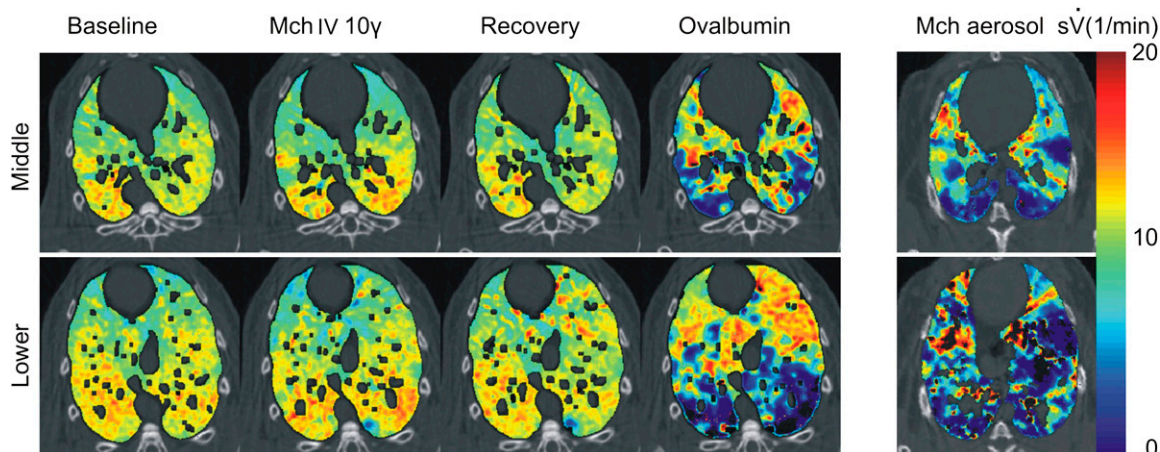


**Figure 4.** Left panel: images of specific ventilation in one representative sensitized rabbit at baseline, during methacholine (Mch) infusion, on recovery, and after ovalbumin provocation.  $\gamma = \mu\text{g/kg/min}$ . Right panel: images of regional ventilation in one representative control rabbit after Mch aerosol inhalation. Black color indicates zero specific ventilation.

of poorly ventilated zones based on the statistical distribution of  $s\dot{V}$  that accounts for changes in the overall  $s\dot{V}$  of the image slice.

Besides direct visualization of the central airways, essential information regarding the severity of ventilation heterogeneity was obtained from the K-edge subtraction images. Accordingly, a model in which airway heterogeneity was taken into account was fitted to the Zrs spectra (23). The model parameters extracted from these fits allow assessment not only of the overall airway narrowing but also of the functional inhomogeneity that develops in the airways during constrictor challenges. The mechanical heterogeneity as estimated by the model fit, however, did not show a significant relation with lung ventilation heterogeneity. This may be because CV of  $s\dot{V}$  reflects alveolar ventilation heterogeneity, predominantly determined by peripheral airway function, whereas SDRaw/Raw reflects the sum of airway heterogeneities within the entire airway tree.

We applied Ova sensitization to produce bronchial hyperactivity and anaphylactic reactions to allergens (20). In line with the previous results, all the animals in the present study exhibited a response to Ova, with marked increases in all mechanical parameters, confirming the presence of allergic sensitization. Nevertheless, despite a tendency to airway hyperresponsiveness to Mch, the differences in the airway responses between the two groups of animals did not reach statistical significance concerning either the mechanical or the imaging parameters. This may be attributed to the large interindividual variability among the sensitized animals and also to the posture of the rabbits. The stationary, horizontal X-ray beam used for functional imaging in the present study required that the animal be positioned vertically. Because this body position is unusual in animal lung function studies, we performed a supplemental methodological investigation in a separate group of rabbits ( $n = 7$ ). The upright body position was associated with systematic decreases in the respiratory mechanical parameters relative to those obtained with the animals in the supine position (Raw:  $17.4 \pm 0.8$  vs.  $20.8 \pm 1.1$  cm H<sub>2</sub>O · s/L, H:  $462 \pm 29$  vs.  $789 \pm 82$  cm H<sub>2</sub>O/L, and G:  $101 \pm 3$  vs.  $170 \pm 12$  cm H<sub>2</sub>O/L, mean  $\pm$  SEM). The larger CAaw in the vertical position may have blunted the airway responses to Mch and this effect may have prevented the statistical detection of airway hyperresponsiveness in the sensitized animals. Because all these effects were similar for both provocation agents, inducing lung constriction via two different pathways, it is unlikely that the posture of the animal affected our results appreciably.

In this study, it was not possible to randomize the order of challenges because in sensitized animals, Ova induces a late-phase response causing a carryover effect that would have biased a subsequent Mch challenge. However, because Mch and antigen provocations act through completely different pathways, the reversible Mch response is unlikely to have significantly biased the response to the subsequent Ova challenge.

#### Pulmonary Responses to Mch

In the present study, we found that both inhaled and the highest dose of intravenous Mch caused significant increases in Raw and also elevated the ratio SDRaw/Raw, suggesting the development of mechanical heterogeneity in the parallel airway branches. The intravenous Mch-induced changes in the respiratory tissue parameters were dissociated, with G remaining essentially at the baseline level, whereas H exhibited slight but systematic decreases. The lack of changes in G is in contrast with previous results obtained during Mch infusions, when the marked increases in Raw were associated with elevated G (9, 10). However, a single-compartment homogenous model was used to separate airway and tissue responses in those studies. The model parameters extracted by the application of this model have been shown to be biased by airway heterogeneities, particularly causing an artificial increase in G. Because the model in the present study incorporates airway heterogeneities, this bias was minimized. The slight decreases in H after Mch in both protocol groups were unrelated to the dose of the agonist. This suggests that the homogenizing effect of the sigh delivered before the Mch infusions may have recruited small atelectatic lung regions. The possible involvement of this phenomenon is further suggested by the reduced scattering in the CV between the baseline and the first Mch dose (Figure 5). Accordingly, this changing pattern in the mechanical parameters indicates that Mch induced central airway constriction, and the effect on the more peripheral airways was sufficient to induce ventilation heterogeneity only at the highest dose and was too limited to induce any detectable passive tissue distortion or lung volume loss that would have caused elevations in H.

Conversely, inhaled Mch caused elevations in both G and H suggesting a strongly heterogeneous peripheral lung response with airway closures. The strikingly different pattern of response from that observed after infused Mch, despite a similar increase in Raw and otherwise identical experimental condi-

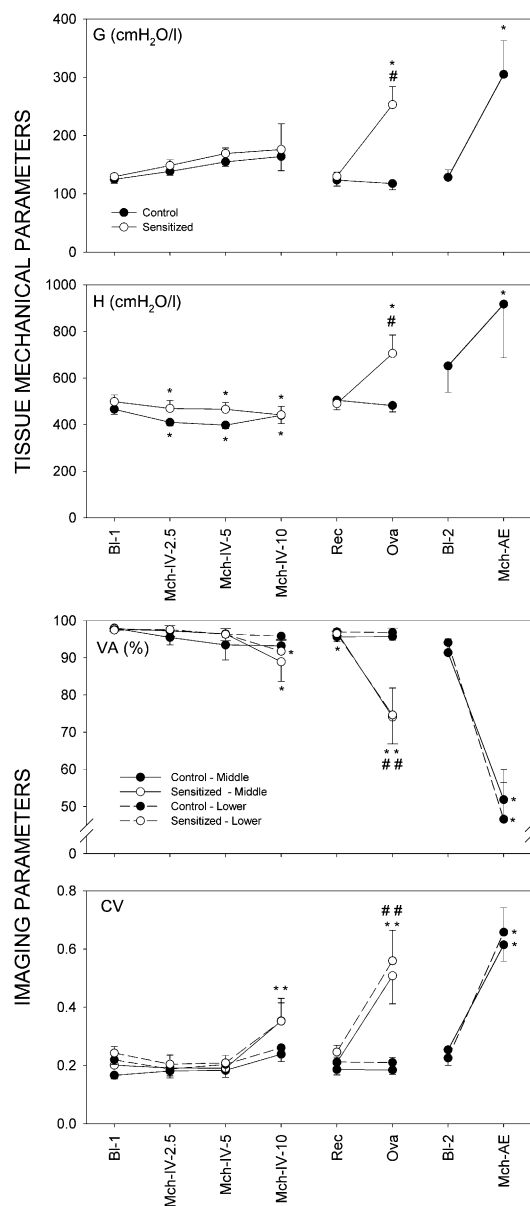


**Figure 5.** Values of the tissue mechanical parameters: damping (G), elastance (H); and imaging parameters: ventilated alveolar area (VA) and coefficient of variation of specific ventilation (CV), in control and sensitized rabbits in the middle and lower image slices at baseline (BL-1 and BL-2), after methacholine (Mch) aerosol inhalation (AE), or during infusion, on recovery, and after ovalbumin (Ova) provocation. Data are means  $\pm$  SEM. \* $P$  < 0.05 versus baseline; # $P$  < 0.05 versus control.

tions, may be due to the pattern of aerosol deposition. This is suggested by previous studies wherein deposition of aerosol particles with a mass median aerodynamic diameter similar to the present study showed significant peripheral heterogeneity under mechanical ventilation (28, 29).

These conclusions are confirmed by the changes in the parameters obtained from the images. Marked decreases in CAaw were observed (Figure 4). The changes in CAaw correlated strongly with those in the airway conductance during Mch challenges (Figure 6). It is noteworthy that the intravenous Mch-induced changes were detectable earlier in the CAaw data (as low as 2.5  $\mu$ g/kg/min) than in the mechanical measurements. In the present study, K-edge subtraction imaging provided direct new evidence of a small but significant increase both in the area

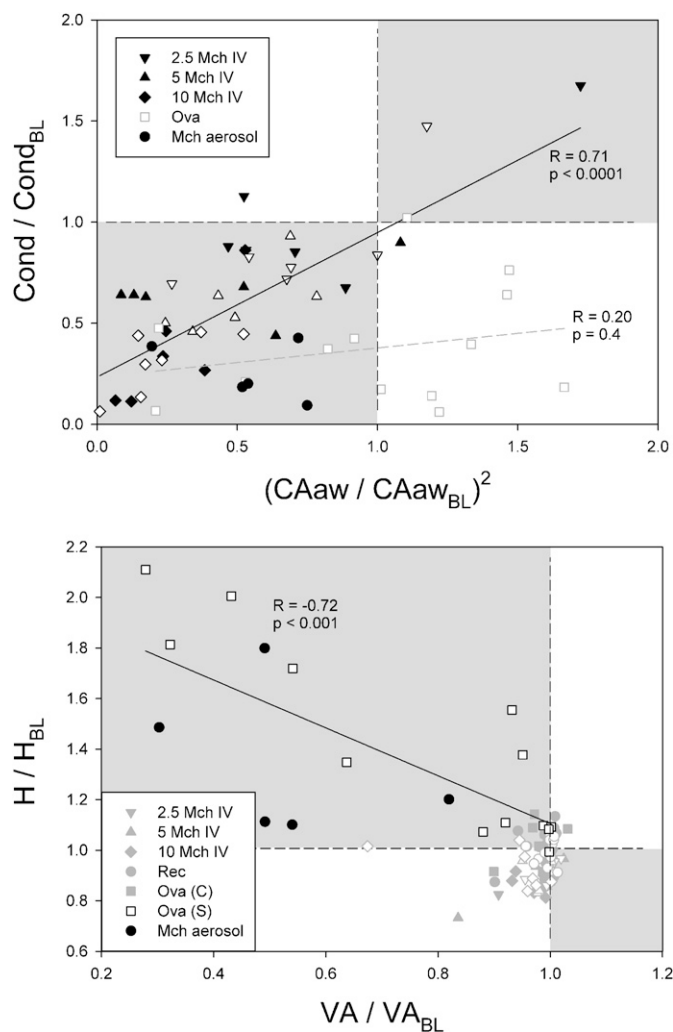


**Figure 6.** Upper panel: correlation between relative change in conductance (Cond) versus the relative change in central airway cross-sectional area (CAaw). CAaw<sub>BL</sub> = central airway cross-section at baseline; Cond<sub>BL</sub> = airway conductance at baseline. Post-Ova CAaw data are expressed relative to recovery values. Lower panel: tissue elastance (H) versus ventilated alveolar area (VA). H<sub>BL</sub> = tissue elastance at baseline; VA<sub>BL</sub> = ventilated alveolar area at baseline. Closed and open symbols indicate data from control and sensitized animals, respectively.

of the poorly ventilated lung zones and in the heterogeneity of peripheral ventilation as measured by the heterogeneity at the highest Mch infusion rate, as well as after the inhalation of this agonist. The appearance of poorly ventilated lung zones was most likely caused by the constriction of clusters of small peripheral airways rather than changes in the local tissue mechanics. Although small peripheral airways could not be visualized directly with K-edge subtraction imaging, we previously observed from a combination of lung mechanics measurements and of central conducting airway imaging that peripheral defects in ventilation may be present immediately after histamine inhalation, together with an elevation in respiratory system resistance, without any constriction in the central conducting airways (20). This finding suggested that the observed peripheral ventilation defects were due to peripheral airway constriction. Tgavalekos and colleagues used a combination of PET imaging of regional ventilation and oscillatory mechanical measurements in patients with asthma provoked with inhaled Mch, with a three-

dimensional airway tree model, to examine the role of large versus small airways in the simultaneous deterioration of mechanical function and ventilation (30). They found that matching both the degree of mechanical impairment and the size and location of the PET ventilation defects requires either the constriction of airways less than 2.4 mm in diameter or the simultaneous constriction of small and large airways (but not merely large airways alone).

Overall, in view of these observations obtained from mechanical measurements and image analyses, it can be concluded that the response to intravenous Mch predominantly occurred in the central conducting airways. However, it was not limited solely to these conducting airways, because a peripheral airway response seemed to appear during a high dose of Mch. The deposition pattern of Mch aerosol is likely to have caused a remarkably different distribution of response through the inhaled route with a much more heterogeneous impact on the lung periphery.

### Lung Responses to Ova

We found a markedly different pattern of response to Ova in sensitized animals as compared with that to Mch. Although the magnitudes of the increases in Raw were similar at the highest dose of Mch and on Ova provocation, the latter changes were associated with much smaller decreases in CAaw. This resulted in a poor correlation between the changes in the airway conductance and CAaw measured from the images (Figure 6, *top*), which suggests that the effect of Ova provocation is predominantly localized to small peripheral airways beyond the generations visualized in the images.

In the sensitized animals, Ova induced significant elevations in G and H, accompanying the increases in Raw and SDRaw/Raw. This pattern suggests the increased peripheral ventilation heterogeneity in combination with true changes in the mechanical properties of the lung tissue (23), most likely due to a loss in lung volume subsequent to peripheral airway closures. This is evidenced by the development of severe ventilation defects after Ova administration (a drop in VA; Figure 5), measured from the ventilation images and further confirmed by the very strong correlation between the changes in H and VA (Figure 6). Besides this effect, the tissue responses observed after Ova provocation in sensitized animals may also be a consequence of interdependence between the peripheral airways and the surrounding lung parenchyma (31, 32).

The reasons for such fundamentally different patterns of response to intravenously administered nonspecific Mch and specific Ova airway provocations are not clear. Both constrictor agents were administered via the same route, and thus the observed pattern of response cannot be due to differences in distribution of the agent. Mch produces smooth muscle constriction by stimulating muscarinic cholinergic receptors. The distributions of different subtypes of muscarinic receptors may be involved in the pattern of response to Mch. In several species, including dog, pig, mouse, and human, M3 muscarinic receptors on the airway smooth muscle mediate large and small conducting airway cholinergic constriction and also submucosal gland stimulation (33). Hence, a potential explanation for the predominant effect of intravenous Mch on the central bronchi in the present study could be a higher density of M3 receptors in these airways. In humans, these receptors have been shown to be more densely distributed in the trachea and proximal central airways (34). Moreover, the total muscarinic receptor density proved to be higher in the trachealis muscle than in the bronchi or lung tissue in dog (35). In rabbits, the presence of the M3 and M4 receptors in the central airway smooth muscle, and of the M4 receptor in the lung periphery, has been demonstrated (36). However, as far

as we are aware, the serial distribution of the cholinergic receptors along the bronchial tree has not been quantified in this species. In addition, the functional significance of M4 receptor subtypes expressed in the bronchiolar smooth muscle and alveolar walls has not been elucidated. Nevertheless, a higher muscarinic receptor density in the central airways remains a plausible explanation of our findings.

On the other hand, antigen-induced anaphylaxis leads to the release of a host of mediators and chemotactic substances through the degranulation of mast cells, eosinophils, and basophils (37). These include histamine, proteases, a number of cytokines, and lipid-derived mediators, such as prostaglandins, leukotrienes, and the platelet-activating factor. The diversity of mediators and possibly the localizations of their receptors both in the airways and in the lung periphery may well be the reason for the far stronger peripheral impact of Ova provocation as compared with that of intravenous Mch. Finally, we cannot exclude the potential contribution of increased mucus secretion, airway edema, and surfactant dysfunction after antigen provocation in the occurrence of airway closures.

### Conclusions

In summary, we have characterized the changes that occur in the pulmonary system during a nonspecific constrictor challenge with Mch and after provocation with the specific allergen in sensitized rabbits. The functional changes were assessed by respiratory mechanical measurements allowing partitioning of the airway and tissue changes. We combined these results with those obtained with a novel imaging technique having the ability to quantify simultaneous changes in the lumen of the conducting airways and the regional lung ventilation. We observed strong associations between the airway and tissue mechanical responses and corresponding imaging parameters, supporting the ability to separate airway and tissue mechanics on the basis of low-frequency Zrs data. Functional localization of the primarily affected lung compartment revealed radical differences between the changes due to Ova and Mch provocations, despite similar magnitudes of increase in Raw. Our results demonstrate that through intravenous infusion, the effect of Mch in the lungs predominates in central airways, with minor changes in the lung periphery. In contrast, after Ova provocation through the same route, both the mechanical and the functional imaging parameters indicated the development of severe ventilation heterogeneity and airway closure in the lung periphery. Inhaled Mch, however, induces a heterogeneous lung response similar to that observed with intravenous allergen, despite different underlying mechanisms.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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### References

- Peták F, Hantos Z, Adamicza A, Asztalos T, Sly PD. Methacholine-induced bronchoconstriction in rats: effects of intravenous vs. aerosol delivery. *J Appl Physiol* 1997;82:1479–1487.
- Zosky GR, von Garnier C, Stumbles PA, Holt PG, Sly PD, Turner DJ. The pattern of methacholine responsiveness in mice is dependent on antigen challenge dose. *Respir Res* 2004;5:15.
- Xu LJ, Sapienza S, Du T, Wasserman S, Martin JG. Comparison of upper and lower airway responses of two sensitized rat strains to inhaled antigen. *J Appl Physiol* 1992;73:1608–1613.
- Kaczka DW, Ingenito EP, Israel E, Lutchen KR. Airway and lung tissue mechanics in asthma: effects of albuterol. *Am J Respir Crit Care Med* 1999;159:169–178.

5. Pellegrino R, Violante B, Nava S, Rampulla C, Brusasco V, Rodarte JR. Expiratory airflow limitation and hyperinflation during methacholine-induced bronchoconstriction. *J Appl Physiol* 1993;75:1720–1727.
6. Ingenito EP, Davison B, Fredberg JJ. Tissue resistance in the guinea pig at baseline and during methacholine constriction. *J Appl Physiol* 1993;75:2541–2548.
7. Lutchen KR, Suki B, Zhang Q, Peták F, Daróczy B, Hantos Z. Airway and tissue mechanics during physiological breathing and bronchoconstriction in dogs. *J Appl Physiol* 1994;77:373–385.
8. Schütz N, Peták F, Barazzzone-Argiroffo C, Sly PD, Habre W. Prevention of bronchoconstriction in sensitized guinea pigs: efficacy of common prophylactic drugs. *Respir Physiol Neurobiol* 2004;141:167–178.
9. Lutchen KR, Hantos Z, Peták F, Adamicz A, Suki B. Airway inhomogeneities contribute to apparent lung tissue mechanics during constriction. *J Appl Physiol* 1996;80:1841–1849.
10. Van de Woestijne KP, Franken H, Caubergs M, Landser FJ, Clement J. A modification of the forced oscillation technique. In: Debrecezi L, Hutás I, editors. *Advances in physiological sciences. Respiration. Proc. of 28th Int. Cong. of Physiol. Sci. Budapest Hungary, June 1980.* Oxford, UK: Pergamon; 1981. Pp. 655–660.
11. Thorpe CW, Bates JH. Effect of stochastic heterogeneity on lung impedance during acute bronchoconstriction: a model analysis. *J Appl Physiol* 1997;82:1616–1625.
12. Sánchez-Cifuentes MV, Rubio ML, Ortega M, Peces-Barba G, Paiva M, Verbanck S, Mangado NG. Lung function and ventilation inhomogeneity in rat lungs after allergen challenge. *J Appl Physiol* 2000;88:821–826.
13. Brown RH, Herold CJ, Hirshman CA, Zerhouni EA, Mitzner W. In vivo measurements of airway reactivity using high-resolution computed tomography. *Am Rev Respir Dis* 1991;144:208–212.
14. Samee S, Altes T, Powers P, de Lange EE, Knight-Scott J, Rakes G, Mugler JP III, Ciambotti JM, Alford BA, Brookeman JR, *et al.* Imaging the lungs in asthmatic patients by using hyperpolarized helium-3 magnetic resonance: assessment of response to methacholine and exercise challenge. *J Allergy Clin Immunol* 2003;111:1205–1211.
15. Vidal Melo MF, Layfield D, Harris RS, O'Neill K, Musch G, Richter T, Winkler T, Fischman AJ, Venegas JG. Quantification of regional ventilation-perfusion ratios with PET. *J Nucl Med* 2003;44:1982–1991.
16. van Beek EJ, Hoffman EA. Functional imaging: CT and MRI. *Clin Chest Med* 2008;29:195–216. [Review.]
17. Porra L, Monfraix S, Berruyer G, Le Duc G, Nemoz C, Thomlinson W, Suortti P, Sovijärvi AR, Bayat S. Effect of tidal volume on distribution of ventilation assessed by synchrotron radiation CT in rabbit. *J Appl Physiol* 2004;96:1899–1908.
18. Bayat S, Porra L, Suhonen H, Nemoz C, Suortti P, Sovijärvi ARA. Differences in the time course of proximal and distal airway response to inhaled histamine studied by synchrotron radiation CT. *J Appl Physiol* 2006;100:1964–1973.
19. Bayat S, Le Duc G, Porra L, Berruyer G, Nemoz C, Monfraix S, Fiedler S, Thomlinson W, Suortti P, Standertskjöld-Nordenstam CG, *et al.* Quantitative functional lung imaging with synchrotron radiation using inhaled xenon as contrast agent. *Phys Med Biol* 2001;46:3287–3299.
20. Peták F, Hantos Z, Adamicz A, Gálicy H, Habre W. Development of bronchoconstriction after administration of muscle relaxants in rabbits with normal or hyperreactive airways. *Anesth Analg* 2006;103:103–109.
21. Monfraix S, Bayat S, Porra L, Berruyer G, Nemoz C, Thomlinson W, Suortti P, Sovijärvi AR. Quantitative measurement of regional lung gas volume by synchrotron radiation computed tomography. *Phys Med Biol* 2005;50:1–11.
22. Suki B, Yuan H, Zhang Q, Lutchen KR. Partitioning of lung tissue response and inhomogeneous airway constriction at the airway opening. *J Appl Physiol* 1997;82:1349–1359.
23. Hantos Z, Daróczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 1992;72:168–178.
24. Csendes T. Nonlinear parameter estimation by global optimization—efficiency and reliability. *Acta Cybernetica* 1988;8:361–370.
25. Snedecor GW, Cochran WG. *Statistical methods.* Blackwell Publishing; 1989.
26. Ramchandani R, Shen X, Elmsley CL, Ambrosius WT, Gunst SJ, Tepper RS. Differences in airway structure in immature and mature rabbits. *J Appl Physiol* 2000;89:1310–1316.
27. Fuld MK, Easley RB, Saba OI, Chon D, Reinhardt JM, Hoffman EA, Simon BA. CT measured regional specific volume change reflects regional ventilation in supine sheep. *J Appl Physiol* 2008;104:1177–1184.
28. Leong BK, Coombs JK, Sabaitis CP, Rop DA, Aaron CS. Quantitative morphometric analysis of pulmonary deposition of aerosol particles inhaled via intratracheal nebulization, intratracheal instillation or nose-only inhalation in rats. *J Appl Toxicol* 1998;18:149–160.
29. Dahlbäck M, Wollmer P, Jonson B. Selective deposition of inhaled aerosols to mechanically ventilated rabbits. *J Aerosol Med* 1994;7:315–324.
30. Tgavalekos NT, Tawhai M, Harris RS, Musch G, Vidal-Melo M, Venegas JG, Lutchen KR. Identifying airways responsible for heterogeneous ventilation and mechanical dysfunction in asthma: an image functional modeling approach. *J Appl Physiol* 2005;99:2388–2397.
31. Mead J, Takishima T, Leith D. Stress distribution in lungs: a model of pulmonary elasticity. *J Appl Physiol* 1970;28:596–608.
32. Winkler T, Venegas JG. Complex airway behavior and paradoxical responses to bronchoprovocation. *J Appl Physiol* 2007;103:655–663.
33. Barnes PJ. Muscarinic receptors in airways: recent developments. *J Appl Physiol* 1990;68:1777–1785. [Review.]
34. Barnes PJ, Basbaum CB, Nadel JA. Autoradiographic localization of autonomic receptors in airway smooth muscle. Marked differences between large and small airways. *Am Rev Respir Dis* 1983;127:758–762.
35. Emala C, Black C, Curry C, Levine MA, Hirshman CA. Impaired beta-adrenergic receptor activation of adenylyl cyclase in airway smooth muscle in the basenji-greyhound dog model of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 1993;8:668–675.
36. Mak JC, Haddad EB, Buckley NJ, Barnes PJ. Visualization of muscarinic m4 mRNA and M4 receptor subtype in rabbit lung. *Life Sci* 1993;53:1501–1508.
37. Kemp SF, Lockey RF. Anaphylaxis: a review of causes and mechanisms. *J Allergy Clin Immunol* 2002;110:341–348.

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**METHACHOLINE AND OVALBUMIN CHALLENGES ASSESSED BY FORCED  
OSCILLATIONS AND SYNCHROTRON LUNG IMAGING**

**ONLINE DATA SUPPLEMENT**

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**MATERIALS AND METHODS***Animal preparation*

Animal care and experimental procedures were in accordance with the Guidelines for the Care and Use of Animals published by the American Physiological Society and approved by the local institutional authorities. The experiments were performed on 27 male New Zealand rabbits ( $2.6 \pm 0.04$  kg, Elevage Scientifique des Dombes, Chatillon sur Chalaronne, France). Thirteen animals were sensitized over a one-month period before the start of the experimental protocol, as detailed previously (Ei). Briefly, intraperitoneal injections of a solution containing 0.1 mg of ovalbumin (Sigma-Aldrich Chimie S.a.r.l., St. Quentin Fallavier, France) and 10 mg of aluminum hydroxide as adjuvant (Merck KGaA, Darmstadt, Germany) were administered 30 and 17 days before the start of the protocol. The animals were then exposed to daily aerosolized ovalbumin (10 mg/ml for 20-min) for 5 days prior the experiments (System LS290, Velleneuve sur Lot, France). For the experiments, a catheter (22 G) was inserted into the marginal ear vein (Cathlon IV, Ethicon, Rome, Italy) under local anesthesia, using 5% topical lidocaine (Emla, Astra-Zeneca, France). Anesthesia was induced by the intravenous injection of thiopental sodium (25 mg/kg IV, Nesdonal, Rhone-Poulenc-Rohrer, Paris, France). The animal was tracheostomized and an endotracheal tube (no. 3, Portex, Berck sur Mer, France) was inserted and secured with a gas-tight seal. A 22 G catheter was inserted into the left carotid artery for blood pressure monitoring and arterial blood sampling for blood gas measurements (Radiometer, ABL77, Copenhagen, Denmark). The left jugular vein was also catheterized for methacholine infusion. The lower extremities of the animal were wrapped in bandage and the animal was immobilized in the vertical position in a custom made cylindrical PVC holder. The chest wall and diaphragmatic motion were free of constraint, because the rabbit was maintained in the vertical position by a plastic attachment below the lower maxillary bone, which supported the weight of the animal. There was very little stress on the forelimbs, which were taped to keep them out of the image field. The chest and

diaphragm were entirely free, and the lower limbs were securely maintained in the cylindrical holder with the use of foam. Anesthesia was maintained with intravenous Midazolam (0.2 mg/kg/h, Aguettant, Lyon, France). Paralysis was induced with intravenous Atracurium (1.0 mg/kg/h, Tracrium, Glaxo-SmithKline, Münchenbuchsee, Switzerland). The respiratory gas flow was monitored by using a heated pneumotachometer (Hans Rudolph, Kansas City MO, USA). The endotracheal pressure was monitored continuously. All monitored signals were amplified, digitized at 400 Hz (Powerlab, ADI Instruments, Oxfordshire, UK), and recorded on a computer.

### *Synchrotron lung CT technique and instrumentation*

The synchrotron CT imaging technique employed in this study, called K-edge subtraction imaging, uses dual X-ray beams at two slightly different energies. Simultaneous acquisition and subtraction of two CT images allows the direct quantitative measurement of xenon gas within the airways, and of the regional gas volume (Eii). Dynamic imaging during xenon wash-in allows the measurement of regional specific ventilation, i.e. the ventilation normalized to voxel volume (Eiii). Visualization and quantitative measurement of the xenon within the airways is based on the property that the attenuation coefficient of xenon increases by a factor of 5.4 when the energy of the incident X-ray beam crosses the energy threshold of 34.56 keV, which is the xenon K-edge (Eiv,Ev) (Figure E1). The changes in the attenuation coefficients of cortical bone and lung tissue are negligible. Imaging is performed simultaneously with two X-ray beams at slightly different energies, above and below the xenon K-edge. Subtraction of these two mono-energetic images on a logarithmic scale allows the observation of small anatomic structures carrying the contrast agent, while removing practically all features due to other structures. The intensity scale of the resulting image is directly proportional to the content of the contrast element per image voxel (xenon density), based on the property that for mono-energetic X-rays the logarithm of the transmitted intensity is proportional to the line integral of the attenuation coefficients. X-rays from a synchrotron radiation source are required since, as opposed to standard X-

ray sources, they allow the selection of monochromatic beams from the full X-ray spectrum while conserving enough intensity for imaging.

All experiments were performed at the Biomedical Beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The mono-energetic beams were produced from the continuous synchrotron radiation spectrum by a bent silicon crystal monochromator and the energy difference between the beams was 250 eV. The beams focused and crossed at the animal position, beyond which they diverged and were recorded by a liquid nitrogen-cooled high-purity germanium dual-line detector (Eurisy Measure, Lingolsheim, France). The horizontal pixel size of the detector was 0.35 mm, and the beam vertical width was 0.7 mm. Since the X-ray beam is stationary, the animal was moved for imaging. For projection imaging, the animal was moved vertically through the beams and for CT imaging the animal was rotated around an axis perpendicular to the beams ( $6.56^\circ$  off-vertical). The detector was read out synchronously with the animal motion to provide 0.35 mm vertical pixel size in the projection images, and  $0.5^\circ$  angular resolution in the CT images. The rotation speed in CT imaging was  $180^\circ/\text{s}$  and images were recorded from 720 angular projections per full  $360^\circ$  rotation. CT reconstruction was performed using the filtered-back-projection algorithm, with the Interactive Data Language (IDL, RSI, Boulogne-Billancourt, France).

The experimental set-up was essentially identical to that used in previous experiments (Eiii, Ev). An extra electromagnetic valve was included, which allowed the switching of the ventilation circuit from the ventilator to the forced oscillatory measurement system. Details of the ventilation system were published previously (Evi). The set-up consisted of a custom-made apparatus, including a T-tube and two electromagnetic valves, used to synchronize mechanical ventilation with the image acquisition. The input gas flow was maintained constant at baseline. The tidal volume was approximately 7.0 ml/kg, with the respiratory rate set to achieve an arterial  $\text{PCO}_2$  close to 40 Torr. Ventilation and the inhaled gas mixture were remotely controlled with electromagnetic valves. Gas

flows were measured continuously, recorded with mass flow meters (Aalborg, Orangeburg, New York) and adjusted prior to data acquisition. During the resting period the animal breathed air, and during imaging it breathed a mixture of xenon (70%) and oxygen (30%). Ventilation was paused in expiration for image acquisition.

### *Image analysis*

Images were processed by using the MatLab programming package (Mathworks Inc., MI, USA). Lung tissue was selected within the monochromatic CT images, by segmentation based on tissue density thresholds. In the one-compartment model, the xenon density for a given region of interest in the K-edge subtraction images increases in the wash-in sequence following mono-exponential dynamics (Eiii). The time constant ( $\tau$ ) of xenon wash-in was calculated from a mono-exponential fit by using

$$d(t) = d_A \{1 - \exp[-(t-t_0)/\tau]\}$$

where  $d_A$  is the asymptotic density and  $\tau$  is the time constant. The arrival time  $t_0$  includes the transfer time from the gas valve to the trachea, and the transfer time from the trachea to the entrance of the acini. The time scale is directly proportional to the number of breaths, because the apnea pauses during imaging were subtracted. The inverse of  $\tau$ , equal to the local specific ventilation ( $s\dot{V}$ ) or ventilation normalized to the voxel volume, was calculated for every point of the CT image to produce regional ventilation maps (Evii). The exponential fit was performed by using multidimensional unconstrained non-linear minimization (Nelder-Mead), and the goodness of fit was assessed by examination of the normalized summed squared residuals (SSRs). Images were smoothed with a 5 by 5 pixel moving average window to produce 1.75 mm x 1.75 mm resolution smoothed  $s\dot{V}$  maps, where the effects of small differences (up to two pixels) in the animal position between successive images are eliminated.

The heterogeneity of ventilation was calculated with a coefficient of variation (CoV) of  $s\dot{V}$ . Average regional ventilation  $s\dot{V}$  and CoV were calculated separately from each  $s\dot{V}$  map.

To determine well-ventilated lung zones, a histogram of  $s\dot{V}$  was calculated. At baseline, the distributions were uni-modal. The statistical distribution of the regional ventilation at baseline is best described by a log-normal function due to the fractal structure of the bronchial tree (Eviii, Eiii). This functional form was used to fit the histograms of regional ventilation, using the Nelder-Mead minimization. The probability density function of the log-normal distribution for a random variable  $x$  is given (Eix) by the equation

$$f(x; \sigma, \mu) = \exp[-(\ln x - \mu)^2 / 2\sigma^2] / [x\sigma(2\pi)^{1/2}]$$

Since the well-ventilated lung is supplied by fractally distributed flow, even after airway challenge, these were estimated from the total distribution of  $s\dot{V}$  by fitting the above function, weighted to the larger values of  $s\dot{V}$ :

$$SSR = \sum_{i=0}^n w_i (y_i - f(x_i))^2$$

$$w_i = \begin{cases} 1 & \text{if } f(x_i) < \mu \\ 2 & \text{if } f(x_i) \geq \mu \end{cases}$$

Where SSR is the sum of square residuals, and  $w_i$  the weighting factor. The goodness of fit was assessed based on the SSRs. The area of well-ventilated zones (VA) was defined as the area where:

$$s\dot{V} > (\mu - 2\sigma)$$

*Measurement of respiratory mechanics*

At end-expiration, the mechanical ventilation was paused, a loudspeaker-in-box system and the tracheal cannula were connected through a polyethylene tube (100 cm length, 0.375 cm ID) and a small-amplitude (1 cmH<sub>2</sub>O peak to peak) forcing signal was delivered into the trachea. The loudspeaker was driven by a computer-generated pseudorandom signal ranging from 0.5 to 21 Hz. Lateral pressures were measured at the loudspeaker end ( $P_1$ ) and the distal end ( $P_2$ ) of the wave-tube with miniature sidearm transducers (ICS 33NA00D). These pressure signals were low-pass filtered (<25 Hz) and digitized at a sampling frequency of 128 Hz. The pressure transfer function ( $P_1/P_2$ ) was created by fast Fourier transformation from the 8-s recording. The input impedance of the respiratory system ( $Z_{rs}$ ) was computed from the pressure transfer function as the load impedance of the wave-tube ( $Z_L$ ) by using the transmission line theory ( $Z_L$ ):

$$Z_{rs} = Z_0 \cdot \text{sh}(\gamma L) / [P_1/P_2 - \text{ch}(\gamma L)]$$

where  $Z_0$  is the characteristic impedance of the wave-tube and  $\gamma$  is the complex propagation wavenumber; these were determined from the geometrical parameters of the wave-tube, and the material constants of the tube and the gas in it.

Three to five  $Z_{rs}$  spectra were ensemble-averaged under the baseline conditions and during steady-state methacholine infusions. Because of the absence of a plateau response after the intravenous ovalbumin challenge, parameters obtained from the  $Z_{rs}$  data collected 2 min after the ovalbumin injections were included in the analysis.

A distributed-parameter model (Exii) that includes airway inhomogeneities in the form of a continuous uniform distribution of airway resistances leading to identical airway inertances ( $I_{aw}$ ) and constant-phase tissue compartments (Exiii) incorporating tissue damping ( $G$ ) and elastance ( $H$ ) was fitted to the averaged  $Z_{rs}$  data. The data at frequencies coinciding with the heart rate and its harmonics were often corrupted, as evidenced by poor coherence and a high SD, and they were omitted from the model

fitting. The minimization of the squared sum of weighted differences between the measured and the modeled impedance data was accomplished by a global optimization algorithm (E13). Examples of the Zrs data and the corresponding model fits obtained under baseline conditions and during lung constriction induced by IV methacholine and ovalbumin in a representative animal are shown in Figure E2. The flow resistance of the airways was characterized by the arithmetic mean of the resistances of the parallel pathways (Raw) and the coefficient of variation (SDRaw/Raw) as the inhomogeneity index.

### *Study protocol*

Following baseline acquisitions of Zrs data and maps of regional ventilation, methacholine was administered by intravenous infusion using 2 protocols, A and B (Figure E3); protocol A: 2.5 followed by 5.0  $\mu\text{g/kg/min}$  in 6 control and 6 sensitized animals; protocol B: 10  $\mu\text{g/kg/min}$  in 6 control and 7 sensitized animals. The total methacholine infusion time was identical in the two groups. After a 30-min recovery from the effect of methacholine, a new set of data was collected prior to ovalbumin provocation (2.0 mg) in both groups, 2 min after which data acquisition was repeated. In order to standardize the volume history, single sighs defined as an inflation to a tracheal pressure of 30 cmH<sub>2</sub>O were performed at baseline, prior to methacholine challenge, upon recovery and before ovalbumin administration.

We compared IV Mch challenge with inhaled Mch (125 mg/ml, 90 s) in a separate group (n=5) of controls (Figure E2, C). In these animals, following baseline Zrs and image data acquisitions, a 125 mg/ml Mch (Sigma, St. Quentin Fallavier, France) solution in normal saline was administered using an ultrasonic nebulizer (SAM LS2000, Villeneuve sur Lot, France) for 90 seconds. The mass median aerodynamic diameter (MMAD) of the aerosol particles was 3.5  $\mu\text{m}$  with a geometrical standard deviation of 2.0, as determined by laser optical diffraction using the Malvern SprayTec, according to the manufacturer.

The stability of the experimental preparation and the mechanical and imaging parameters was studied in 2 sham-treated animals. Both mechanical and imaging parameters proved to be stable throughout the experiment in these animals.

### *Statistical analysis*

The scatters in the parameters were expressed by the SEM values. The Kolmogorov-Smirnov test was used to test data for normality. Both the mechanical and imaging parameters were normally distributed. Accordingly, two-way repeated measures analysis of variances (ANOVA), with variables of group (C and S) and methacholine dose was used to evaluate the changes in the mechanical and functional imaging parameters. Identical tests were applied to assess the effects of ovalbumin on the lung functional changes. Pairwise comparisons were performed by using Student-Newman-Keuls multiple comparison procedures. The parameters obtained from the lung histology were not distributed normally; the Mann-Whitney rank sum test was performed for these variables. Statistical tests were carried out with the significance level set at  $p < 0.05$ .

## FIGURES

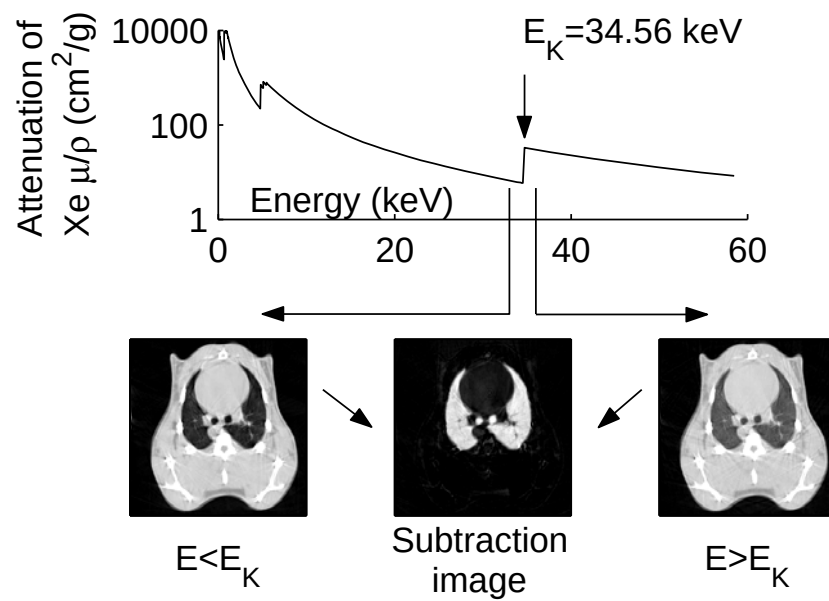


Figure E1.

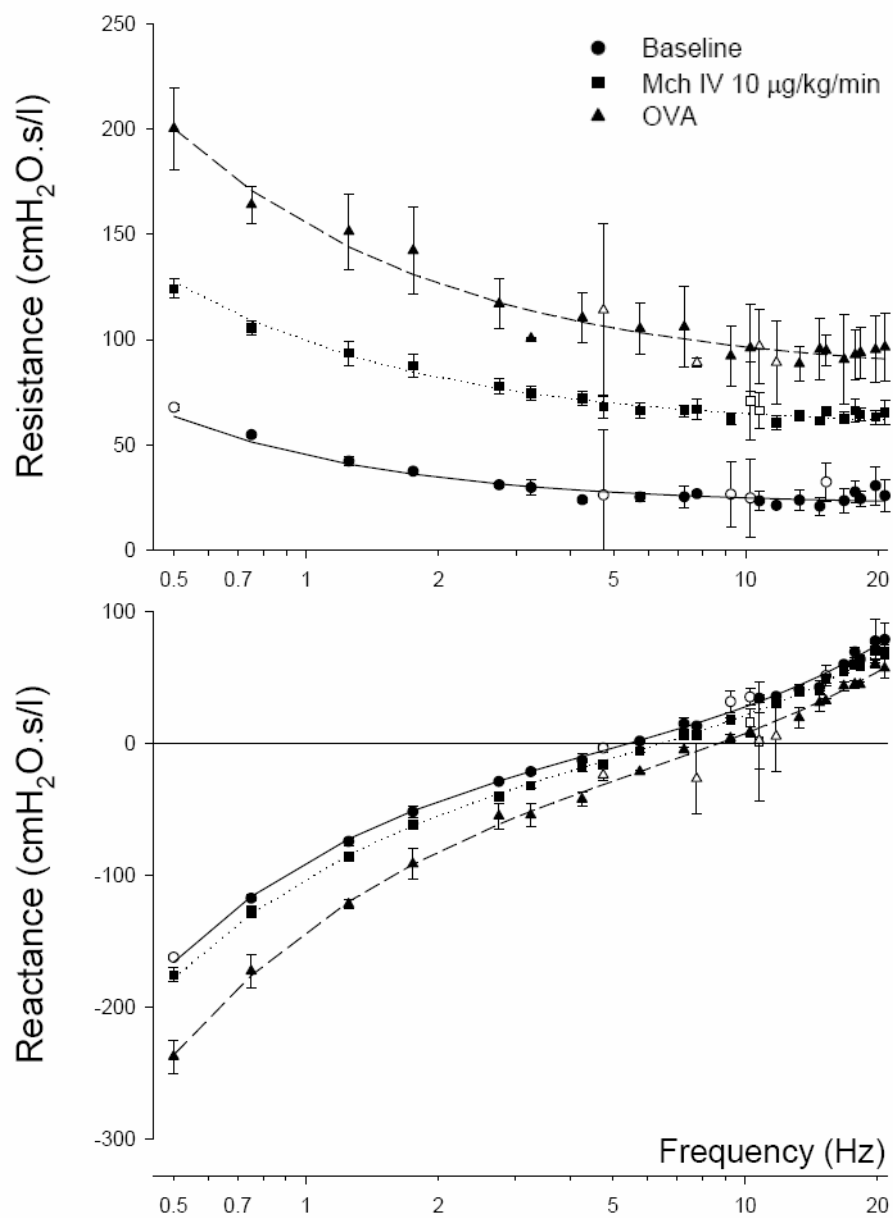


**Figure E2.**

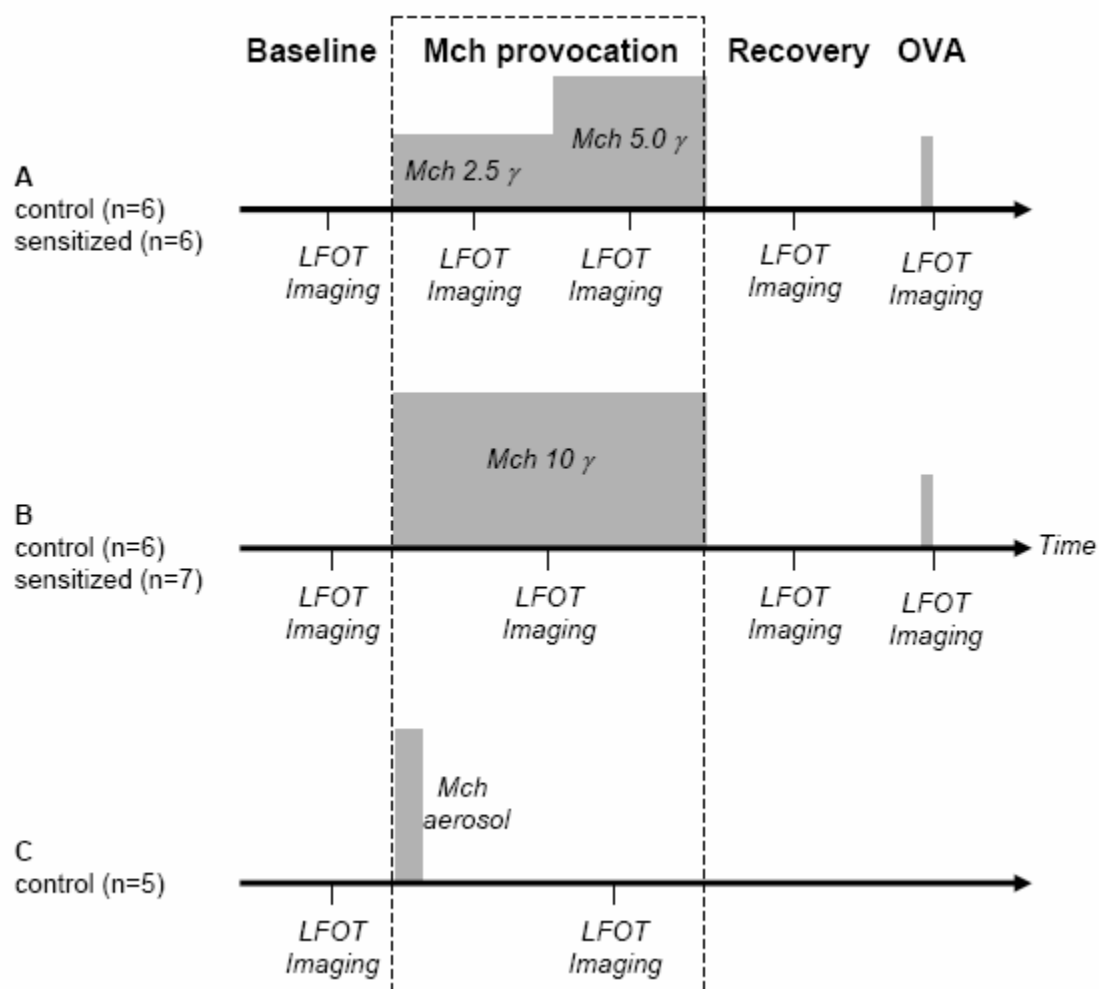


Figure E3.

**FIGURE LEGENDS**

**Figure E1:** In K-edge subtraction imaging (KES), two simultaneous CT images are acquired using two X-ray beams at two different energies above and below the K-edge of xenon. Absolute quantity of the contrast agent is determined directly on any given point of a lung CT image after subtracting these two images on a logarithmic scale. Reproduced with permission from (Evi).

**Figure E2.** Examples of the Zr data (symbols) and the corresponding model fits (lines) under baseline condition and following lung constriction induced by IV methacholine and ovalbumine obtained in a representative animal. Open symbols represent data points that were omitted from the model fit because of cardiac artifact..

**Figure E3.** Experimental protocol; LFOT: low-frequency forced oscillation technique; Mch: methacholine aerosol or infusion challenge; OVA: ovalbumin provocation.

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**REFERENCES**

- Ei. Peták F, Hantos Z, Adamicza A, Gálity H, Habre W. Development of bronchoconstriction after administration of muscle relaxants in rabbits with normal or hyperreactive airways. *Anesth Analg* 2006;103:103-109.
- Eii. Monfraix S, Bayat S, Porra L, Berruyer G, Nemoz C, Thomlinson W, Suortti P, Sovijärvi AR. Quantitative measurement of regional lung gas volume by synchrotron radiation computed tomography. *Phys Med Biol* 2005;50:1-11.
- Eiii. Porra L, Monfraix S, Berruyer G, Le Duc G, Nemoz C, Thomlinson W, Suortti P, Sovijärvi AR, Bayat S. Effect of tidal volume on distribution of ventilation assessed by synchrotron radiation CT in rabbit. *J Appl Physiol* 2004;96:1899-1908.
- Eiv. Giacomini JC, Gordon H, O'Neil R, Van Kessel A, Cason B, Chapman D, Lavendar W, Gmur N, Menk R, Thomlinson W, Zhong Z, Rubenstein E. Bronchial imaging in humans using xenon K-edge dichromography. *Nucl Instr and Meth in Phys Res A* 1998; 406:473-478
- Ev. Bayat S, Le Duc G, Porra L, Berruyer G, Nemoz C, Monfraix S, Fiedler S, Thomlinson W, Suortti P, Standertskjöld-Nordenstam CG, and Sovijärvi ARA. Quantitative functional lung imaging with synchrotron radiation using inhaled xenon as contrast agent. *Phys Med Biol* 2001;46:3287–3299.
- Evi. Bayat S, Porra L, Suhonen H, Nemoz C, Suortti P, and Sovijärvi ARA. Differences in the time course of proximal and distal airway response to inhaled histamine studied by synchrotron radiation CT. *J Appl Physiol* 2006;100:1964–1973.
- Evii. Simon BA, Marcucci C, Fung M, Lele SR. Parameter estimation and confidence intervals for Xe-CT ventilation studies: a Monte Carlo approach. *J Appl Physiol* 1998;84:709-716.
- Eviii. Majumdar A, Alencar AM, Buldyrev SV, Hantos Z, Lutchen KR, Stanley HE, Suki B. Relating airway diameter distributions to regular branching asymmetry in the lung. *Phys Rev Lett*. 2005;14;95(16):168101.
- Eix. Limpert E, Werner AS, Abbt M. Log-normal distributions across the sciences: keys and clues. *Bioscience* 2001;51:341-352.
- Ex. Peták F, Hantos Z, Adamicza A, Asztalos T, Sly PD. Methacholine-induced bronchoconstriction in rats: effects of intravenous vs. aerosol delivery. *J Appl Physiol* 1997; 82:1479-1487.
- Exi. Van de Woestijne, K. P., H. Franken, M. Cauberghs, F. J. Landser, and J. Clement. 1981. A modification of the forced oscillation technique. In: *Advances in Physiological Sciences. Respiration*. (Debreczeni L. and Hutás I, editors.) Proc. of 28th Int. Cong. of Physiol. Sci. Budapest Hungary, June 1980. Pergamon, Oxford, UK. 655-660.
- Exii. Suki B, Yuan H, Zhang Q, Lutchen KR. Partitioning of lung tissue response and inhomogeneous airway constriction at the airway opening. *J Appl Physiol* 1997;82:1349-1359.

Exiii. Hantos Z, Daróczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 1992;72:168-178.

