

**X-ray based lung function measurement reveals persistent loss of lung tissue elasticity in mice  
recovered from allergic airway inflammation**

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**Running head**

Loss of elastic recoil in lung tissue of recovered AAI mice

## **Abstract**

Chronic asthma patients experience difficulties even years after the inciting allergen. Although studies in small animal asthma models have enormously advanced progress in uncovering the mechanisms of inception and development of the disease, little is known about the processes involved in the persistence of asthma symptoms in the absence of allergen exposure. Long term asthma mouse models have so far been scarce or not been able to reproduce the findings in patients. Here we used a common ovalbumin induced acute allergic airway inflammation mouse model to study lung function and remodeling after a four months recovery period. We show by x-ray based lung function measurements that the recovered mice continue to show impaired lung function by displaying significant air-trapping compared to controls. High resolution synchrotron phase contrast computed tomography of structural alterations and diaphragm motion analysis suggest that these changes in pulmonary function are due to a pronounced loss in lung elasticity. Histology of lung sections confirmed that this is most likely caused by a decrease in elastic fibers, indicating that remodelling can develop or persist independently of acute inflammation and is closely related to a loss in lung function. Our findings demonstrate that this x-ray based imaging platform has the potential to comprehensively and non-invasively unravel long term effects in preclinical mouse models of AAI and thus benefits our understanding of chronic asthma.

## **Key words**

X-ray lung function, allergic airway inflammation, lung tissue elasticity, air trapping

## Introduction

The pathomechanism that leads to chronic asthma is still not completely understood. Preclinical research is therefore still vital to unravel processes underlying this complex and heterogeneous disease (2). The predictive value of the commonly used allergic airway inflammation (AAI) mouse models with regard to clinical outcome is controversially discussed. A major concern is that we compare a chronic situation in patients that has developed over years with an acute response in mouse models (15). While AAI mouse models have provided important insights in the mechanism of asthma exacerbation and are valuable tools to test novel treatments (12), they have largely failed to address long term effects (16). In chronic asthma patients structural changes in the lung are sometimes observed years after the inflammatory response has resolved, including increased thickness of the subepithelial reticular basement, hyperplasia and hypertrophy of smooth muscle cells as well as changes of the extracellular matrix (21). These characteristics of airway-remodeling are poorly examined in preclinical models as they require chronic and long-term investigations. In the few mouse models that do, the observed remodeling is primarily based on the development of fibrosis rather than on the cellular and structural changes known from patients data (16, 24).

Another important aspect in human chronic asthma is a persistent or long lasting loss in lung tissue elasticity even years after the last acute asthma attack as described by Gelb et al. (13). These patients still experience a reduction in their lung function and a corticosteroid treatment is not effective as it acts primarily on the inflammatory pathway of the disease (8).

We recently established a non-invasive lung function method based on planar cinematic x-ray imaging of the chest, x-ray based lung function (XLF), which we have successfully used

previously to assess the lung function of isoflurane anesthetized mice with AAI (11). For quantification of lung remodeling, the application of high resolution imaging tools has received increasing attention. In particular, inline free propagation synchrotron phase contrast CT (pSR $\mu$ CT) provides a more than 10-fold increased contrast-to-noise ratio than absorption based CT and has been effectively employed for in-situ mouse lung imaging (10, 17). Here we apply these novel techniques on AAI mice that recovered 4 months from allergen exposure. We demonstrate that a long term reduction in lung tissue elasticity can be found in these mice similar to the condition described in humans. Moreover, we show that this effect can be measured in vivo using the novel approach of XLF (11). We furthermore confirm the in vivo findings by observation of alterations in morphological structures using pSR $\mu$ CT and classical histology.

## **Methods**

### *Allergic airway inflammation (AAI) model*

Female BALB/c mice (6 - 8 weeks old) were used to generate an ovalbumin (OVA) (Hyglos) induced experimental allergic airway inflammation (AAI) model to mimic severe acute asthma (N=6) as described in Dullin et. al (10) and schematically outlined in Figure 1. Control mice received phosphate-buffered saline (PBS) instead of OVA during immunization and for the first 2 challenges (CN, N=6). Both groups were then kept without any additional treatment for 121 days. All animal in vivo procedures were performed in compliance with the guidelines of the European (2010/63/EU) and the German ethical laws and were approved by the administration of Lower Saxony, Germany (Nr. G15.1747).

*XLF – low dose x-ray based planar cinematic lung function measurement*

XLF was performed as previously described by Dullin et al. (11) by acquiring planar cinematic x-ray images of the chest of unrestrained but 2% isoflurane anesthetized mice using a low dose in vivo microCT (QuantumFX, Perkin Elmer). Briefly, the CT produces a movie from a sequence of 2D radiographs capturing the chest movements during breathing, depicting the x-ray transmission of the chest area. In principal, any x-ray tube with an attached camera and anesthesia could be used for this method. In these generated x-ray movies, regions of interests are placed on the left and the right lung lobes at the time point of maximum contraction of the chest and within the back ground. The x-ray transmission over time is calculated as average intensity in lung regions corrected and expressed as relative difference to the average intensity of the background region to account for short-term fluctuations of the x-ray tube. Thus the function is without a physical unit. In order to account for long term shifts in the x-ray production, the function is corrected for a slow varying background. The breathing frequency during acquisition is adjusted to one breathing event in 1400 ms and movies are captured for 34 s over about 21 breathing cycles for each mouse (Figure 2B). Each breathing peak is parameterized and the average of all cycles within the captured time frame is used to characterize the lung function of the mouse. Table 1 indicates the functions that each parameter relates to.

**Table 1: X-ray transmission function (XTF) and parameters derived from the XTF.** The relationship of the parameters to lung functions is indicated. AAI related changes in parameters are indicated in the last column. AuC=area under the curve of the breathing peak; L=length; BP=breath periodicity;

AnIso=anisotropy-index; ATrp=air-trapping

| $XTF(t) = b_2(t-b_1)^3 - b_3(t-b_1)^2 + b_4(t-b_1) + b_5$ |                                                  |        |
|-----------------------------------------------------------|--------------------------------------------------|--------|
| Parameter                                                 | Relates to/is influenced by:                     | AAI    |
| b1                                                        | shift of the curve/time of inhalation            | ▲      |
| b2                                                        | assymetry of the curve                           | ▼      |
| b3                                                        | speed of air exchange                            | ▼      |
| b4                                                        | assymetry of the curve                           | ▼      |
| b5                                                        | air volume/inflammation/air-trapping             | ▼      |
| L[s]                                                      | breath length                                    | ▲      |
| AuC                                                       | total air volume/inflammation                    | ▼      |
| AnIso                                                     | anisotropy-index (time of inhalation/exhalation) | ▼      |
| BP                                                        | periodicity of the breathing events              | const. |
| tpeak[s]                                                  | time to reach maximum inhalation volume          | ▼      |
| tin[%]                                                    | relative time of inhalation (tpeak/L)            | ▼      |
| ATrp                                                      | air-trapping                                     | ▲      |

In addition to the parameters  $b_1$  -  $b_5$  reported by us previously (11), we recorded the mean length of the breathing events **L** and analyzed the average x-ray transmission of the mouse chest between the breathing events (**ATrp**) in order to quantify air-trapping (Figure 2A). XLF was performed shortly before the 1. challenge as well as two days and 121 days after the last challenge (Figure 1). The applied software program for XLF is available at DOI 10.17605/OSF.IO/BYS3H.

### *Diaphragm motion analysis*

Diaphragm motion analysis was performed by calculating the average and the standard deviation (Std) of the brightness per pixel over time in the acquired x-ray movies. As the motion of the diaphragm causes the movement of the lower border of the lung it can be detected as an

area of a strong Std (Figure 4A and B, black arrows). The width of this area represents the average diaphragm movement ( $s_{dia}$ ). Because the duration of the breathing event is shorter than the periods between the breathing events, the average of the x-ray transmission over time predominantly depicts the size of the lung in exhalation ( $A_{Lung}$ ).

#### *In-situ lung imaging by synchrotron inline free propagation phase contrast CT (pSR $\mu$ CT)*

Following the last XLF measurement, mice were sacrificed. Tracheotomies were then performed and the lungs were filled with air at a constant pressure of 30 cm water column. The entire mice were then embedded in 1% agarose gel in 50 ml tubes as described by Dullin et al. (9). In order to prevent alterations in the elastic properties of the lung tissue no fixatives such as formalin or ethanol were added. Before acquisition, the temperature of all samples was equalized to room temperature. The samples were then imaged at the SYRMEP beamline (Synchrotron Light Source 'Elettra', Trieste, Italy) with the following parameters: E = 22 keV, 360° off-center scan with 1800 angular projections, detector exposure time per projection 2s and a sample-to-detector distance of 30 cm to allow for the formation of phase effects. Prior to 3D reconstruction a single distance phase retrieval algorithm (14) was applied (delta-to-beta ratio 1950) resulting in the generation of 3D data sets with a voxel size of 9×9×9  $\mu\text{m}^3$  predominately expressing the distribution of the  $\delta$ -part of the complex refractive index within the sample (about 10 times more sensitive in lungs than using standard absorption based (18). In the reconstructed data sets the soft tissue **vol.ratio** was quantified by calculating the average volume ratio of the lung soft-tissue in relation to the total volume of eight non-overlapping

volumes-of-interests (VOIs) of 2 mm × 2 mm × 2 mm that were placed uniformly in the peripheral region of the lung as described in (10).

#### *Diaphragm motion analysis*

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#### *Histology*

Following pSRμCT, the lungs were excised, fixed in 10% buffered formalin and embedded in paraffin. Three μm-thick paraffin lung sections containing main stem bronchi were used for staining procedures. Periodic-acid Schiff (PAS) staining was performed according to the manufacturer's protocol (Merck). A Masson trichrome kit (Sigma Aldrich) was used to stain collagen for assessment of airway fibrosis. Sections were microscopically scored from 0 to 3 in a blinded fashion by two observers to evaluate the amount of mucus producing goblet cells (designated PAS-score) and the amount of collagen in the Masson trichrome staining (MT score), with 0 = no mucus and no collagen, 1 = low mucus and collagen, 2 = medium mucus and collagen, 3 = high mucus and collagen. An Elastica van-Gieson kit (Morphisto) was used for

staining of elastic fibers, which were counted in respiratory bronchioles and alveolar ducts in 10 separate views per histological slide (AAI-R, N=5; CN-R, N=5). Alpha smooth-muscle actin was stained by anti-mouse- $\alpha$ SMA antibody (Abcam, ab5694, 1:500).  $\alpha$ SMA expression was quantified in the following way: 10 small bronchioles per section were selected with approximately the same size. Using ImageJ the area of the epithelial cells surrounding a bronchus was segmented based on its color. The cross-section of the bronchus was approximated with an ellipse. In order to calculate the thickness of the  $\alpha$ SMA positive epithelial layer, the segmented area was divided by the circumference of the calculated ellipse.

#### *Data processing and statistics*

For statistical analysis, the unpaired Welch t-test for same mean implemented in MS Excel 2010 was used with a *P*-value of 0.1 as margins for statistical significance. In order to measure correlation, the Pearson correlation coefficient  $R^2$  implemented in MS Excel 2010 was used. The XLF algorithm is implemented in Matlab 2011b (7.13.0.564).

#### **Results and Discussion**

Long-term effects of acute allergic inflammation are scarcely investigated in animal models and the long lasting loss in lung tissue elasticity in humans is not well understood. To elucidate the long-term effects of AAI we used several x-ray based methods to examine the lungs of mice after 4 months recovery period from an acute allergic ovalbumin induced reaction. XLF is an approach which allows a very sensitive evaluation of functional alterations in lung disease in-vivo over time. Due to the combined influence of functional and morphological alterations on

the measured parameters, XLF provides additional information when compared to other lung function methods such as plethysmography. pSR $\mu$ CT provides 3D and high resolution images of anatomical changes.

#### *XLF reveals sustained air-trapping after recovery from AAI*

In order to assess the lung function of mice over time we used XLF, a novel non-invasive technique that we have recently employed for the same experimental asthma mouse model (11). During the process of inspiration and expiration, the air content of the lung changes over time, which modulates the x-ray transmission over the chest region, exemplified in the radiographs in Figure 2A. This change in x-ray transmission can be captured as a parameterized x-ray transmission function (XTF, Methods, Table 1) over about 21 breathing cycles (Figure 2B). We performed XLF on isoflurane anesthetized mice with allergic airway inflammation (AAI, N=6) in comparison to control mice (CN, N=6) before the first challenge (AAI-BC and CN-BC) and two days after the last challenge (AAI-AC and CN-AC). The same groups were then left to recover for four months and lung function was measured once more by XLF (AAI-R and CN-R) before sacrifice of the mice (Figure 1) (11). An example of one breathing cycle of a mouse from each of the 4 groups (AAI-AC, AAI-R, CN-AC, CN-R) is shown in Figure 2A (middle panel). There are significant differences in the x-ray transmission curves of the different groups, which are reflected in modifications of the shape and area under the curve of the XTF during the breathing events and changes of the baseline of the function in exhalation state.

Figure 2C shows the results of the measured XLF parameters as relative differences of AAI lungs to their respective controls. We found a significant decrease in parameters **b<sub>2</sub>-b<sub>5</sub>** as well as an

increase in  $L$  between AAI-BC and AA-AC lungs. As the parameters  $b_3$  and  $b_5$  describe how much air is transported through the lungs in which time, the reduction in  $b_3$  and  $b_5$  values seen in the acute inflammatory phase of AAI-AC mice is related to lower air content and a longer exchange time within the lung due to an increase in soft-tissue. This increase in soft tissue content is most likely the result of a combination of airway thickening, goblet cell hyperplasia/hypertrophy and immune cell infiltration, all processes observed by us and others in this model (10, 20).

The parameters  $b_2$  and  $b_4$  both account for asymmetry in the XTF. They reflect differences in the ratio between the inhalation and exhalation time as well as changes in the slope of decay of the XTF in the exhalation period. Both parameters were significantly decreased in the AAI-AC mice (acute phase of AAI) when compared to the time point before challenge (AAI-BC), reflecting a change in the ratio between inspiration and exhalation time (Figure 2C). The observed altered value of  $L$ , representing the change in breathing length, supports this result, by showing a significantly longer total breathing time in AAI-AC mice (Figure 2C). Combined, these results are in agreement with our earlier XLF findings (11) and reflect the acute decrease of lung function that occurs two days after challenge with OVA, due to the morphological changes that take place during the inflammatory phase, such as swelling of the airways and cell infiltration, and that affect the x-ray transmission function.

As a new parameter we determined **ATrp**, which describes the average x-ray transmission in the exhalation state. If air-trapping occurs, the air content is higher in the exhalation state, resulting in increased x-ray transmission and thus in a higher **ATrp** value (Figure 2A, middle panel). The increased **ATrp** parameter in AAI-AC mice (Figure 2C) indicates the presence of air-trapping at the acute state of the disease. However, the thickening of the airway walls due to the present

inflammatory response causes a reduction of the x-ray transmission and therefore interferes with the measurement of the air-trapping parameter. This probably results in lower **ATrp** values than could be expected for this acute phase of AAI mice, albeit still substantially higher than in AAI-BC mice.

When we examined the same mice by XLF four months after the last challenge (AAI-R), we found that most of the XLF parameters had indeed “recovered”, showing levels in parameters **b<sub>2</sub>-b<sub>5</sub>** and **L** similar to those in AAI-BC mice before challenge (Figure 2C). This is in good agreement with the expectation that pulmonary inflammation has subsided, leading to a decrease of tissue swelling and therefore an increase in the air content (4). Remarkably, the parameter **ATrp** did not change after four months of recovery in AAI-R mice and was found to be significantly elevated compared to **ATrp** before challenge in AAI-BC mice (Figure 2C). This strongly suggests that the recovered AAI-R mice still have abnormal retention of air in the lungs after expiration, indicative of air-trapping, a characteristic feature which is commonly known from patients with chronic severe asthma (5, 24). All other XTF parameters (Methods, Table 1) were not significantly changed in the recovered group.

Our XLF results also illustrate the advantages of this new imaging method for long-term in vivo experiments, as it provides the possibility of non-invasive lung function measurement of the same animal over time. Preclinical lung function analysis to date has had its limitations, because currently used plethysmography is either invasive or insensitive in the case of unrestrained plethysmography (17). By contrast, XLF was able to sensitively detect subtle changes in pulmonary function caused by remodeling of the lung long after inflammation has resolved. To

our knowledge, air-trapping long after resolution of inflammation has not been detected in vivo in experimental asthma mouse models before.

*pSRμCT reveals loss of lung elasticity after recovery from AAI*

To assess whether our XLF findings in recovered mice are associated with subtle structural changes, we performed synchrotron inline free propagation phase contrast CT (pSRμCT) on in situ lungs, a method that exploits the coherence properties of synchrotron radiation. In contrast to classical x-ray based techniques which depict differences in the x-ray attenuation within the sample, pSRμCT in combination with phase retrieval algorithms produces data sets predominately demonstrating differences in the refractive index within the sample. It has been demonstrated, that especially for low absorbing tissue with strong gradients in the refractive index such as the lung, pSRμCT outperforms classical CT in terms of contrast-to-noise ratio (18). Thus, using pSRμCT we produced high resolution images of the in-situ mouse lung (10), as exemplified in Figure 3A. In previous experiments using pSRμCT on lungs of mice with AAI we repeatedly demonstrated a significant increase in the soft-tissue volume ratio (**vol.ratio**) of AAI lungs compared to controls at the acute phase of AAI (2 days after challenge), owing to the inflammation derived thickening of the bronchi and infiltration of immune cells (10, 11). Interestingly, we found a significantly reduced **vol.ratio** in recovered AAI-R lungs compared to CN-R controls (Figure 3B), suggesting more air in recovered lungs than in those of controls. This may seem like a contradiction in terms, but is actually plausible on closer inspection of our preparation method: the lungs of the mice are inflated with a constant pressure and are embedded in this state in agarose before measurement by pSRμCT. The unexpected result of a

higher air volume in AAI-R then strongly suggests that the lungs of recovered mice are easier to inflate because they are less elastic than the control lungs, i.e. had less elastic recoil, leading to higher air content in the lungs. As the mice are all age and gender matched, they should have similar lung volumes. Moreover, as confirmed by H&E and PAS staining of lung sections, airway inflammation has largely resolved by this time (data not shown). Therefore, soft tissue volume changes are not present and even if they were, they would increase the **vol.ratio** of AAI-R instead of decreasing it. The observed relative increase in air volume is therefore most likely explained by a loss in lung tissue elasticity in the recovered mice. We believe, these pSRμCT results reflect the persistence of airway remodeling after resolution of the inflammatory response as found in chronic asthma patients (7). As the measured soft-tissue includes the circulatory vessels and airways we cannot entirely exclude that the change in the ratio could reflect or be influenced by a change in the vasculature or bronchial walls. However, as we measure the vol.ratio in the periphery of the lung where blood vessels and airways are very small, we believe that both of these are likely to be minor or non-contributing.

We further found a high degree of correlation between the pSRμCT **vol.ratio** and the XLF **ATrp** results of recovered mice, with a coefficient of determination  $R^2$  as high as 0.8 (Figure 3C), suggesting that both parameters are influenced in the same way by changes in the lung elasticity ( $ATrp \sim k1 * vol.ratio^{-1}$ ). This is further corroborated by the observation that two mice in our AAI-R cohort concurrently revealed high pSRμCT values and low ATrp values close to those of the control group.

*Reduced diaphragm motion persists after recovery from AAI*

To elucidate the persistent loss of lung elasticity in recovered mice, we performed a diaphragm motion analysis using the same data sets that we acquired for XLF. While analysis of the movement of the lung itself is not possible from the acquired movies, the movement of the diaphragm-lung interface can be detected even at a low dose, because it resembles a simple gray value edge. Due to the strong contrast of the edge, its movement causes a strong standard deviation of the x-ray attenuation over time. Figure 4 representatively displays the average (Figure 4A and B, left panels) and the standard deviation (Figure 4A and B, right panels) of the x-ray transmission over time for one AAI and one CN mouse before challenge (BC), after challenge (AC) and after 121 days of recovery (R). It was apparent that in comparison to controls, mice with acute AAI had a nearly 20% larger lung area  $A_{\text{Lung}}$  in exhalation two days after challenge (AAI-AC, Figure 4C), which is composed of a 12% increase in the length of the lung  $h_{\text{Lung}}$  (Figure 4D) but an only 2% increase in width  $w_{\text{Lung}}$  (Figure 4E), as measured in the average x-ray transmission images. This suggests that in AAI-AC the diaphragm must sit in a lower position, most probably due to the reduced counterforce that the less elastic lung provides.

The diaphragm motion analysis also revealed that AAI-AC mice try to counteract this effect by activating the intercostal muscles (white arrow in Figure 4A).

In the lungs of the recovered mice AAI-R, the lung area ( $A_{\text{Lung}}$ ) including width and length of the lung ( $w_{\text{Lung}}$ ,  $h_{\text{Lung}}$ ) appear normal again. However, the motion of the diaphragm ( $s_{\text{dia.}}$ ) as measured in the standard deviation images (black arrow heads) is significantly reduced by 15% when compared to controls (Figure 4F), pointing to a persistent loss of lung tissue elasticity. While previous reports in humans have attributed a change in diaphragm position to a change in

lung volumes (14), we believe that cause and effect are reversed: the diaphragm is lower because of the loss in elastic recoil and this drop causes a larger lung volume and consequently air-trapping at exhalation.

From these results, we propose the following model, schematically represented in Figure 4G:

When air is drawn into the lungs, the muscles in the diaphragm contract and pull the central tendon inferiorly into the abdominal cavity. This enlarges the thorax and allows air to inflate the lungs. When the diaphragm relaxes, air is exhaled by elastic recoil of the lung and the tissues lining the thoracic cavity. If the lungs lose elastic recoil, the diaphragm will drop, because it cannot move back the complete distance. As a result, less air is exchanged and more air remains in the lungs. This condition appears to persist long after the acute allergen challenge (Figure 4H).

*Histologic alterations confirm loss of lung elasticity after recovery from AAI*

We then went on to investigate mechanisms underlying the observed loss of elasticity in AAI-R mice by performing a panel of histological stainings on lung paraffin sections.

PAS staining showed that neither CN-R nor AAI-R lungs contained any mucus or hyperplasia of goblet cells (data not shown). As well, no significant difference in infiltrating immune cells in the lung was found by H&E staining (data not shown). Both these results confirmed that the acute allergic inflammation had resolved four months after recovery.

We found no apparent presence of fibrosis in AAI-R lungs as judged by the amount of Masson trichrome stained collagen, which was not significantly different to that found in control CN-R lungs (Figure 5, upper row). The expression level of  $\alpha$ -SMA, which we quantified in small

airways, was found to be significantly decreased in AAI-R lungs (Figure 5, middle row). This result would appear to contradict earlier reports that describe an increase in airway smooth muscle mass as well as  $\alpha$ -SMA expression in both asthmatic humans and mice with AAI (3). However, none of the previous studies examined the lungs at a time-point long after recovery from the acute inflammatory process. In fact, a decreased  $\alpha$ -SMA expression as we found in recovered AAI-R mice would be in line with the reduced lung elasticity that we observed by pSR $\mu$ CT and diaphragm motion analysis. While an increase in  $\alpha$ -SMA during the acute phase of AAI (as previously found) may well be a result of inflammatory stimuli (21), a decrease at a time point months after recovery may be a long lasting effect of an active muscle degradation that could take place after inflammation.

Most importantly, we found a highly significant reduction in numbers of elastic fibers in respiratory bronchioles and alveolar ducts of AA-R lungs (Figure 5, lower row). This result suggests that the persistent loss in lung elasticity in recovered mice may – at least in part – be due to a loss in elastic fibers. In COPD the destruction of elastin through the release of neutrophil elastase and metalloproteinases from inflammatory cells is well known as a leading mechanism of alveolar destruction in the lungs (6). In asthma, there have been conflicting reports of the state of elastin in the airway wall, with some describing increased elastin and others decreased or unchanged elastin in some layers of the airway (23). There are, however, several studies that indicate an association of neutrophil elastase activity with asthmatic subjects: It was found that in sputum obtained from asthmatic patients, the levels of both total and active elastase were significantly increased as compared with those of control subjects and were significantly correlated with the percentage of neutrophils (26). Moreover, a systemic

upregulation of neutrophil protease expression was found in patients with neutrophilic asthma (1) and paediatric asthma patients with epithelial damage displayed a particularly high concentration of neutrophil elastase (27). Monteseirin et al. challenged neutrophils in vitro with the specific allergens that produced clinical symptoms in asthmatic patients and found that these elicited a release of elastase in a highly allergen specific manner (19). These reports indicate that an upregulation of elastase during exacerbation may well contribute to a degradation of elastic fibers. Our findings indicate that at least at the layer of alveolar ducts a reduction of elastic fibers is evident and could be caused by a permanent damage during the acute phase of the allergic reaction.

In summary, our data indicate that - at least in mice - measurable sustained loss of lung function and remodeling is not dependent on chronic repeated allergen exposure but present 4 months after a single episode of allergen challenges. Hence our results support former suggestions (24), that remodeling can develop or persist independently of acute inflammation and is more closely related to lung function than inflammation is.

## **Conclusion**

In conclusion, our work shows that AAI mice display air-trapping, most likely due to a loss of lung elasticity, after months of recovery from allergic inflammation, mirroring the observation found in patients with chronic asthma. Moreover, we suggest that a persistent loss in lung elasticity in recovered mice may – at least in part – be due to a loss in elastic fibers. With this work, we demonstrate that our non-invasive XLF imaging approach is a reliable and

389 comprehensive in vivo technique for longitudinal assessment of lung function in experimental  
390 asthma.

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## Figure legends

**Figure 1: Timeline of AAI model.** Immunisation and challenge scheme as well as time points of XLF measurements are shown in days (d). OVA = ovalbumin; AIOH = Aluminiumhydroxide; XLF = x-ray lung function; AAI-BC = time point before challenges; AAI-AC = time point after challenges; AAI-R = time point after 121 d of recovery; i.p. = intraperitoneal; i.n. = intranasal.

**Figure 2: XLF of recovered AAI-R mice.** (A) Representative x-ray images of a mouse lung in both, exhalation state (left panel) and inhalation state (right panel) and an example of a x-ray transmission curve derived from one breathing cycle of each AAI-AC, AAI-R, CN-AC and CN-R mouse (middle panel). The time points of the depicted breathing states are marked in the CN-AC curve. (B) 21 breathing cycles were recorded for each mouse. (C) Relative differences within the parameters of XLF ( $b_2$ - $b_5$ , L, ATrp) of mice with AAI before challenge (AAI-BC), after challenge (AAI-AC) and after 4 months of recovery (AAI-R) are shown in comparison to the average in the control group at the same time points (red line). Parameters  $b_2$ - $b_5$  of AAI-R show similar values to those of AAI-BC, suggesting normal lung function. ATrp of AAI-R is significantly higher to AAI-BC, indicating persistent air-trapping in AAI-R mice. N numbers are shown in brackets. [au]: arbitrary units. \*  $P < 0.1$ ; \*\* $P < 0.05$

**Figure 3: pSR $\mu$ CT of recovered AAI-R mice.** (A) Volume rendering of a phase-retrieved data set of an AAI-R lung by pSR $\mu$ CT with a pixel size of 9 $\mu$ m exemplary shows that structural changes within mouse lungs can be studied in-situ at very high resolution. (B) The soft tissue vol.ratio

was significantly higher in CN-R lungs than in AAI-R lungs due to the loss of elasticity in AAI-R lungs and the consequential increase in inflation. (C) The soft tissue vol.ratio measured by pSR $\mu$ CT highly correlated with the lung function parameter ATrp. Red crosses represent AAI-R lungs; blue triangles represent CN-R lungs.

**Figure 4: Diaphragm motion analysis** of AAI mice and CN mice before challenge (AAI-BC; CN-BC), after challenge (AAI-AC; CN-AC) and after 4 months of recovery (AAI-R; CN-R). (A-B) Representative average x-ray transmission images (left panels) and standard deviation (SD) images (right panels) of AAI mice (A) and of CN mice (B) are shown. Black arrows show the SD used to calculate the motion of the diaphragm  $\Delta s_{dia}$ . White arrow shows that AAI-AC mice counteract the larger lung volume/loss of elasticity by activating the intercostal muscles. (C-E) The lung area  $A_{lung}$ , the length of the lung  $h_{Lung}$  and the width of the lung  $w_{Lung}$  are calculated from the average x-ray transmission images and are presented relative to the mean of the respective control animals (red line). AAI-AC mice have a nearly 20% larger  $A_{lung}$  than AAI-BC mice (C), which is composed of a 12% increase in  $h_{Lung}$  (D) and an only 2% increased width  $w_{Lung}$  (E). (F) The motion of the diaphragm ( $s_{dia}$ ) was determined from the Std images and was significantly reduced by 15% in AAI-R mice, pointing to a persistent loss of lung tissue elasticity. (G) Model showing that due to the loss of lung elastic recoil, the diaphragm of AAI mice is unable to move back to its natural position. (H) While inflammation subsides shortly after challenge (AAI-AC), a loss of elasticity is most probably caused during the acute allergic reaction and persists long after recovery (AAI-R).

537 **Figure 5: Presence of collagen,  $\alpha$ -SMA, and elastic fibers in lung sections.** A representative  
538 image of Masson trichrome (MT) staining (upper row), anti- $\alpha$ -SMA immunohistochemistry  
539 (middle row) and Elastica van-Gieson staining (lower row) is shown for AAI-R (left panel) and  
540 CN-R (middle panel) lung sections. Quantification of the different stainings (box plots, right  
541 panel) reveals the significant increase in  $\alpha$ -SMA expression and in number of elastic fibers in  
542 AAI-R lungs (N=5) as compared to CN-R (N=5). Stars show examples of counted elastic fibers. aw  
543 = airway; FOV = field of view; A = area; u = circumference. \*\*\* $P < 0.005$ ; \*\*\*\* $P < 0.001$ .

Figure 1

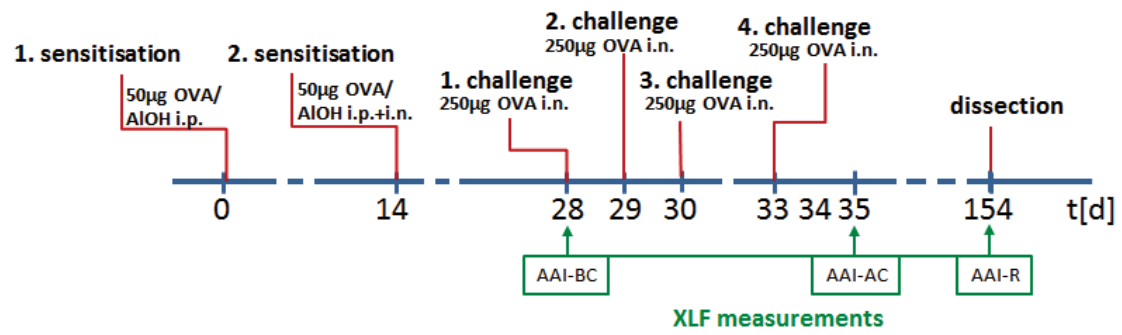
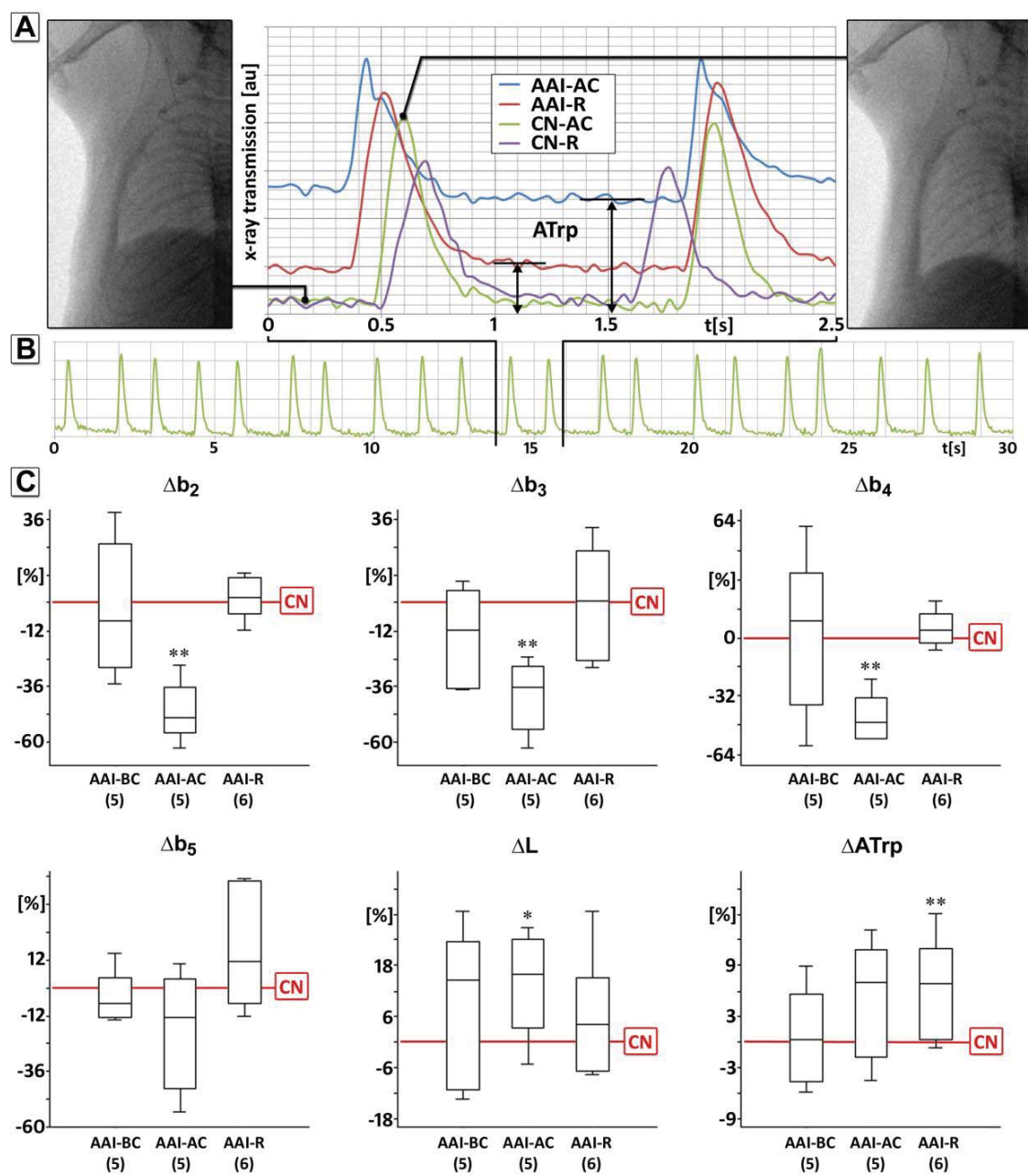


Figure 2



### Figure 3

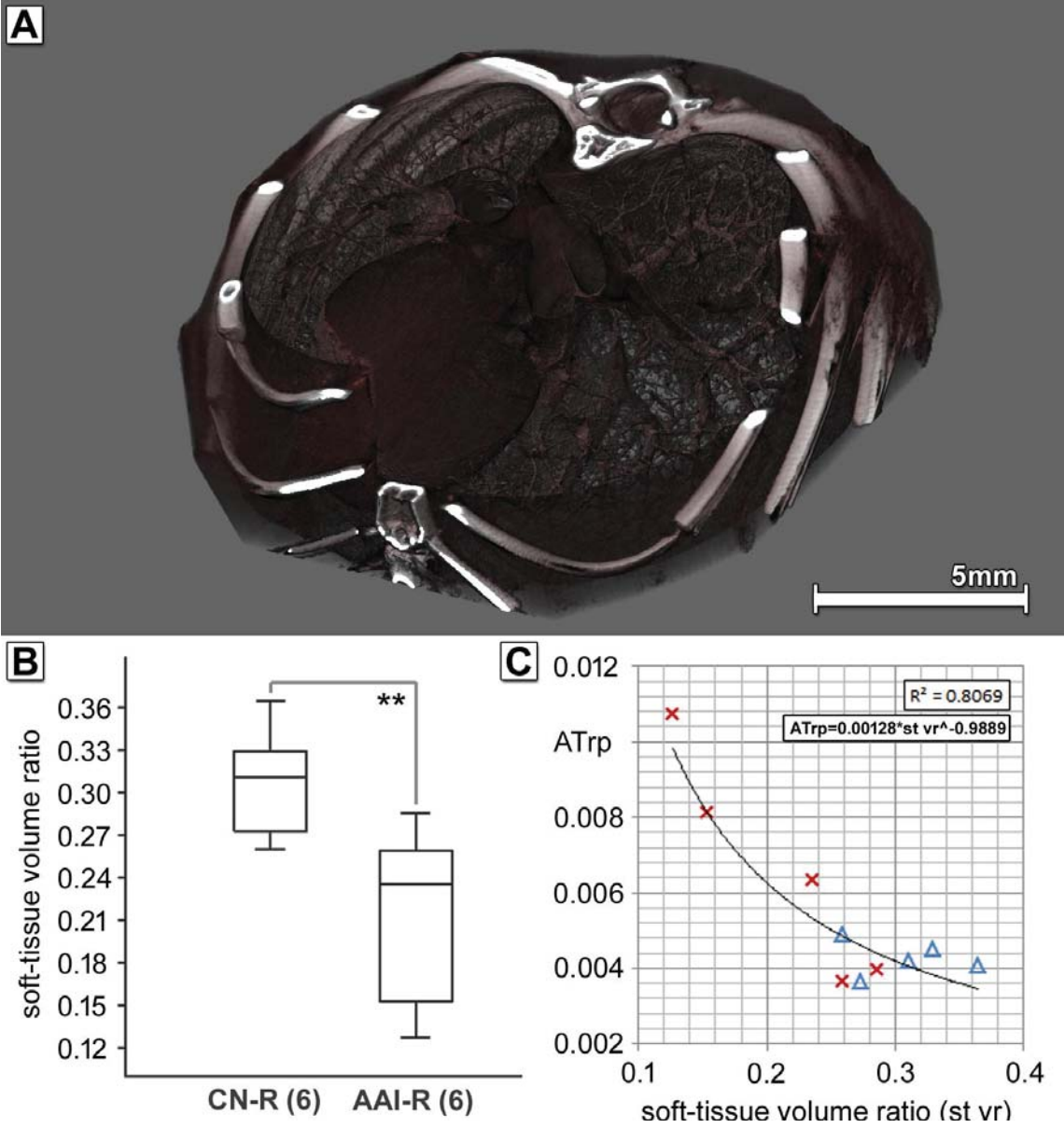
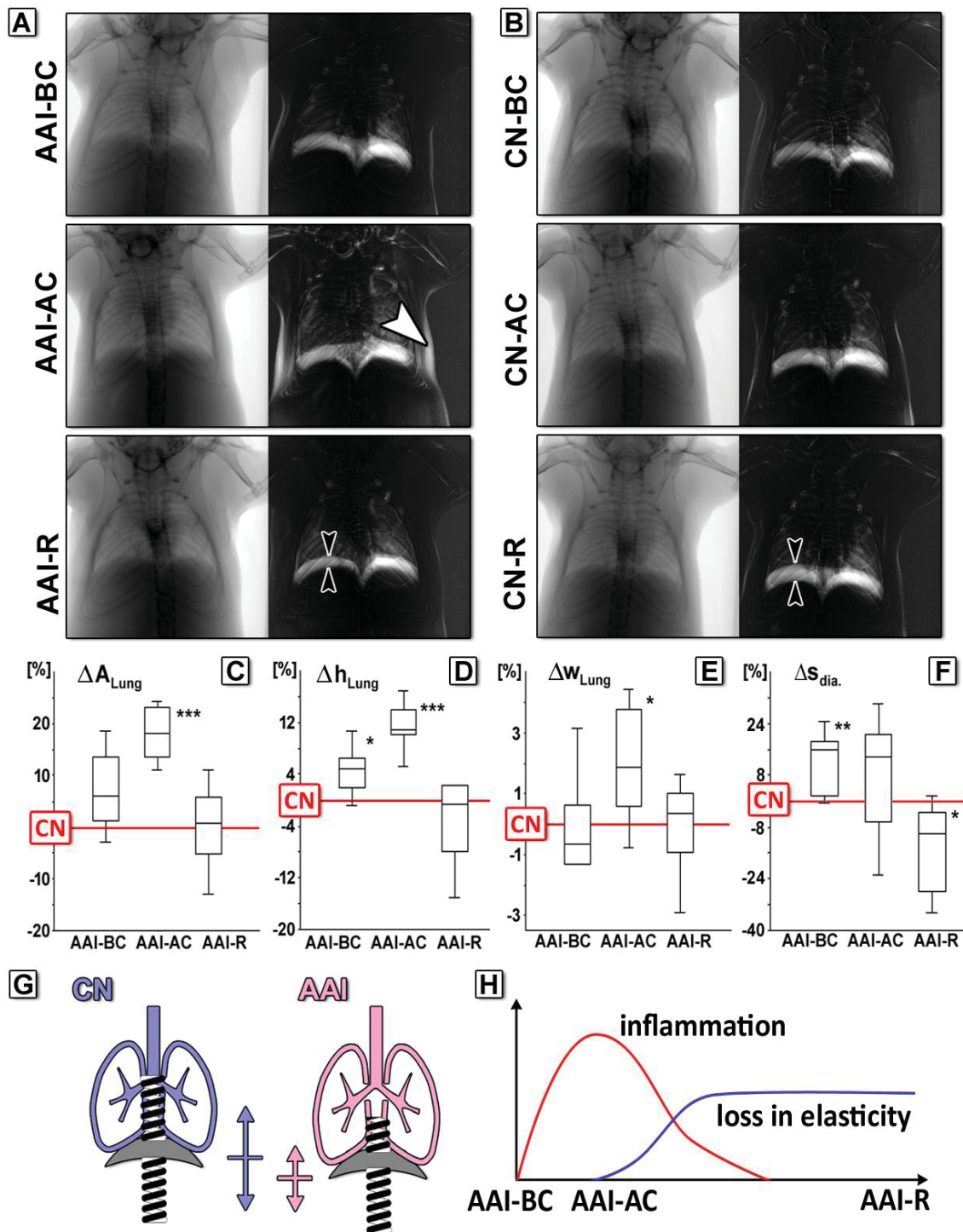


Figure 4



### Figure 5

