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2008

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### *Citation for published version (APA):*

Rydell-Törmänen, K. (2008). *Remodeling of the pulmonary circulation - a novel response to allergic airway inflammation*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Inst för Experimentell Medicinsk Vetenskap, Lunds Universitet.

### *Total number of authors:*

1

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# Remodeling of the pulmonary circulation - a novel response to allergic airway inflammation

Akademisk avhandling

av

**Kristina Rydell-Törmänen**

Som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap offentlig försvaras i Belfragesalen, Biomedicinskt centrum, Klinikgatan 30, Lund, torsdagen den 20 mars 2008, kl 9.00.

## **Fakultetsopponent**

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Department of Anatomy  
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UNIVERSITET**  
Medicinska fakulteten

Organization LUND UNIVERSITY Department of Experimental Medical Science Unit of Airway Inflammation BMC D12, S-221 84 Lund		Document name DOCTORAL DISSERTATION	
Author(s) Kristina Rydell-Törmänen		Date of issue March 20, 2008	
		Sponsoring organization	
Title and subtitle Remodeling of the pulmonary circulation - a novel response to allergic airway inflammation			
Abstract Asthma is characterized, not only by inflammation but also by airway and vascular remodeling. Airway remodeling is established early in disease, structural alterations have been found in children, and is thought to contribute to asthma symptoms. Unfortunately, airway remodeling is considered difficult to reverse and it seldom resolves completely. Studies of vascular involvement in asthma have mainly focused on the tracheal and bronchial microcirculation, as these vessels are relatively easy to obtain. Some scattered studies have investigated bronchial and pulmonary arteries, using autopsy specimens. The overall aim of the project was to investigate vascular remodeling by utilizing two different animal models of allergic airway inflammation (where chicken egg albumin (OVA) or house dust mite (HDM) extract were used as allergens). More specifically the structural alterations of remodeling, the time frame of development and the resolution were investigated. The studies show that vascular remodeling is a feature of allergic airway inflammation, despite which allergen used to initialize the inflammatory response. Both the systemic tracheal microvessels as well as all parts of the pulmonary circulation undergo changes. The structural alterations of vascular remodeling induced by allergic airway inflammation, was similar to features as seen in airway remodeling. Vascular remodeling appears to be only partially reversible, as some structural alterations seem to remain even when the allergic inflammation is resolved.  In summary this thesis describes the involvement of the vasculature in airway inflammation, characterizes vascular remodeling and shows that similar structural alterations are induced by two different allergens.			
Key words: Vascular remodeling, asthma, OVA, HDM, animal models, $\alpha$ -smooth muscle actin, procollagens I and III, proliferation			
Classification system and/or index termes (if any):			
Supplementary bibliographical information:		Language English	
ISSN and key title: 1652-8220		ISBN 978-91-85897-88-9	
Recipient's notes		Number of pages 180	
		Price	
		Security classification	

Distribution by (name and address)

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Signature Kristina Rydell-TörmänenDate February 14, 2008

# **Remodeling of the pulmonary circulation - a novel response to allergic airway inflammation**

Doctoral Thesis

by

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**LUND  
UNIVERSITY**  
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Lund, Sweden, 2008

ISSN 1652-8220  
ISBN 978-91-85897-88-9

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Lund University, Faculty of Medicine Doctoral Dissertation Series 2008:35

*To Markus & Albin*

The front of the cover illustrates vascular remodeling of small solitary vessels, triple stained for  $\alpha$ -smooth muscle actin (red), procollagen I (green) and the nucleus marker H33342 (blue), following 7 weeks of HDM-exposure. Yellow cells are co-positive for  $\alpha$ -smooth muscle actin and procollagen I.

The back of the cover illustrates vascular remodeling of small solitary vessels, triple stained as described above, following 20 weeks of HDM-exposure.

***The only way to discover the limits of the possible is  
to go beyond them into the impossible***

*Arthur C. Clarke*



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## Original papers

This thesis is based on the following papers, which will be referred to in the text by their respective roman numerals

- I. **Tormanen, K.R.**, Uller, L., Persson, C. G. A., Erjefält, J.S. Allergen exposure of mouse airways evokes remodeling of both bronchi and large pulmonary vessels. *Am J Respir Crit Care Med.*, 2005, 171:19-25
- II. **Rydell-Törmänen, K.**, Uller, L. Erjefält, J.S. Remodeling of extra-bronchial lung vasculature following allergic airway inflammation. *Respir Res.*, 2008, 9:18
- III. **Rydell-Törmänen, K.**, Uller, L. Erjefält, J.S. Vascular plasticity and partial resolution of vascular remodeling induced by allergic airway inflammation. *Clin Exp All.*, 2008. *Resubmitted*
- IV. **Rydell-Törmänen, K.** Johnson, J. R., Fattouh, R., Jordana, M., Erjefält, J.S. 2007. Induction of vascular remodeling in the lung by chronic house dust mite exposure. *Am J Respir Cell Mol Biol.*, 2008. *Accepted for publication*

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## **Peer-reviewed papers, not included in the thesis**

**Rydell-Törmänen K**, Uller L, Erjefält JS. Neutrophil cannibalism--a back up when the macrophage clearance system is insufficient. *Respir Res.*, 2006 Dec 14;7:143.

**Rydell-Törmänen K**, Uller L, Erjefält JS. Direct evidence of secondary necrosis of neutrophils during intense lung inflammation. *Eur. Respir. J.*, 2006 Aug;28(2):268-74

Uller L, Lloyd CM, **Rydell-Tormanen K**, Persson CG, Erjefält JS. Effects of steroid treatment on lung CC chemokines, apoptosis and transepithelial cell clearance during development and resolution of allergic airway inflammation. *Clin Exp Allergy.*, 2006 Jan;36(1):111-21.

Uller L, **Rydell-Tormanen K**, Persson CGA, Erjefält JS. Anti-Fas mAb-induced apoptosis and cytolysis of airway tissue eosinophils aggravates rather than resolves established inflammation. *Respir Res.*, 2005 Aug 8;6(1):90

# Abbreviations

<b><math>\alpha</math>-SMA</b>	Alpha smooth muscle actin
<b>BM</b>	Basement membrane
<b>BSA</b>	Bovine serum albumin
<b>COPD</b>	Chronic obstructive pulmonary disease
<b>DAB</b>	3,3 diaminobenzidine tetrahydrochloride
<b>ECM</b>	Extracellular matrix
<b>EPO</b>	Eosinophil peroxidase
<b>FEV<sub>1</sub></b>	Forced expiratory volume in 1 s
<b>GM-CSF</b>	Granulocyte macrophage colony stimulating factor
<b>HDM</b>	House dust mite
<b>IHC</b>	Immunohistochemistry
<b>IL-</b>	Interleukin
<b>i.n.</b>	Intranasal
<b>i.p.</b>	Intraperitoneal
<b>IPAH</b>	Idiopathic pulmonary arterial hypertension
<b>MMPs</b>	Metalloproteinases
<b>OVA</b>	Ovalbumin
<b>PAH</b>	Pulmonary arterial hypertension
<b>PAS</b>	Periodic acid schiff labeling
<b>PBS</b>	Phosphate buffered saline
<b>PINP</b>	Procollagen 1, N-terminal
<b>PIIINP</b>	Procollagen 3, N-terminal
<b>PSR</b>	Picro Sirius red (Direct red 80)
<b>SAL</b>	Saline
<b>SLE</b>	Systemic lupus erythematosus
<b>SSc</b>	Systemic sclerosis, scleroderma
<b>TBS</b>	Tris buffered saline
<b>TGF-<math>\beta</math></b>	Transforming growth factor beta
<b>TIMP</b>	Tissue inhibitor of matrix metalloproteinases
<b>VEGF</b>	Vascular endothelial growth factor



# INTRODUCTION

## Asthma

Asthma is a chronic inflammatory disease that affects approximately 5-10 % of the population of developed countries (1, 2). Estimations suggest that nearly 300 million people in the world suffer from the disease, and in the United States approximately 5000 people die every year with asthma reported as the underlying cause of death (3). An overview of some of the features of an asthma attack is illustrated in Figure 1.

Asthma was defined 1997 by the National Heart, Lung and Blood Institute (4) ” *Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Moreover, recent evidence indicates that subbasement membrane fibrosis may occur in some patients with asthma and that these changes contribute to persistent abnormalities in the lung function*”.

Asthma is considered a complex disease, and is most likely a collection of several different phenotypes with similar symptoms and effects rather than a single disease with one pathologic mechanism (5). Attempts have been made to distinguish between different types of asthma; however, no exactly defined division of subtypes is currently accepted, although guidelines suggested by the ATS-sponsored workshop is regarded a good start (3, 6).

## *Introduction*

### **The normal lung and airways**

The respiratory organ consists of the upper airways (the nose, mouth, larynx and pharynx) and the lower airways (the trachea, bronchi and alveoli). This thesis focuses on the lower airways; however, it is important to remember that the entire respiratory system is interlinked, a theory known as “united airways” (7), and that allergy is a systemic disease (8, 9). It has been shown that asthma and allergic rhinitis are often connected (9), and by treating one the other condition may improve (10).

### **The systemic and pulmonary circulation**

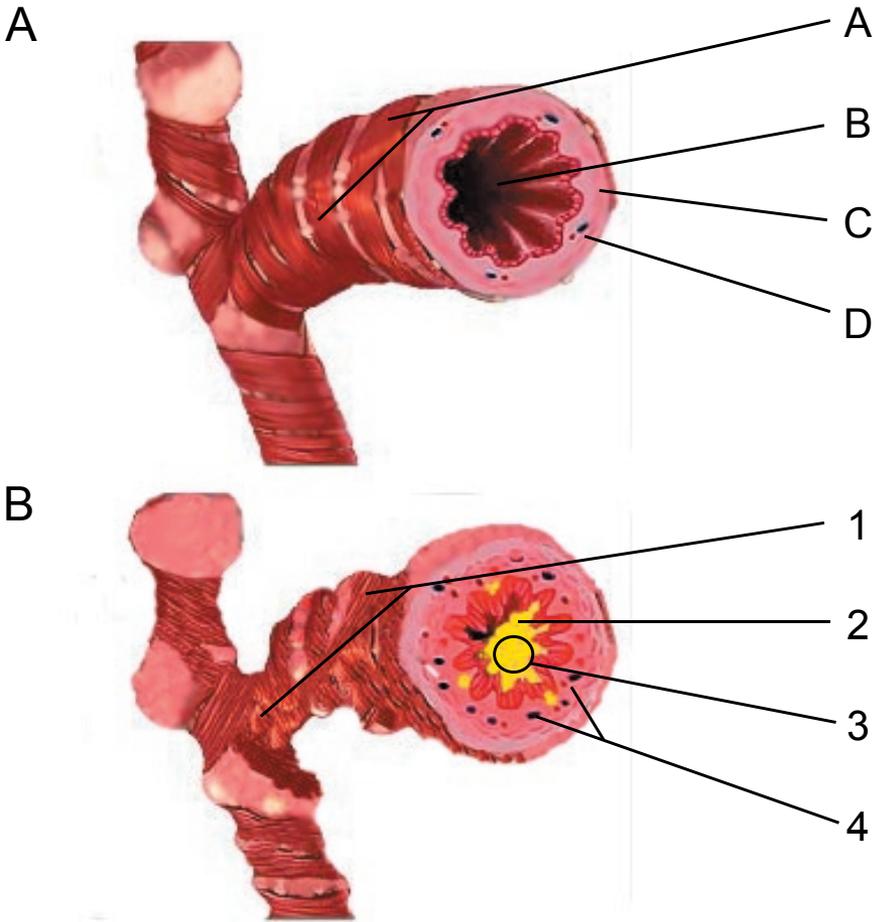
The blood vessels in the lung and adjacent to the airways belong both to the systemic and the pulmonary circulation. An overview of the anatomy of humans is presented in Figure 2.

### **The systemic circulation**

The systemic circulation supplies the upper airways, trachea and bronchi with blood, and one of the largest differences between mice and humans is the extension of the systemic circulation into the lung. In humans systemic vessels accompany airways down to the bronchioles and supply the supporting tissues (11). In mice, however, the systemic vessels do not extend very far into the lung, meaning that most vessels accompanying airways in a mouse lung belong to the pulmonary circulation. Both the bronchial arteries and the tracheal and bronchial microcirculation, are affected in humans in response to asthma, shown in post mortem material (12), as well as in biopsy material (13, 14) and by bronchovideoscope (15). Similar effects can also be seen in other species and models, for example in the tracheal microcirculation in mice (16-18) and rats (18) following a chronic inflammation induced by *Mycoplasma pulmonis*.

### **The pulmonary circulation**

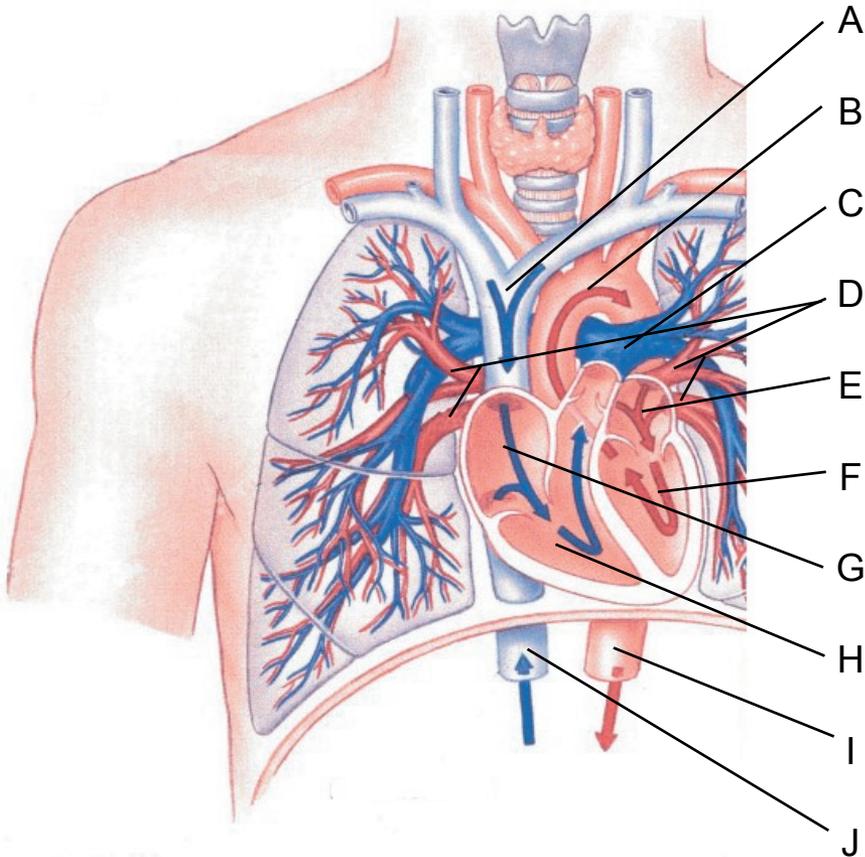
The pulmonary circulation has several differences compared to the



- A - Smooth muscle
- B - Airway lumen
- C - Airway wall
- D - Bronchial microcirculation

- 1 - Smooth muscle contraction
- 2 - Increased mucus production
- 3 - Decreased airway lumen
- 4 - Increased bronchial microcirculation

**Figure 1:** Schematic illustration of a normal healthy airway (A) and the changes occurring in the airways during an asthma attack (B).



- A - Superior vena cava
- B - Aortic arch
- C - Pulmonary artery
- D - Pulmonary veins
- E - Left atrium
- F - Left ventricle
- G - Right atrium
- H - Right ventricle
- I - Descending aorta
- J - Inferior vena cava

**Figure 2:** Overview of the pulmonary circulation and heart. In humans blood flow into the lungs through small bronchial arteries originating from the systemic circulation. These arteries support the supporting tissues of the lung, as well as the large and small bronchi. In mice, this function is instead performed by the pulmonary circulation.

systemic circulation, primarily regarding the pre-capillary vessels. These differences make the pulmonary circulation a unique vascular bed, and not directly comparable to the systemic circulation, although similarities exist. The primary differences being that (11):

- The pulmonary arteries are thin, with a wall thickness of about 1/3 of that of the aorta.
- The arteries and arterioles have larger internal diameters than their systemic equivalents.
- The arteries are distensible giving them the capacity to accommodate large volumes.
- The main pressure of the pulmonary artery averages about 25 mmHg, in contrast to ~100 mmHg in the aorta.
- The pulmonary arteries contract in response to hypoxia.

The pulmonary circulation, in similarity to the systemic circulation, differs between species. Not only are there differences regarding the circulatory supplementation, there is also a question of size, since mice are very small compared to humans. Naturally, there is also a discrepancy in the number of branches, as well as the diameter of vessels that has to be incorporated into any translations from mice to humans. Despite these differences, it has been shown that several characteristics of the lung vasculature found in animals are also found in humans, and despite the fact that the vessels may belong to different circulatory systems, an effect on the vessels can be found in response to inflammatory stimuli. Interestingly it has been shown that pulmonary vessels adjacent to bronchi are affected by airway inflammation, although they themselves are not directly exposed to the allergen (19).

### **Vessels investigated within the thesis**

The focus in this thesis has mainly been on the pulmonary circulation; however, the systemic tracheal microcirculation has been briefly investigated

## ***Introduction***

in Paper I. Although mice are a commonly used species in animal models, surprisingly little is to be found regarding the pulmonary circulation and how to define the different vessels, although some unofficial definitions have been found (20, 21). Due to these difficulties we turned to descriptions of the rat (22-24) and human pulmonary circulations (11) however, although rodents may be relatively closely related, they differ in this aspect. Based on the information obtained in these other species we concluded that the large bronchial-associated vessels are pulmonary arteries (located closely to an airway, with two elastic laminae), the mid-sized solitary vessels are most likely pre-septal veins (one elastic lamina and located in solitude within the parenchyma). The small vessels were very difficult to exactly differentiate into arterioles or venules, and are thus usually classified as microvessels due to this. However, as no exact definitions were found, we used the more neutral terms bronchial-associated (BA), mid-sized solitary (MSS) and small solitary (SS) vessels. Unfortunately, the methods used in this thesis were not sensitive enough to allow any analysis of the alveolar capillaries; however, the studies monitor both the pre- and post-capillary vessels. An illustration of the three types of vessels investigated within this thesis is presented in Figure 3.

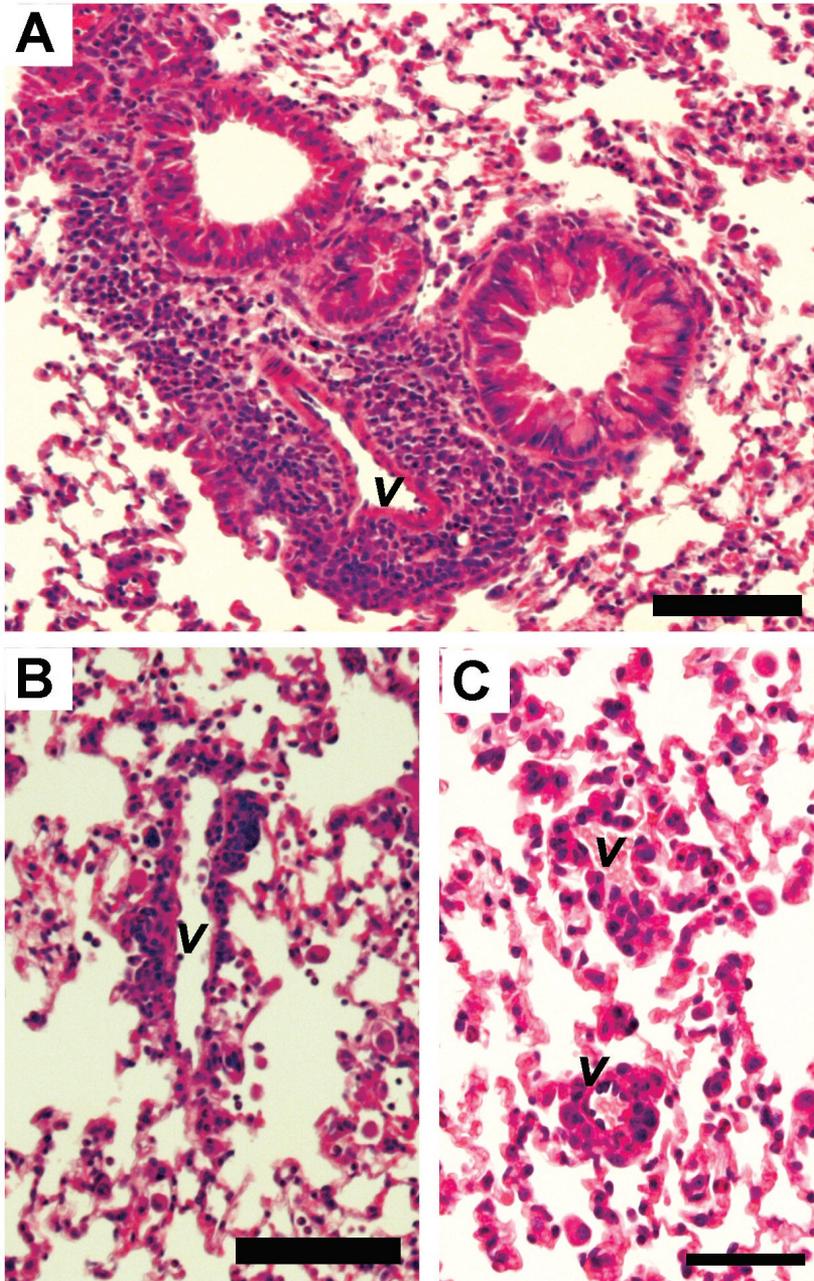
## **Airway remodeling**

Airway remodeling consists of multiple structural alterations (13, 25-29) affecting the airways in several ways. Some of the hallmarks and structural alterations, such as epithelial and basement membrane alterations, increased smooth muscle and fibrosis, is described below.

## **Features of airway remodeling in asthma**

### ***Epithelial alterations***

Increased mucus production and airway obstruction due to mucus plugs in the airways of asthmatics is well known, and directly contributes to symptoms during an attack (28, 30). Asthmatics display an altered distribution of



**Figure 3** - Representative images of the different types of vessels analyzed in the studies. Bronchial associated vessels (A) were defined as being  $\geq 250 \mu\text{m}$  in perimeter and in close proximity to an airway. Mid-sized solitary vessels (B) was defined as vessels  $250\text{-}500 \mu\text{m}$  in perimeter;  $\geq 150 \mu\text{m}$  from the closest bronchi, and small solitary vessels (C) was defined as vessels  $50\text{-}249 \mu\text{m}$  in perimeter;  $\geq 150 \mu\text{m}$  from the closest bronchi. The images are taken at 1 day resolution after seven daily allergen challenges, and stained with HTX/eosin. Scale bars represent  $100 \mu\text{m}$  in A-B, and  $50 \mu\text{m}$  in C.

## ***Introduction***

mucus glands, as they are found to be present in the more peripheral bronchi (27), and in cases of fatal asthma mucus plugs are very often present (27). Another feature of epithelial remodeling during asthma is the epithelial damage and shredding (31), which most likely is accompanied by repair which have been suggested to be impaired and a contributing factor in the disease (32). Furthermore, epithelial stress and associated collagen deposition was shown to occur in children (33), suggesting that epithelial alterations are present from the beginning of disease.

### ***Increased basal membrane (BM) thickness and fibrosis***

Increased thickness of the BM is a well known and frequently studied (27). Subepithelial fibrosis is the thickening of the basement membrane (28). The increased mass is due to increased accumulation of fibronectin and collagens I, III and V (34), and the increased fibrosis is correlated with a decline in FEV<sub>1</sub> (28). Studies have found proteoglycans to be correlated with airway responsiveness (35), and increased deposition of collagen in the submucosal area is present even in cases of mild asthma (29). Subepithelial fibrosis is however not a direct determinant of asthma severity as increased subepithelial fibrosis has been found in patients with mild asthma as well as in non-asthmatic rhinitis patients (28).

### ***Increased muscularization***

The increased amount of airway smooth muscle is a prominent feature of airway remodeling (36), and is thought to contribute to asthma symptoms as a larger muscle mass increases the contractile capacity of the airways (27). Smooth muscle is an active participant in the disease, not only by contracting airways during an asthma attack, but also by elevated production and release of a number of inflammatory cytokines and extracellular matrix proteins, as well as increased expression of cell surface molecules that engage inflammatory cells (37). Furthermore, increased smooth muscle mass has also been implicated in bronchial hyperresponsiveness often found in asthmatics (37).

### Increased vascularization

An increased vascularization of the bronchial wall is a well-described feature of asthma (13). In asthma the proportion of the airway wall occupied by vessels is increased, a feature described in both histological investigations of bronchial tissue as well as bronchoscopic examination of the airways (12-14). Whether this feature is due to angiogenesis or remodeling of existing vessels, or a combination of both is currently unclear (13).

### Eosinophils

Eosinophils are thought to be very involved in the process of remodeling. The presence of eosinophilia is usually considered a characterizing feature of asthma, and the importance of these cells in remodeling has been highlighted by the finding of abolished airway remodeling in IL-5-knock out mice (38) and decreased deposition of ECM-proteins in mild asthmatics after treatment with anti-IL-5 antibodies (39). Eosinophils are thought to contribute to remodeling in several ways. For example by production and release of pro-inflammatory proteins and molecules implicated in remodeling processes such as TGF- $\beta$ , MMP-9, TIMP-1 and VEGF (39), or indirectly by stimulating other cells to produce these mediators. Furthermore, subepithelial fibrosis appears to be connected to the eosinophilia, as therapeutic treatment with anti-IL-5 antibodies reduced the expression of tenascin, lumican and procollagen III in the reticular basement membrane in asthmatic patients (39). Interestingly, the administration of the anti-IL-5 antibody did not completely abolish eosinophilia (median decrease was 55 %), but rather reduced the TGF- $\beta$  production by eosinophils (39).

## **The induction and maintenance of airway remodeling**

### Why and how is remodeling initiated?

Remodeling has been suggested to be the result of several different processes, and no theory has been presented that fully and completely

## *Introduction*

explains the processes behind these tissue alterations. Although it is of no doubt that eosinophils, TGF- $\beta$  and other growth factors as well as Th2-associated cytokines influence and possibly also initiate remodeling (see for example (39-42)), so far no complete explanation, addressing all aspects of remodeling has been published. In mice, IL-5 is of utmost importance for remodeling as knock-out mice do not develop remodeling in response to allergic airway inflammation (38). However, when data were translated to humans by employing treatment with neutralizing antibodies, the same positive results were not observed, although the treatment did have effect on the eosinophil content in the blood of the patient (43), and the deposition of ECM in mild asthmatics (39). The importance of the epithelium has been highlighted as the epithelium had been suggested to be dysfunctional in asthma (31), and this abnormality might be responsible for triggering activation and damage of the epithelial cells more easily than in healthy individuals (43). As damage needs repair, and most reparative mediators are also proliferative this might account for remodeling, implying that dysregulated healing of the epithelium is the basis for remodeling (31, 32, 44). Interestingly, studies in mice utilizing HDM as allergen, have found some components of HDM-extract to be proteolytic and possess the ability enhance epithelial permeability (45). Another well-described mediator is VEGF, being one of the most potent known angiogenic factors (40), and the release of VEGF from a variety of cells, including endothelial cells, eosinophils and fibroblasts is stimulated by TGF- $\beta$ , GM-CSF, IL-6 and IL-8 as well as TNF- $\alpha$  (40), mediators which have been associated with the airway inflammatory response to HDM exposure (46). In support of this, a correlation between GM-CSF and the number of eosinophils, as well as increased levels of GM-CSF in BAL, has been found in patients with systemic sclerosis (47), a disease known for the occurrence of vascular remodeling (48). VEGF and angiopoetin, are overexpressed in the airways of asthmatics (49) and these angiopoetic mediators have lately been shown to be of great importance in the process of remodeling of different compartments of the lung (49-51).

### Remodeling in children

Previously remodeling was considered a late feature of disease, an event occurring after multiple attacks and / or many years of active disease. That is unfortunately not the case, as remodeling has been shown to occur early in disease. It has even been shown to occur in children, before the onset of symptoms (43, 52, 53). This therefore suggests that remodeling might even be a primary feature of the disease that occurs in parallel with lung inflammation. This is further indicated by a study investigating endobronchial biopsies from children (6 – 17 years of age) with severe asthma (54, 55), where clear features of remodeling were present. In contrast, no remodeling features were found in wheezing infants with reversible airflow obstruction (56), even in the presence of atopy.

### Correlation between remodeling and severity of disease

Interestingly there seems to be a limited correlation between the severity of disease and the degree of remodeling (55). For example, in a study of asthmatic children the degree of basement membrane thickening did not correlate with FEV<sub>1</sub> (54), and remodeling has been found in children with intermittent asthma (57). Investigations on collagen deposition in large airways in mild and severe asthmatics did not show any correlation between collagen deposition, eosinophilia, and TGF- $\beta$  producing cells when comparing asthmatics against healthy individuals (58). However as the study monitored bronchial biopsies from larger airways, it is possible that correlations exist in small airways, which are not evaluated in the biopsy material analyzed. Interestingly, another study using the same approach found evidence that structural abnormalities allowed distinguishing of patients with severe disease from patients with milder disease (59).

### **Is remodeling reversible?**

Remodeling is considered difficult to resolve completely (43), however recent studies have shown that some processes may be resolved if treated in time and with appropriate medications (57). A study on corticosteroid

## ***Introduction***

(CS) therapy and a long-term version of the OVA model found that CS decreased peribronchial fibrosis and inhibited myofibroblast accumulation, whereas the peribronchial smooth muscle hypertrophy was unaffected (60). Unfortunately, the CS therapy was introduced 6 h before the first OVA challenge, suggesting that CS inhibited the development of remodeling features rather than resolving established alterations. The same model was utilized in a subsequent study investigating the effect of allergen avoidance and CS as well as the combination of both (61). The results show that allergen avoidance alone has effect on mucus production and on the inflammatory response, whereas no effects on remodeling were evident. The combination of allergen avoidance and CS is effective in reversing both fibrosis and smooth muscle hypertrophy; however fibrosis decreased also without CS administration giving rise to questions about the chronic nature of the model. These findings may be supported by a study (62) investigating patients with severe asthma and the effect of allergen avoidance (measured non-invasively), which suggested that allergen avoidance in combination with CS resulted in better control than with CS alone.

## **Vascular remodeling**

### **Vascular remodeling in asthma**

In 1922, Huber and Koessler (63) described several cases of fatal asthma, where effects on the pulmonary vasculature were recorded. This paper did not include any physiologic measurements, but it did describe a feature of asthma that has been largely neglected since. The study investigated both systemic bronchial arteries as well as pulmonary arteries, and interestingly found them both to be subjected to remodeling. The descriptions of vascular remodeling include diffuse thickening of the pulmonary veins as well as a thickened pulmonary artery and some bronchial arteries. They also described several cases of a dilated and thickened right heart (ventricle), and this feature was found to be present even in a small child aged 15 months. As the authors state “*These observations made it plain that in*

*man, at least, the allergic reaction of the tissues is not confined alone to the smooth muscle fiber system, but involves also the whole organ system which serves exudative processes, endothelium, epithelium, capillaries and glands”.*

In the 1950s, 60s and 80s some papers connecting asthma with heart failure and / or pulmonary hypertension were published (64-68). These very interesting studies suggested that asthma can, in some cases, but not commonly, be associated with *cor pulmonale* or other signs of pulmonary hypertension, suggesting vascular remodeling within the pulmonary circulation. Interestingly, one of these papers reported that two patients with pulmonary hypertension actually became symptom free when asthma was treated (64). Another study reports on three patients suffering from asthma and presenting with *cor pulmonale* where the cardiovascular symptoms dramatically improved as their asthma was treated (65), although the cause of these reversal was unknown.

Why pulmonary vascular remodeling in asthma has remained a white spot on the map for so long is unknown, perhaps as treatments got better, these symptoms became less common? Maybe the mantra *asthma is not associated with pulmonary hypertension* became a “truth”? It is a fact however that patients still die from asthma today (69) and some patients do not respond or respond inadequately to any form of treatment presently available (6). Patients suffering from severe asthma might in fact be relatively similar to the patients described in the study from 1922 (63), with the difference that patients today are usually medicated with steroids and other drugs in an attempt to conquer disease.

### **Features of vascular remodeling**

Prior to the papers included in this thesis, the involvement of the vasculature in asthma was only known from descriptions of autopsy material by a few authors (12, 19, 63), and until very recently no studies on remodeling of the

## *Introduction*

pulmonary vasculature following allergic airway inflammation in animals had been published. Recently however, a study by Daley *et. al.* showed that vascular remodeling occurs in response to two long-term allergen exposure protocols (70). Another paper describes parenthetically how increased peribronchial collagen deposition extends around the adjacent blood vessels and surrounds them (71). Descriptions (with the exception of the results described in this thesis) of the involvement of the vasculature in asthma and allergic airway inflammation, include the following characteristics:

### *Increased wall thickness*

The overall thickness of the vessel wall is increased (63); however whether this increase was due to intimal, medial or adventitial thickening is not clear. In contrast, another study (19) did not find any thickening of the vessel wall, but described instead a perivascular distribution of inflammatory cells. In recent years a study by Green and colleagues (12) found that bronchial arteries displayed classical features of vascular remodeling, including increased wall thickness. Furthermore, the study by Daley (70), clearly showed that increased smooth muscle mass and increased smooth muscle cell proliferation is a feature following allergic airway inflammation.

### *Increased ECM deposition*

Increased collagen deposition was found to extend from the airways and surround both airways and the adjacent blood vessels (71). The deposition was detected by Masson's trichrome, and the type of collagen in this fibrosis was therefore not determined: however, another study utilizing roughly the same model found that the increased peribronchial collagen deposition primarily consisted of collagen type I (72).

### *Perivascular eosinophilia*

Perivascular eosinophilia is not a structural event in itself, as eosinophils are not a component of the lung architecture. It has been shown however that eosinophils are important in the remodeling process as IL-5 knock out

mice do not develop airway remodeling (38). The occurrence of prominent perivascular eosinophilia, in addition to peribronchial eosinophilia was highlighted by Saetta and colleagues (19), and further confirmed in paper I of this thesis (73).

### *Smooth muscle alterations*

Recently, a paper describing two long-term models utilizing soluble antigens was published; describing increases in smooth muscle area and increased numbers of proliferating smooth muscle cells (70).

### **Why and how is vascular remodeling initiated?**

As the occurrence of pulmonary vascular remodeling in association with allergic airway inflammation or asthma is a relatively new finding the causes have not been extensively studied. It has been suggested to be the result of a spill over from the inflamed airways (19), as the airway epithelium is exposed to the allergen whereas the vessels are not. The interaction between endothelial cells and matrix (74) provides an intriguing possibility as the interaction between epithelial cells and mesenchymal cells is supposed to be important in the inflammatory processes within airways (75). TGF- $\beta$  has in animal studies been shown to increase the mRNA for collagens I and III, as well as increase the amount of total collagen (76), indicating the importance of this mediator. Furthermore, the importance of eosinophils in airway remodeling is most likely also applicable to vessels as eosinophilia is both peribronchial and perivascular, and the importance of eosinophils on airway remodeling has been established, although it might not provide the sole solution to the remodeling enigma. Unfortunately, no descriptions or theories, except the spill over-theory, have been presented; however as the vascular remodeling is similar to the airway remodeling it is plausible to assume that the two processes are interlinked and / or that similar mechanisms are responsible.

## *Introduction*

### **Vascular remodeling in other diseases**

Vascular remodeling is a novel finding in asthma; however it is a hallmark of several pulmonary diseases where vascular remodeling is either the primary problem, or a secondary symptom. Some of the diseases where vascular remodeling is known to occur are chronic obstructive pulmonary disease and pulmonary arterial hypertension as well as several autoimmune diseases, such as systemic sclerosis and systemic lupus erythematosus.

#### *Pulmonary arterial hypertension – PAH*

PAH is defined as sustained elevation of pulmonary arterial pressure to more than 25 mm Hg at rest (48). PAH is not one but a collection of diseases with the pulmonary arterial hypertension as a common feature together with histologic manifestations but the cause of these symptoms vary (48, 77). PAH is usually associated with medial hypertrophy and increased endothelial proliferation, the later often resulting in lumen occlusion. Unfortunately the pathogenesis of most of these diseases is unknown or inadequately known, although the importance of inflammation and inflammatory processes has been highlighted recently (78, 79).

#### *Systemic sclerosis and systemic lupus erythematosus – SSc and SLE*

In cases of both SSc and SLE, the disease can cause pulmonary arterial hypertension, which is frequently less responsive to pulmonary vasodilator therapies. Remodeling of the pulmonary arteries is well-known and presents as intimal fibrosis, increased medial thickness, pulmonary arterial occlusion and plexiform lesions (48). Recently, remodeling of pulmonary veins as well as capillaries was established as a feature of severe disease (80), suggesting the ability of the entire pulmonary circulation to undergo vascular remodeling.

One of the problems with PAH initiated by autoimmune diseases is the difficulty in diagnosing it until it is too late. Due to the lung's ability to adapt, the symptoms often do not show until the disease has progressed

significantly (81), and the stress on the heart presents as symptoms of right heart failure. Due to this, it has been suggested that patients undergo regular testing in order to discover pulmonary hypertension and start treatment as soon as possible (48).

### *Chronic obstructive pulmonary disease – COPD*

Patients with COPD, display thickening of the vessel wall, a feature present early in disease as thickening of the intima and as the COPD worsens, the amount of smooth muscle and extracellular matrix further thickens the vascular wall (82, 83). Even asymptomatic smokers display structural anomalies (84). This early vascular remodeling has been suggested to be associated with smoking and the inflammatory processes this initiates (85, 86).

## **Modeling human disease in animals**

### **Animal vs. human**

Animals are often used as models of human disease. The advantages are of course considerable, but it is always important to remember that animals are not humans and the results obtained from an animal cannot be directly translated to human disease. Due to this, it is of utmost importance to include human tissue or subjects in the study of a disease, and not solely rely on results obtained in animal models. Some of the more relevant differences when investigating asthma / allergic airway inflammation is the presence of submucosal glands, airway smooth muscle bundles and a pseudostratified columnar epithelium in humans (87). Furthermore, Th1 and Th2 responses differ greatly as mice display a distinct Th2 polarized inflammation in response to allergen exposure, whereas the response in humans is more mixed and responses to some cytokines also differ between species (87). Despite these differences there are also similarities and the use of animals as models of human disease are of great importance in medical research. It is vital to remember that in most models some kind of artificial intervention

## ***Introduction***

is needed to initiate the “disease” and this is often something very different from the human situation (where the initiating factors are very often partly or totally unknown). This is both an advantage and a disadvantage, as the initiation may be different from the human situation, but can be controlled and applied at a set time.

### **The benefits of animal studies**

Animal models, and in particular mouse models, have several advantages compared to exploration of human material or human subjects (88). For example, with the ability to manipulate the genetics of mice, we can investigate genetic bases of a disease and allow us to model specific disease phenotypes. Furthermore, research animals are usually inbred, minimizing genetic variation between individuals (although some individual variations cannot be excluded) and thereby the large differences usually seen in human patients can be avoided. Mice can furthermore be very well matched in age, sex, exposure to pathogens, pollutants etc, which can be difficult when using human subjects, and may be of great importance.

### **The benefits of human studies**

It is a truth universally acknowledged that to study human disease the most suitable research objects is *Homo sapiens*. No matter how well an animal model mimics the features of human disease, it is still an approximation. The primary advantage of studies on humans or human material is the study of the species that we are interested in with direct implications on human health (87).

### **OVA vs HDM as models of asthma / allergic airway inflammation**

The OVA model is well-described and by now it is well known. It is not an ideal model of asthma, but it generates a robust and consistent inflammatory response (45). The OVA model used in this thesis is a modified version of the model developed by Brusselle and colleagues (89), which has been well studied and described both in terms of the inflammatory response and airway

remodeling (see for example (89-93). OVA models have been suggested to be good models of airway hyperresponsiveness, but not of acute physiological responses to allergen, as they do not induce bronchoconstriction (94). In combination with the fact that mice easily develop tolerance against OVA (95-97), OVA models can be thought of as good models of the more acute features of asthma / allergic airway inflammation, although are not as good at mimicking the chronic inflammation characteristic of human asthma.

The HDM model is not as extensively studied as the OVA model, as it was more recently described and characterized (45, 46, 98-100). The features of the model are relatively similar to the features of the OVA model: however, the mechanisms behind the development of remodeling and inflammation are fundamentally different. The HDM model is based on an extract from mites, making the allergen very complex, and is a known allergen also in humans that is associated with asthma (45). Furthermore, HDM induces airway allergy by mucosal sensitization (101), and the airway remodeling seen in this model is not only similar to asthma (102), but some aspects of it appear to be irreversible (100), in similarity to human disease.



## **AIMS**

The overall aim of my project was to investigate vascular remodeling following allergic airway inflammation and describe the histologic features of the phenomenon, as well as to explore the plasticity and resolution of this allergic inflammation.

### **Paper I**

The aims of this study were to investigate whether allergen-induced bronchial remodeling is associated with remodeling of the adjacent pulmonary vessels, and if present, to characterize the features of remodeling.

### **Paper II**

The aim of this paper was to investigate whether, during an allergic airway inflammation, remodeling also occurs in vessels distant from any allergen-exposed airway and if so, to characterize the remodeling changes and investigate how they related to the vascular remodeling of the large bronchial-associated vessels.

### **Paper III**

The aims of this study were to investigate whether vascular remodeling was reversible, and if the plasticity of the vessels were similar as to previously described in other models of airway inflammation

### **Paper IV**

The aim of this paper was to study how chronic airway inflammation affects the lung vasculature, and investigate whether any vascular remodeling in this setting is reversible.

# METHODS

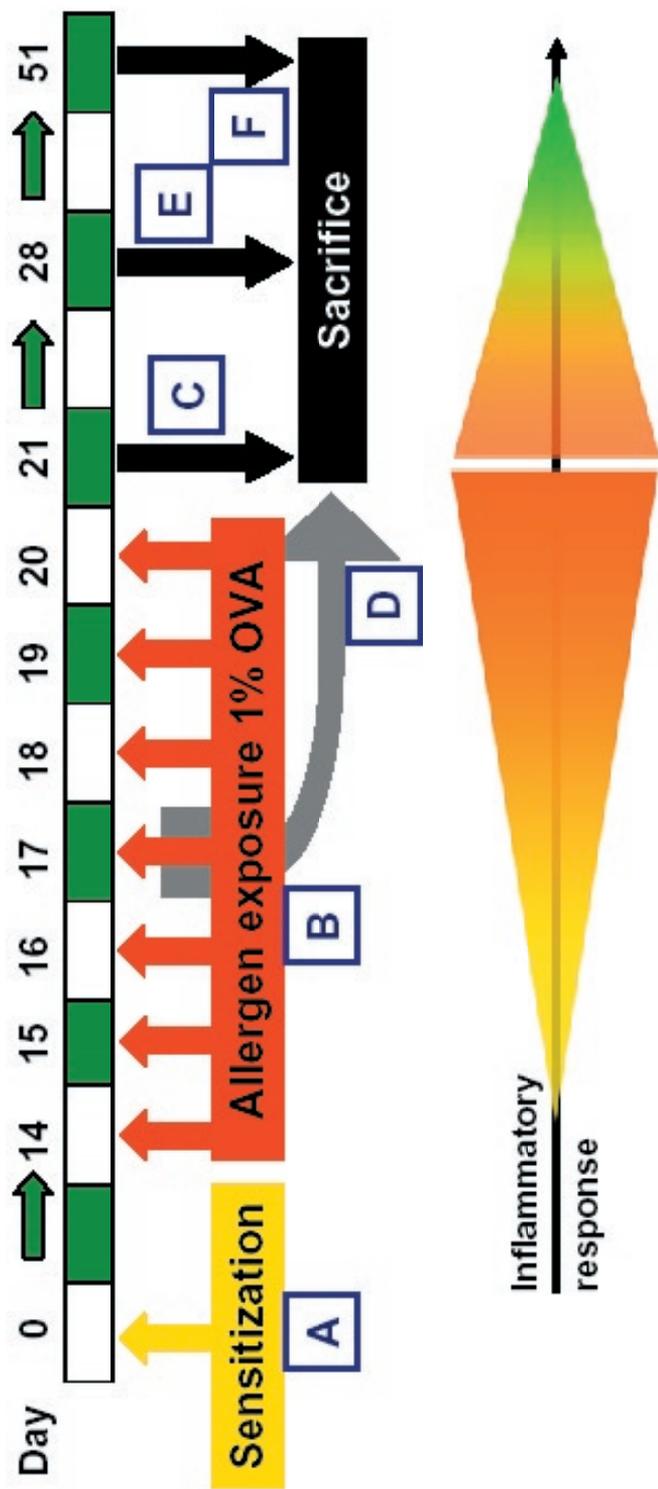
## ***In vivo* procedures**

Two different animal models were used in this thesis. For all animal studies female Balb/c mice were used, and were housed in special animal facilities following a 12 light-dark cycle and provided food and water *ad libitum*. All experimental protocols were approved by the regional ethics Committee in Malmö/Lund, Sweden or the Animal Research Ethics Board of McMaster University, Hamilton, ON, Canada.

### **1. Ovalbumin-induced airway inflammation (Papers I-III)**

The experimental method of inducing allergic airway inflammation by immunization and challenge with chicken egg albumin (OVA, Sigma, St. Louis, MO, USA) is well known, and well described (92), and an overview of the study protocol used is presented in figure 4. The protocol extends for 21 days and starts by immunization of the mice with 10 µg OVA in 1 mg alum adjuvant, administered as an i.p. injection. 14 days later the mice are exposed to 1% OVA for 30 minutes daily, for 7 subsequent days. The aerosol was generated by a nebulizer, driven at 4 bars (an illustration of the equipment used for allergen challenges are shown in figure 5). Control animals were immunized and challenged with saline. Previous experiments have shown that no inflammatory response is present following sham-sensitization, a sham-challenge or a double-sham approach (92), and therefore the double-sham protocol was used for controls. The OVA used in the study is not stated by the manufacturer to be endotoxin free, resulting in a slight possibility for reactions to be caused by endotoxin. However, as animals sham-sensitized but exposed to OVA do not develop airway inflammation (92), this possibility seems unlikely.

In papers I and II, the animals were sacrificed 24 h after the last challenge and



A – Sensitization, 10  $\mu$ g OVA + 1 mg Al(OH)<sub>3</sub>, i.p. administration

B – Daily allergen exposures, 1% OVA aerosol, 30 minutes.

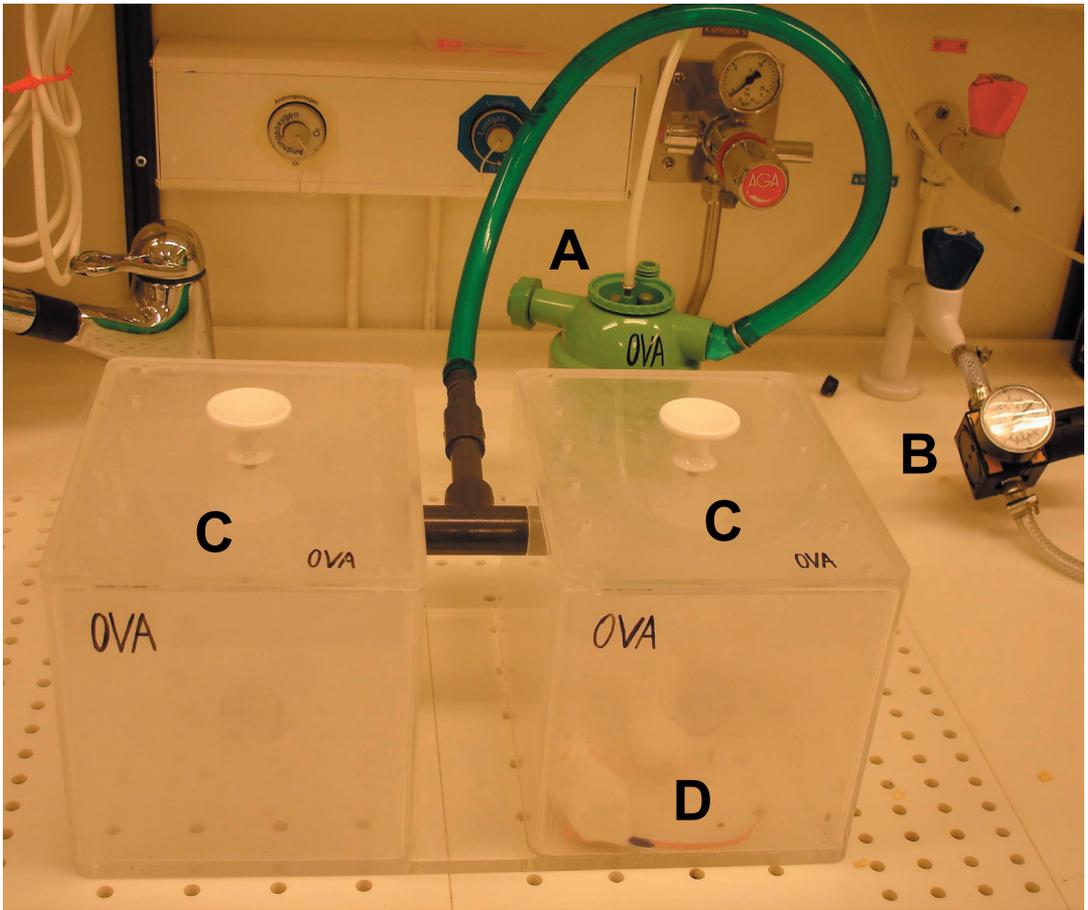
C – Sacrifice 24 h (1 day) after last challenge (Papers I - III)

D – Sacrifice 24 h after 3 days of exposure (Paper I)

E – Sacrifice 1 week after last allergen challenge (Paper III)

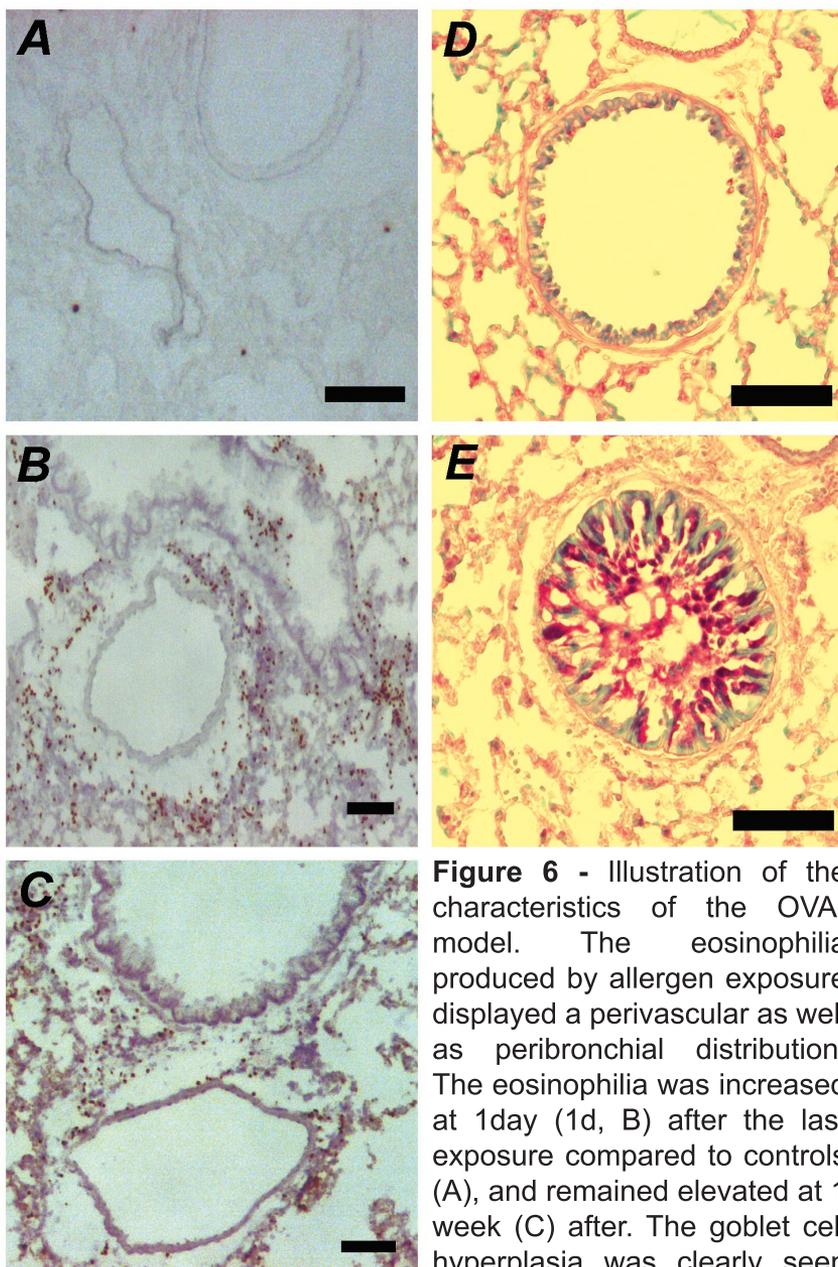
F – Sacrifice 1 month after last allergen challenge (Paper III)

**Figure 4** - Overview of the OVA-model, including the different end points used in the studies utilizing this model (Paper I-III).



- A - Nebulizer
- B - High pressure valve
- C - Exposure chambers
- D - Mice

**Figure 5** - Illustration of the equipment used in the OVA-model.



**Figure 6** - Illustration of the characteristics of the OVA-model. The eosinophilia produced by allergen exposure displayed a perivascular as well as peribronchial distribution. The eosinophilia was increased at 1day (1d, B) after the last exposure compared to controls (A), and remained elevated at 1 week (C) after. The goblet cell hyperplasia was clearly seen in OVA-exposed animals (E) compared to controls (D). Scale bars represent 100  $\mu\text{m}$  in A, 200  $\mu\text{m}$  in B-C and 100  $\mu\text{m}$  in D-E.

## **Methods**

in Paper III the animals were sacrificed both 24 h after last challenge (1 day after, 1d) as well as 1 week (1w) or 1 month (1m) following the last allergen challenge. In paper I, two groups (one control and one OVA-exposed) were sacrificed 24 h after only 3 days of exposure. The animals were sacrificed with an overdose of pentobarbital sodium (Pentobarbitalnatrium, Apoteket AB, Umeå, Sweden), administered by i.p. injection.

In paper I an investigation of the tracheal microcirculation was conducted. To visualize these vessels we injected 0.1 ml solution of FITC-labeled lectin (from *Lycopersicon esculentum*, tomato (Sigma)) 3 minutes before termination into the tail vein of the animals. Lectin binds to the endothelial cells in the body, making the vessels clearly visible.

This mouse model is characterized by peribronchial and perivascular eosinophilia and goblet cell hyperplasia (illustrated in figure 6) as well as structural alterations and proliferation of different cell types (73), and is frequently used as a model of allergic airway inflammation. Due to the mice developing tolerance, this model is considered a more acute model of allergic airway inflammation. Some altered protocols have been developed however, which may mimic a more chronic situation.

## **2. House dust mite-induced airway inflammation**

The protocol extends for 5-20 weeks, and the animals are continuously responsive to the allergen and the remodeling features are to a large extent irreversible. The model is known to result in several structural changes affecting the airways, such as goblet cell hyperplasia as well as subepithelial collagen deposition and increased contractile tissue (airway smooth muscle and myofibroblasts (100)). The method has been described in depth elsewhere (46, 100, 103). Briefly, mice were subjected to intranasal administrations of HDM extract five days in a row, followed by 2 days of rest before a new cycle. This cycle was repeated for 5-20 weeks. Controls were allergen naïve animals, as previous experiments have shown no

difference between saline-exposed animals and naïves (46). Significantly, a number of dust mite allergens possess proteolytic activity (104, 105), capable of disrupting the integrity of airway epithelial cells through the degradation of the tight junction adhesion proteins occludin and ZO-1 (106, 107). Moreover, HDM proteases have direct proinflammatory effects, since HDM-purified Der p 1, 3, and 9 have been shown to induce the production of GM-CSF, IL-6 and IL-8 from airway epithelial cells (108) through the activation of protease-activated receptor-2 (PAR-2) (109). It has also been suggested that HDM may enhance IgE synthesis and privilege the generation of a Th2-polarized response (110, 111). Following the exposure period, anesthetized animals were sacrificed by exsanguination before tissue specimens were obtained for paraffin embedding by immersing them in 10% formalin. Purified HDM whole-body extract was purchased from Greer Laboratories (Lenoir, NC, USA).

### **Bronchoalveolar lavage (BAL)**

In Papers I-II and Paper IV, BAL was performed on the animals directly after sacrifice.

### **Procedure**

We used a standard protocol, described before (112). Briefly the trachea is cannulated and a constant pressure of 10 cm H<sub>2</sub>O is used to passively allow PBS into the lungs for 2 minutes. Following the 2 minutes the fluid is allowed to drain for 1 minute. This process is repeated twice, and the BAL fluid obtained is centrifuged. The supernatant is removed and the cell pellet is resuspended in 200 µl PBS and kept on ice until counting and cyto-spin.

### **Quantification of luminal cells**

The total amount of cells was counted in either a hemocytometer or an automatic cell counter.  $1 \times 10^5$  cells were cyto-spun onto microscope slides. The slides were stained with May-Grünwald Giemsa, and differential

## ***Methods***

cell counts were obtained. The percentage of eosinophils, neutrophils, macrophages and lymphocytes were determined by counting 200 cells in a blinded manner.

## **Histology – processing tissue**

Following BAL the lungs were removed from the thoracic cavity and tissue samples were obtained for immunohistochemistry by immersing the samples in different fixatives.

### **Fixation**

In Paper I-III, tissue samples were obtained for cryo-sectioning and paraffin-embedding. Tissue destined for cryo-sectioning was fixed in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1M PBS, pH 7.2), rinsed in Thyrode buffer (PBS buffer supplemented with 10% sucrose) and frozen embedded in TissueTek® (Histolab AB, Gothenburg, Sweden). Tissues intended for paraffin-embedding were fixated in buffered 4% paraformaldehyde (pH 7.2) and rinsed in 70% EtOH before dehydration and embedding. In paper IV, the tissue specimens were fixed in 10% formalin at a standard pressure of 20 cm H<sub>2</sub>O, stored in 70% EtOH and dehydrated prior to embedding.

### **Sectioning**

Paraffin-embedded tissue was sectioned using a microtome, into 5 µm thin sections. Frozen tissue was sectioned using a cryostat, where the TissueTek embedded tissue can be sectioned without thawing. After sectioning the slides were either re-hydrated (paraffin embedded sections) or thawed (frozen sections), before allowing to equilibrate in buffer (PBS or TBS (TRIS-buffered saline)) for 5-15 minutes.

### **Antigen retrieval**

Some antibodies require pre-treatment of the tissue section in order to

bind onto the section. Different retrieval methods can be used depending on tissue, fixation and antibody. The methods used herein are stated in association with the antibodies.

### **Blocking of unspecific labeling**

In order for some antibodies to label the correct structures blocking of unspecific labeling is needed. This can be carried out in different ways but the following was used when needed in Papers II-IV. Following equilibration, dried milk (10% w/w, for fluorescence labeling) or normal-sera solution (10% w/w, for non-fluorescence labeling) was applied for 20 minutes. The excess dry milk / normal-sera solution were then shaken off and the primary antibody was added directly onto the sections.

### **Whole mount-preparation**

To visualize capillaries in the trachea (Paper I), the animals were injected with FITC labeled lectin as previously described. After sacrifice, the tracheas were dissected free, cut laterally and mounted as whole-mount preparations on slides using PBS / Glycerol as mounting medium. The preparation was viewed and photographed under a fluorescence microscope and the length and width of the small capillaries between the cartilage segments were measured.

### **Immunohistochemical labeling**

For all immunohistochemistry (IHC) a standardized protocol were used. Briefly, the antibody was diluted to an appropriate dilution in dilution buffer (PBS or TBS + 10 % BSA), and kept cold until application on slides. Following equilibration the slides were removed from the buffer, excess fluid dried off and antibody was applied (when blocking for unspecific labeling was used the antibody was applied directly after blocking, without rinsing in between). Antibodies were incubated either for 1 h at room temperature or 4°C overnight. The slides were then washed (3 x 5 minutes

## **Methods**

in PBS or TBS); a secondary antibody applied (if necessary) and incubated for 45 minutes at room temperature. Following the secondary antibody, the slides were again washed for 3 x 5 minutes in TBS or PBS before mounting. If a double labeling was preformed, a second primary antibody was applied following the washing, and the cycle repeated. Several different detection systems are used for visualization of the labeling; both fluorescence and light microscopic markers were used. Which one used to visualize the different antibodies is stated under respective antibody (Tables 1 and 2). Some antibodies are directly conjugated to a marker, meaning a secondary antibody is unnecessary. These slides were instead mounted after washing off the primary antibody. Different mounting techniques were used, depending on the detection system; slides dyed with a fluorescent secondary antibody were mounted in a 50 / 50 solution of PBS and Glycerol, whereas slides with light microscopic visualization were mounted in Kaiser's medium. A few staining techniques used in the papers were mounted in Pertex, in these cases the slides were dehydrated in ethanol and cleared in xylene before mounting.

Details on primary and secondary antibodies as well as non-antibody labeling is provided in tables 1, 2 and 3.

### **Hoechst 33342 (H33342)**

To label all cell nucleus the sections were stained with H33342, a cell membrane permeable DNA-marker which labels all DNA fluorescent blue. This was used in combination with other antibodies to verify that the labeled areas were indeed cells.

### **$\alpha$ -Smooth Muscle Actin**

This antibody labels cells containing  $\alpha$ -SMA, mostly smooth muscle cells, but also myofibroblasts (detected by co-labeling with procollagen I). The antibody used is a mouse monoclonal antibody, but due to the fact that it is directly conjugated to a fluorescent dye (Cy3) or alkaline phosphatase

Table 1- Primary antibodies used in the studies

Antigen/ Labelling	Code	Raised in	Dilution	Source
Proliferating Cell Nuclear Antigen / PCNA	U7032	Mouse	Ready-to-use	Dako A/S; Glostrup, Denmark
PCNA	sc-9857	Goat	1:100	St.Cruz Biotechnology, Santa Cruz, CA, USA.
KI-67 (clone TEC-3)	M7249	Rat	1:200	Dako A/S
TUNEL, ApoTag	-----	-----	Kit	Intergen, Purchase, NY, USA
$\alpha$ -Smooth Muscle Actin / $\alpha$ -SMA; Cy3- or AP-conjugated	C6198 (Cy3) C5691 (AP)	Mouse Mouse	1:5000 1:200	Sigma
von Willebrand factor (vWf)	A0082	Rabbit	1:640	Dako A/S
Procollagen I NP (PINP)	-----	Rabbit	1:1-200	Prof. Juha Risteli and Saana Karttunen, Oulo University.
Procollagen III NP (PIIINP)	-----	Rabbit	1:200	Prof. Juha Risteli and Saana Karttunen, Oulo University.

Definition of abbreviations: TUNEL = terminal deoxy RNase nick end labeling, AP = alkaline phosphatase

## Methods

**Table 2 – Secondary antibodies used in the studies.**

Secondary antibody	Visualization system	Product code	Dilution	Manufacturer
Pig anti rabbit	FITC	F0205	1:100	Dako A/S, Glostrup, Denmark
Donkey anti rabbit	Texas Red	711-075-152	1:300	Jackson ImmunoResearch West Grove, PA, USA
Goat anti-rabbit	HRP	111-035-144	1:200	Jackson ImmunoResearch
Donkey anti-rabbit	Alexa 488	A21206	1:200	Molecular Probes, Eugene, OR, USA
Goat anti rabbit	Alexa 555	A21428	1:300	Molecular Probes,
Donkey anti rat	Cy3	712-165-153	1:300	Jackson ImmunoResearch
Rabbit anti rat	Biotin	E0468	1:200	Dako A/S
Rabbit anti goat	AP	305-055-045	1:200	Jackson ImmunoResearch
Rabbit anti-digoxigenin (DIG)	-----	D5105	1:150	Dako A/S

*Definition of abbreviations:* FITC = fluorescein isothiocyanate, HRP = horse raddish peroxidase, AP = Alkaline phosphatase.

**Table 3 – non-antibody labeling and visualization techniques.**

Antigen/Labeling	Code	Dilution	Purpose	Source
Hoechst 33342 (H33342)	B2261	20 mg/ml	Visualization of cell nucleus	Sigma, St Louis, USA
Lectin from <i>Lycopersicon esculentum</i> (tomatoe)	L0401	1 mg/ml	Visualization of tracheal microvessels	Sigma
DAB / 3,3-diaminobenzidine tetrahydrochloride	SK-4100	Kit	Visualization of HRP-conjugated antibodies	Vector Laboratories, Burlingame, CA, USA
New Fuch sine	K698	Kit	Visualization of AP-conjugated antibodies	Dako A/S, Glostrup, Denmark
Picro Sirius Red (Direct red)	365548	0.1 g/100 ml	Visualization of total content	Sigma
ABC-complex	PK-6100	Kit	Amplifier of biotin labeling	Vector

(AP) it can be used on mouse tissue. The Cy3 dye results in a bright red fluorescence, and AP visualized with New Fuchsin results in reddish labeling.

### **Proliferation**

To detect proliferating cells antibodies directed against Proliferating Cell Nuclear Antigen (PCNA) and Ki67 were used. Two different antibodies against PCNA were used in different papers. The first one (Papers I and II), was obtained from Dako and was directly conjugated to HRP and visualized using DAB. The second antibody was obtained from St Cruz Biotechnology (St Cruz, CA, USA), and un-conjugated. This antibody was used in double-labeling to detect proliferating smooth muscle cells (Papers I-IV) and proliferating endothelial cells (Papers I-II). It was also used solitarily to detect proliferating endothelial cells in combination with H33342 (Papers III-IV).

### **Apoptosis**

Programmed cell death, apoptosis, was detected using the TUNEL technique (Intergene, New York, NY, USA), as described before (113). Visualization of apoptotic cells was made by an anti-DIG antibody combined with New Fuchsin, resulting in apoptotic cells being labeled reddish pink.

### **Von Willebrand factor (vWf)**

To detect blood vessels in lung sections anti-von Willebrand factor antibody was used. vWf is present on the walls of blood vessels (114). Vessels were visualized in all vessels larger than capillaries being detectable in tissue sections (capillaries were labeled but are too small to be detected and analyzed in histological sections). The secondary detection was done using a fluorescent secondary antibody.

### **Collagen synthesis**

Antibodies directed against the N-terminus of procollagens I and III were

## ***Methods***

used for detection of collagen synthesis (115). The antibodies differed in expression pattern, procollagen I being located intracellularly, whereas procollagen III accumulates both intracellularly as well as closely around the cells synthesizing it (116). Due to this discrepancy we chose to use procollagen I in combination with a marker for smooth muscle to identify myofibroblasts (defined as cells co-positive for procollagen I and  $\alpha$ -SMA). As the N-terminal is cleaved off when the collagen is assembled, the antibody no longer detects it (117). This makes procollagens I and III good markers of collagen synthesis, rather than collagen content. To visualize the staining secondary antibodies labeled with either Texas red, FITC or HRP (visualized by DAB) were used. In papers III and IV, antigen retrieval was used before labeling. This was done enzymatically by subjecting the slides to 0.4 % pepsin in 0.01 M HCl for 15 -30 minutes in 37°C. The antibodies were obtained as a gift from Professor Juha Risteli, Oulu University, Oulu, Finland.

## **Total collagen content**

The total collagen content in the tissue was detected in paper IV by a histochemical staining that marks all collagen – Picro Sirius Red (PSR). Briefly, PSR (by the name of Direct Red, Sigma) was dissolved in saturated picric acid. The slides were dewaxed and rehydrated, and allowed to equilibrate in buffer before being incubated in the dye-solution for 1h, followed by a quick rinse in water, dehydration and mounting in pertex. PSR is displayed in tissue sections under polarized light as a bright, almost fluorescent red, labeling.

## **Eosinophils**

Eosinophils were visualized by histochemical staining of cyanide-resistant eosinophil peroxidase activity (92) on frozen sections. Positive cells stain grey-black, and are clearly visible against an uncolored background. The labeling was quantified as positive cells per area unit. This staining can also be used on paraffin embedded sections, however then the incubation time

has to be extended to 60 minutes and the labeling is significantly weaker.

### **Goblet cells**

Mucus producing cells, also known as goblet cells, were stained with periodic acid-Schiff reagent (PAS) on paraffin-embedded tissue. The sections were dewaxed and rehydrated, before immersing them in 1% periodic acid for 5 minutes, rinsing them in distilled water for 10 min, followed by Schiff's reagent in 15 minutes, another 10 minute-rinse in water, followed by Fast Green for 30 s and finally a very short rinse in distilled water. The slides were then dehydrated and mounted in Pertex.

### **Computerized image analysis**

All quantification was carried out on digital images, since florescence was often used and the fading makes it impossible to do any quantification directly in the microscope.

### **Photographing**

For digital imaging a microscope equipped with a high resolution digital camera and a computer was used. For each section, multiple pictures were taken in a blinded manner and saved for later quantification. When double or triple fluorescence labeling was used, two or three pictures of each area were obtained – one for each color, and overlaid. When double staining for light microscopy was used a single picture of each area was obtained as the two labels could be analyzed in the same image.

### **Quantification**

Of each section 3-9 (depending on the size of the section, the presence of airways, the parameter studied, the presence of vessels and type of vessels analyzed etc) areas were photographed. The imaging was performed in a blinded manner, although it was sometimes possible to guess treatment due to significant inflammatory responses. The number of labeled cells or the

## ***Methods***

labeled area around a blood vessel was counted / measured and related to the length of the basement membrane. The total proliferation, total apoptosis and tissue eosinophilia was measured by counting the number of positive cells on 3-5 representative and randomly chosen areas (images) and related to the area. The image analysis was made by the program ImageJ (ImageJ, Wayne Rasband, NIH, Bethesda, MD, USA) which can be downloaded from <http://rsb.info.nih.gov/ij/>).

## **Statistical analysis**

Due to the relatively low n-numbers non-parametrical tests were performed, the Wilcoxon rank sum-test was predominantly used, except in Paper III where the Kruskal-Wallis ANOVA was used. Data were either given as Mean  $\pm$  standard error of the mean (SEM, Papers I, II and IV) or Median  $\pm$  inter quartile range (IQR, paper III). A value of  $p < 0.05$  was considered significant. In paper III all controls from three different but equally treated groups were pooled together in order to receive a better statistical stability in the larger numbers. The variation between the controls of different groups was small and no statistically significant differences were found between the three control groups.

## RESULTS AND DISCUSSION

### **Allergen exposure of mouse airways evokes remodeling of both bronchi and large pulmonary vessels – Paper I**

#### **Background**

Airway remodeling and peribronchial eosinophilia are well known and documented features of asthma (25-27). As tissue samples primarily are obtained from asthmatics via bronchial biopsies, focus has been on the large airways although the biopsies may not reflect the overall state within the lung (118). Though, increasing evidence suggests that asthma affects the whole lung, including the small airways, bronchial and pulmonary circulation. The latter have however been poorly investigated in asthma and animal models of asthma. Saetta *et. al.* (19) reported that pulmonary arteries exhibited inflammatory features, suggesting the pulmonary vasculature to be involved in the inflammatory process. Further involvement has been described in passing by Henderson (71), illustrating fibrosis extending from the airways and surrounding the adjacent pulmonary vessels.

#### **Aims**

The aims of this study were to investigate whether bronchial remodeling is associated with remodeling of the adjacent pulmonary vessels in a model of allergic airway inflammation and, if present, to characterize the features of vascular remodeling. To investigate if remodeling occurs in the pulmonary and systemic circulation, the tracheal microcirculation was also investigated.

#### **Results & Discussion**

A well-known OVA protocol was used (92), but in addition to the normal 7 days of challenge two groups (one control and one allergen-challenged) received only 3 days of allergen before sacrifice (to investigate when the

## ***Results and discussion***

remodeling begins). Interestingly, after 7 days of allergen exposure, we found similar remodeling changes in bronchi as in the adjacent pulmonary blood vessels. In addition to the remodeling of the pulmonary circulation we also found changes in the systemic tracheal microcirculation indicating that the effects of allergen exposure may affect the systemic circulation as well as the pulmonary, at least in the parts of the systemic circulation in contact with the allergen. Following 3 days of allergen exposure, remodeling features began to appear; however, no significant increases compared to controls were found. The study showed that virtually all structural changes known to occur in allergen-exposed airways were also present in the large pulmonary blood vessels adjacent to the bronchi. Vascular remodeling has been described and characterized in other diseases of the lung, such as COPD (82, 119), IPF (120) and PAH (48), as well as SSc and SLE (81). The vascular remodeling in these diseases is similar to the changes we describe and is known to have an effect on the individual. Asthma is not usually associated with systemic or pulmonary hypertension, although some reports connecting asthma and pulmonary hypertension (PH) have been published. Rothman and Kulik (64) reported of 2 cases of PH where they concluded that PH was most likely associated to asthma, and long-term treatment of the asthma resulted in a normalization of the PH. Salako and Ajayi (121) concluded that a transient elevation of the systemic blood pressure may occur during an asthma attack, and that the frequency of hypertension among asthmatics is quite high.

*In summary*, this study confirmed that allergic airway inflammation is associated with vascular remodeling of both the systemic tracheal microcirculation and the bronchia-associated vessels. These changes are very similar to airway remodeling occurring in the same model and included increased smooth muscle, increased procollagen I and III production and proliferative responses.

## **Remodeling of extra-bronchial lung vasculature following allergic airway inflammation – paper II**

### **Background**

As the first study focused on large bronchial-associated blood vessels, and demonstrated allergen exposure-induced vascular remodeling of these vessels (73), we sought to investigate the smaller, non-bronchial-associated blood vessels. These vessels are not in close proximity with the allergen exposed airways, and since remodeling of the large bronchial-associated vessels was suggested to be the result of spill-over of inflammatory mediators from the inflamed bronchi, we hypothesized solitary vessels would be less affected than the bronchial-associated vessels.

### **Aims**

The aim of this paper was to investigate whether vascular remodeling extended into the lung, if it affected the parts of the pulmonary vasculature not being in direct contact with the bronchi, and to characterize these remodeling changes.

### **Results & Discussion**

We found that, in similarity to the large bronchial associated blood vessels, the solitary blood vessels displayed features of vascular remodeling. In addition to the features found in large vessels (increased proliferation of smooth muscle and endothelial cells, increased smooth muscle mass, increased procollagen expression), we also found that small vessels that normally have no or very little smooth muscle-positivity in their walls, expressed increased amounts following allergen exposure (i.e. they changed into a more muscularized phenotype). Muscularization of normally non-muscularized blood vessels is a well-known feature of remodeling caused by COPD (119). Furthermore, we found cells determined to be myofibroblasts (defined as cells co-positive for  $\alpha$ -SMA and procollagen I), in association with small solitary vessels, which were not observed in controls. The origin of the myofibroblasts is not clear, but several interesting possibilities

## ***Results and discussion***

exist; it might be a case of dedifferentiation of a naturally occurring cells (for example resting fibroblasts), an endothelial version of the epithelial-mesenchymal transition (75), or fibrocytes migrating into the parenchyma (122). Unfortunately, a full explanation of this interesting discovery was beyond the scope of the paper: however, we feel that any of the three suggestions or a combination of them is a plausible explanation.

*In summary* this paper demonstrated that vascular remodeling affects the entire pulmonary vasculature, not only the bronchial-associated vessels, suggesting that airway inflammation affects the entire lung.

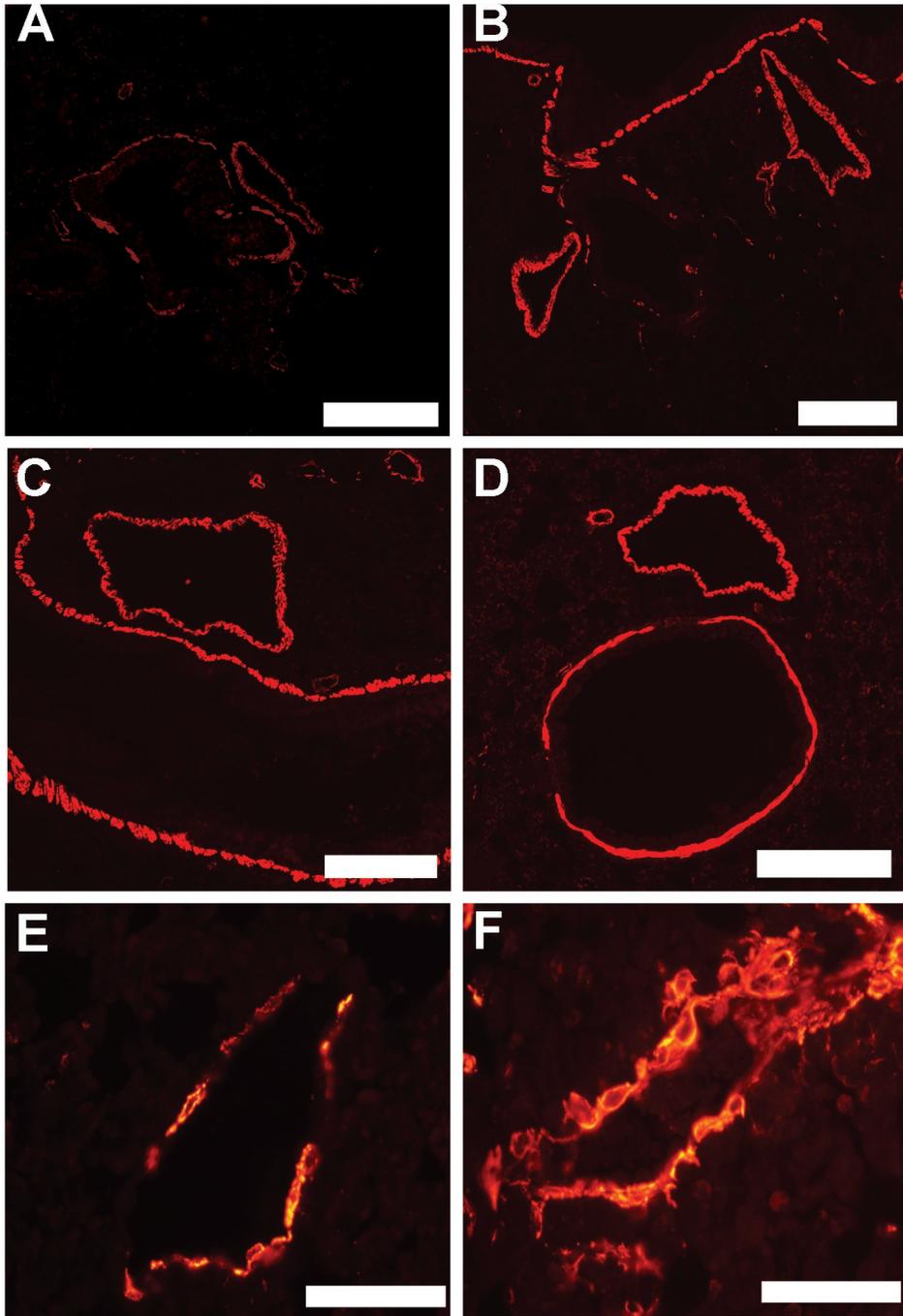
## **Vascular plasticity and partial resolution of vascular remodeling induced by allergic airway inflammation – paper III**

### **Background**

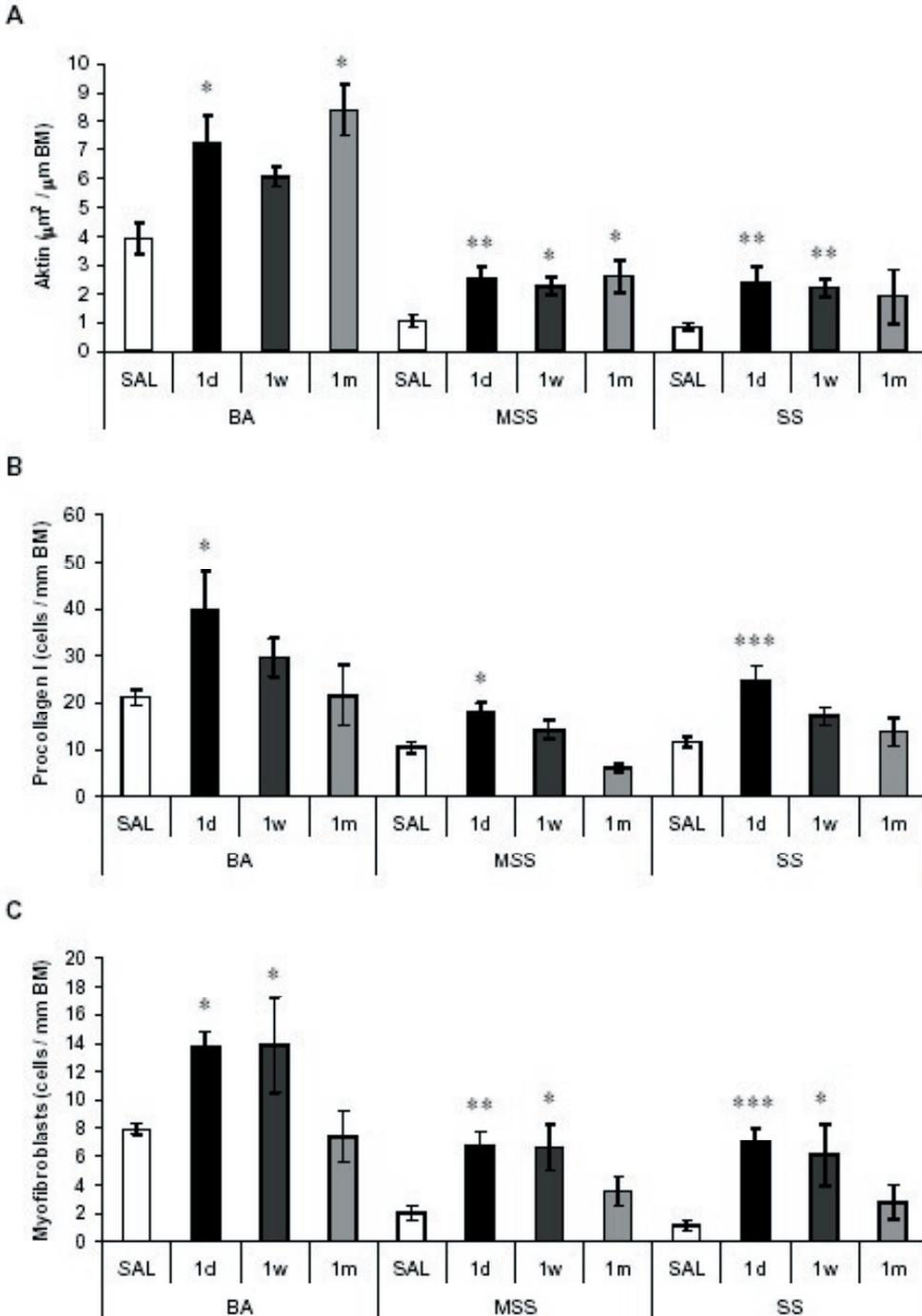
The previous two studies investigated vascular remodeling in three types of vessels in the lung and assessed the structural alterations directly after cessation of allergen administration. However, whether vascular remodeling could be reversed following discontinuation of allergen exposure remained unknown. The plasticity of vascular alterations in response to chronic inflammation has been described before, both the fast reaction upon an inflammatory stimulus and the quick resolution after cessation of stimulation (16, 123). To investigate the resolution process following allergic airway inflammation, animals were sacrificed at 1 day (1d), 1 week (1w) and 1 month (1m) after last allergen exposure. This study was designed to allow for the separation of short-term reversible changes from more permanent alterations, since the effect of seven daily allergen challenges (sacrifice one day after the last challenge, 1d) have been characterized before (Paper I and II).

### **Aims**

The aims of this study was to investigate if vascular remodeling initiated



**Figure 7** - Illustration of the differences in the area of  $\alpha$ -smooth muscle actin labelling at different time points, in bronchial associated vessels. In controls (A), actin is present, but significantly increased following seven days of allergen exposure (B). 1 week (C) and 1 month (D) after the last exposure. Also in mid-sized solitary vessels an increase compared to controls (E) was found following seven daily allergen challenges (F). Scale bars represent 200  $\mu\text{m}$  in A-D and 50  $\mu\text{m}$  in E-F.



**Figure 8** - Summary of the expression and changes of  $\alpha$ -smooth muscle actin (A), procollagen I (B) and myofibroblasts (C) during the different resolution periods in bronchial associated (BA), mid-sized solitary (MSS) and small solitary (SS) vessels. Differences were analyzed by the Kruskal Wallis ANOVA with Bonferroni and  $p < 0.05$  was considered significant. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$  and \*\*\* indicates  $p < 0.001$ .

by 7 days of OVA-exposure was reversible, and if the plasticity of the pulmonary vasculature was similar to previously described in other models of airway inflammation.

## **Results & Discussion**

Tissue eosinophilia was used as a parameter of inflammation, and the eosinophilia peaked at 1d, was still elevated at 1w but had returned to baseline at 1m indicating that the acute inflammatory response was resolved by that time. We found that several of remodeling induced by the inflammation appeared to accompany eosinophilia and reversed as eosinophilia resolved. This apparent correlation had two exceptions: vascular smooth muscle mass (Figures 7 and 8) and endothelial proliferation. These two parameters stayed elevated even after a one month resolution period, during which eosinophilia resolved completely, suggesting that some aspects of vascular remodeling are reversible, while others are not. The vessels display a great plasticity in similarity to previous descriptions, since not only can remodeling be induced in a very short period of time (demonstrated in paper I), but it may also resolve in a fairly short time, although some aspects of remodeling remained.

*In summary*, most features of vascular remodeling appear to be as quickly resolved as previously described by other authors (16, 123): however, some of the alterations appear to be irreversible or resolve much slower than the others. Interestingly, increased endothelial proliferation did not result in any occlusion of the vessel lumen, which could be either a sign of increased cell turnover, or may suggest that an even longer resolution period would have resulted in occlusion of the lumen.

## **Induction of vascular remodeling in the lung by chronic house dust mite exposure – paper IV**

### **Background**

The previous papers describe how the lung vasculature reacts to an acute

## ***Results and discussion***

airway inflammation by undergoing vascular remodeling, which mostly reverses as the inflammation is resolved, although some features remain even after one month of resolution. However, as the airway inflammation previously studied primarily mimics an acute situation, it does not simulate more chronic conditions as seen in human asthma. To study vascular remodeling from a chronic perspective, the HDM model was utilized. This model mimics several features of asthma (100) and is, compared to many other models, relatively similar to the human situation since no adjuvant is needed, no tolerance against the allergen develops (46, 100) and many of the characteristics of asthma are present in HDM-exposed animals (100).

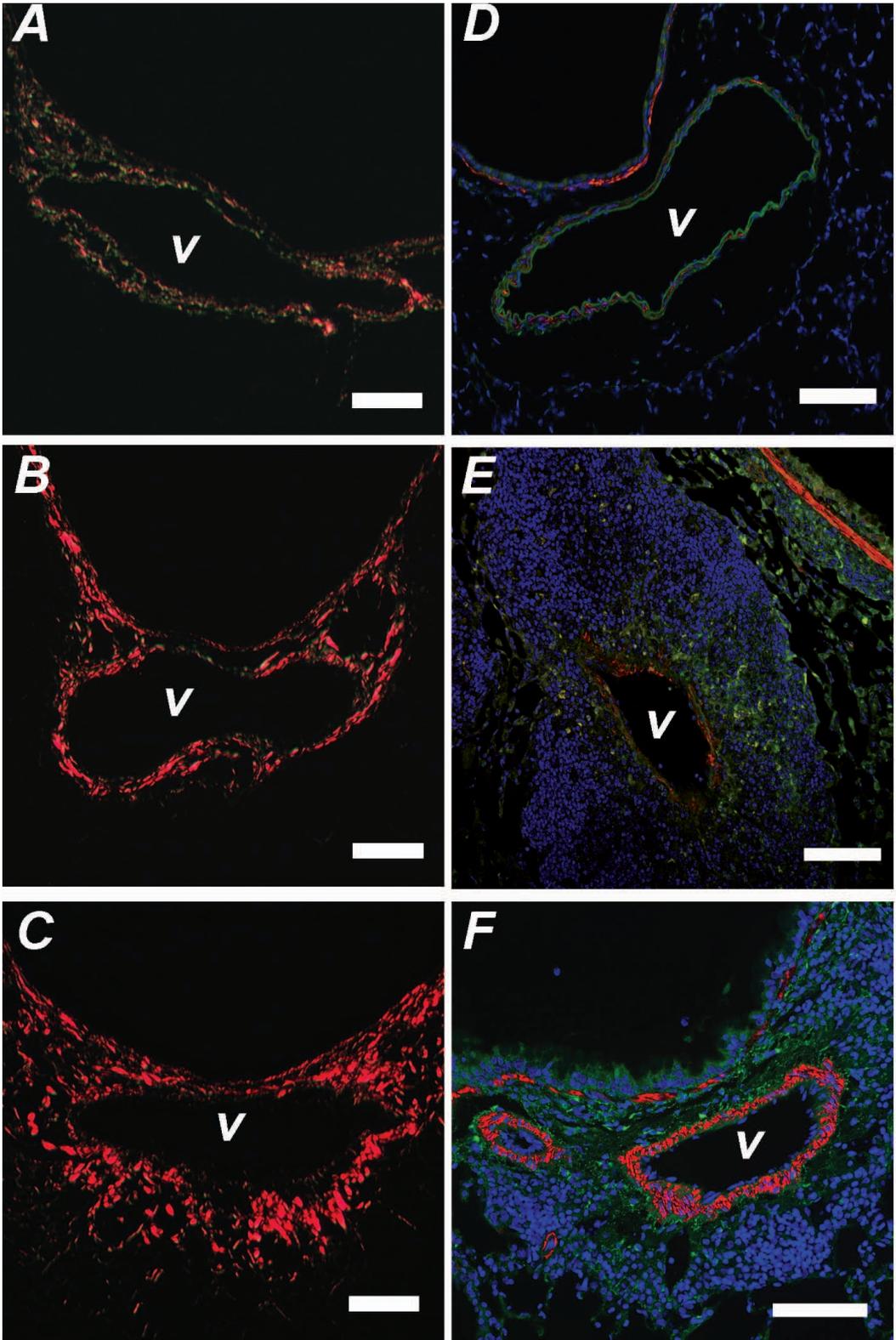
### **Aims**

The aim of this paper was to study how chronic airway inflammation affects the lung vasculature, and to investigate if this remodeling resolves when allergen exposure is ceased.

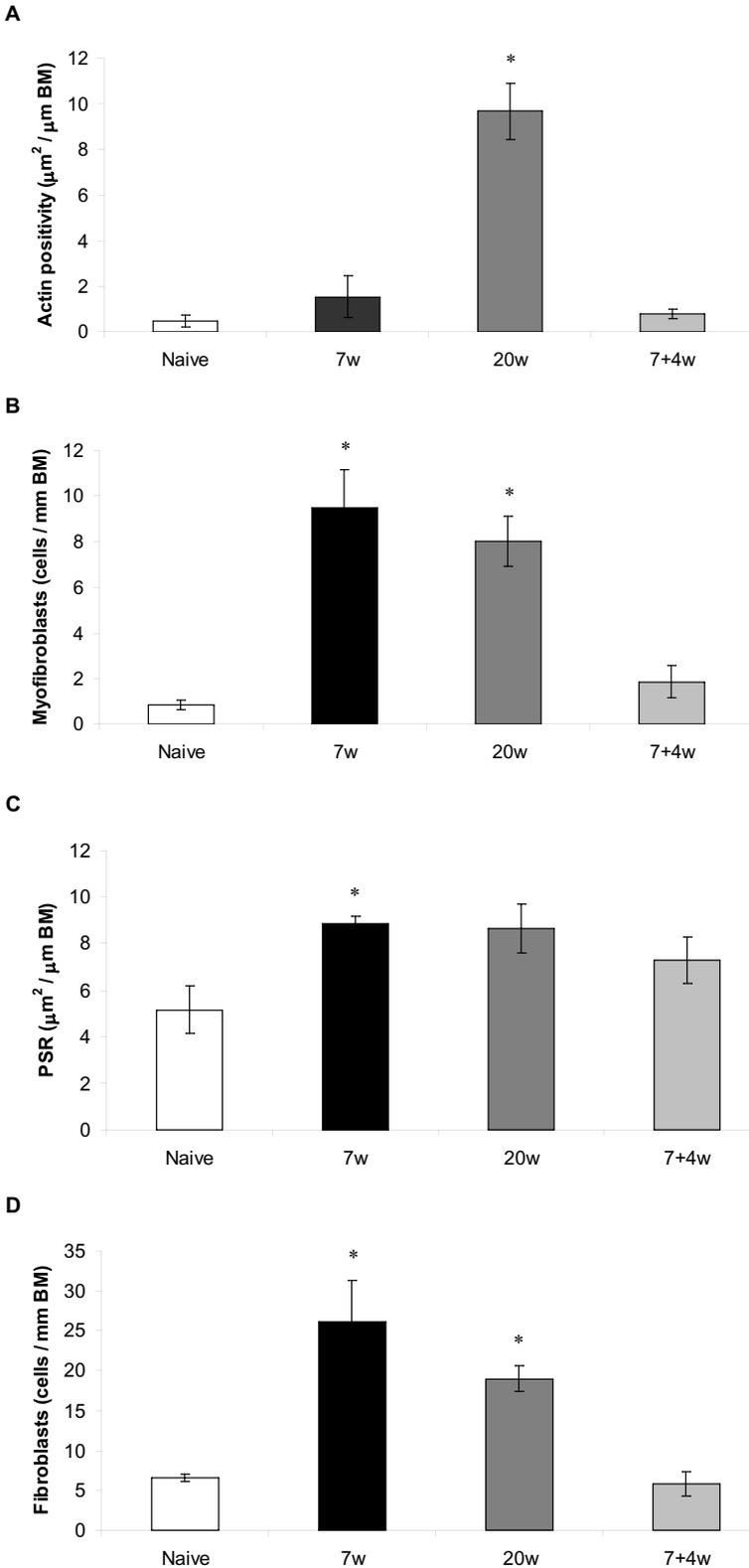
### **Results & Discussion**

This study concludes that vascular remodeling, as expected, take place also in chronic allergic airway inflammation (Figures 9 and 10). Structural alterations are similar to airway remodeling initiated by OVA exposure and some remodeling features seem to be irreversible, even after four weeks of resolution. Interestingly, in contrast to airway smooth muscle (100), no effect of resolution was found on the vascular smooth muscle mass; however this is most likely due to the fact that 7 weeks of HDM exposure did not seem

**Figure 9** - Illustration of remodeling in the HDM-model. Total collagen content increased compared to naïve (A) following 7 (B) and 20 (C) weeks of intranasal HDM-administration. Also the expression of  $\alpha$ -smooth muscle actin (red) and procollagen I (green) increased following 7 (E) and 20 (F) weeks of administration compared to controls (D). Total collagen content is visualized by using Picro Sirius Red (PSR), visible under polarized light as red staining. Smooth muscle was visualized using an antibody directed against  $\alpha$ -smooth muscle actin in combination with an antibody against procollagen I (N-terminal); cells co-positive for these two were defined as myofibroblasts. Hoechst 33342 was used to identify the cell nucleus. Scale bars represent 100  $\mu$ m.



## Results and discussion



**Figure 10:** Summary of the expression and changes of  $\alpha$ -smooth muscle actin (A), myofibroblasts (B), total collagen content (C) and procollagen I (D) in bronchial-associated vessels from naïve and HDM-exposed animals. The Wilcoxon Rank sum test was used for statistical analysis; all groups were compared against naïve. \* indicates  $p < 0.05$  compared to naïve.

to be sufficient to result in a significant increase in this parameter compared to controls. If the 4 week resolution period would have followed 20 weeks of HDM exposure, we suspect that the results would have been different. This is interesting since a previous study (100), found significant and irreversible changes in airway smooth muscle mass after 7 weeks of HDM exposure. This indicates that although remodeling appears to be similar in airways and vessels, the time frames of development differ. Comparing the results of 7 and 20 weeks revealed that the remodeling appears to increase to plateau, and once there the remodeling changes are sustained. This is an expected feature, and most likely also the case in asthmatics, as the entire lung would consist of remodelled tissue otherwise. In animals exposed to HDM for 20 weeks we found some interesting abnormalities, who were not quantified as they did not appear with any regularity, yet we felt a need describe the occurrence. Structurally, they appeared to be occlusions of bronchial-associated vessels, with extensive smooth muscle labelling and smaller amount of procollagen I-labelling within what appeared to be a grossly thickened vessel wall. Furthermore, we found small scattered co-localizations of procollagen I and total collagen (PSR), in the lung parenchyma.

*In summary*, this study shows that chronic allergic airway inflammation, induced by HDM exposure, initiates remodeling of the pulmonary vasculature, similarly to changes occurring in the airways in the same model and in similarity to the vascular remodeling found in the OVA model. The features were similar, although the magnitude and time-frame differed from mice subjected to OVA challenges. Furthermore, some of the alterations were reversible, but total collagen deposition appeared to be irreversible, as these changes remained after four weeks of resolution. In addition, we describe some structural abnormalities of unknown aetiology found to be present after 20 weeks of HDM exposure.



## GENERAL DISCUSSION

### **Allergic airway inflammation induces remodeling of both the systemic bronchial and pulmonary vasculature**

The fact that allergic airway inflammation is associated with vascular remodeling as well as airway remodeling was surprisingly unfamiliar when a literature search in association with Paper I was performed. Only scattered descriptions of vascular remodeling were found, mainly older publications such as the famous and extensive study from 1922 by Huber and Koessler (63). The 1991 study by Saetta and colleagues (19) was the only (to our knowledge) modern description of involvement of the pulmonary vasculature in asthma, and the involvement did not include vascular remodeling but rather descriptions of significant perivascular eosinophilia. Based on these findings the authors suggested an association between blood vessels and airways, and proposed the vascular involvement to be the result of a spill-over of inflammatory mediators from the airways. In contrast to the pulmonary circulation, the involvement of the systemic tracheal microcirculation has been extensively investigated in asthma (see for example (13, 124-126)), but in 2006 a study investigating bronchial arteries showed the presence of vascular remodeling in these vessels (12). Vascular remodeling was found to be present both in cases of fatal asthma, but also in cases of non-fatal asthma (documented asthmatics, but dying from other causes), suggesting that vascular remodeling is present not only in very severe asthma or at the end-stage of disease. In combination with the study by Saetta (19) this study confirms the findings of Huber and Koessler; both the systemic bronchial arteries and the pulmonary arteries are involved in asthma. Furthermore, these results offer a parallel between the human disease and animal models of the disease, as the results presented in this thesis shows that both the systemic airway-associated vasculature and the pulmonary circulation is affected.

## ***General discussion***

*In summary* the findings presented in this thesis suggest that the relative proximity to the inflamed airways acts as determinant of vascular remodeling, and direct contact does not appear to be necessary. Whether vascular remodeling is a result from “spill over” as previously suggested (19), or simply a natural effect of inflammation is currently unknown, although these possibilities are not mutually exclusive.

### **Asthma is not associated with pulmonary hypertension – vascular remodeling without pulmonary hypertension?**

Asthma is not associated with pulmonary hypertension, or rather; pulmonary hypertension is not normally a symptom of asthma. Furthermore, pulmonary hypertension is normally associated with vascular remodeling, although vascular remodeling is not always associated with pulmonary hypertension (86). However, the plasticity of the pulmonary vascular bed is great, and as long as the vessels are able to compensate no symptoms are presented (81). A physiological effect of vascular remodeling is most likely to be transient, since asthma usually displays a periodical pattern, most likely allowing some healing in between the attacks. The studies included in this thesis show that some features of remodeling are reversible and take some time to develop, suggesting that the remodeling may not be fully established directly but increase gradually and even resolve somewhat in between attacks. Results which may explain why asthma is not associated with pulmonary hypertension have recently been published. Daley *et. al.* (70) found OVA-challenged mice to be more sensitive (greater increase in right ventricle systolic pressure (RVSP)) to hypoxia than controls, whereas no correlation between RVSP and pulmonary arterial remodeling was found. Interestingly, Witzenrath *et. al.* (127) found that vessels from allergen sensitized and challenged mice react in a similar way, displaying a greater reactivity than controls to for example, endothelin-1 and serotonin. Furthermore, a study investigating responses of isolated bronchial arteries from OVA-sensitized rabbits (128) found similar increased responses.

Interestingly, in the studies by Daley (70) and Witzentrath (127), limited histological analysis was conducted, and the results show that vascular remodeling was present, with alterations very similar to the findings presented in this thesis.

*In summary* this suggests an interesting scenario, where airway inflammation induces vascular remodeling, which (via an hereto unknown process) in turn results in a hyperreactive vasculature (in response to hypoxia, serotonin, endothelin etc (70, 127)), offering an explanation to why asthma normally is not associated with PH, even though vascular remodeling is present. Interestingly, if these findings are applicable to humans, vascular remodeling may very well have an aggravating effect on an asthma attack, and this phenotypic alteration may also be a contributing factor to the overall disease.

## **Vascular remodeling in other diseases – comparisons to asthma?**

As very few descriptions of vascular remodeling (except of the tracheal microcirculation) in asthma or allergic airway inflammation have been published, an investigation on other diseases associated with vascular remodeling was initiated. Vascular remodeling was found to be a well-known and rather well described feature in many of these diseases. It is therefore interesting to consider the findings presented in this thesis, obtained in a model of allergic airway inflammation, with inflammatory diseases affecting the human lung by inducing vascular remodeling. Vascular remodeling is a feature of several diseases such as IPAH, SSc, SLE and COPD (48, 81, 119, 120). However, as asthma is usually periodic, in contrast to for example IPAH and COPD, differences in the remodeling profile (time frame, remodeling features, reversibility etc.) may therefore differ.

## *General discussion*

How the pulmonary vasculature reacts to chronic airway inflammation was investigated in Paper IV, and the features were similar to what is normally found in for example IPAH; increased smooth muscle, increased proliferation, fibrosis, etc., features normally found in the OVA model. Interestingly, when analyzing the results, some cases of occlusion of bronchial-associated blood vessels was found, which is a feature known to occur in PAH (80), but has to our knowledge not been reported in asthma or in models of allergic airway inflammation before. This indicates that the HDM model is not only a chronic model of allergic airway inflammation, but also a model of chronic inflammation.

PAH, regardless if it is primary or secondary, is a disabling disease with poor survival prognosis. Until recently, the potential for inflammatory processes to be a contributing factor was unrecognized. However, the / an inflammatory component has now been brought into focus, as recent studies indicate a role for inflammation in the vascular remodeling (78, 79). Due to the findings of Paper IV, and in accordance with the literature (78, 79) it is plausible to suggest that IPAH is the result of long-term inflammation, which results in pulmonary hypertension and is usually not noted until vascular remodeling is so extensive that symptoms are evident. At that time, remodeling is most likely well established and resolution is difficult. Interestingly, one of the most important drugs used to treat PAH is the endothelin-1 receptor antagonist (129), and as Witzenrath et al (127) found allergen sensitized and challenged animals to display a greater reactivity for endothelin-1 within the pulmonary vasculature, this suggesting a possible connection.

PAH is also a symptom of some of the autoimmune diseases, such as SSc and SLE (81) as well as some fibrotic diseases (80). Vascular remodeling found in these diseases is the result of chronic inflammation, and once symptoms of pulmonary hypertension are evident, the prognosis is not good (81) even when the patient is treated with different medications. The robust

remodeling found in Papers III and IV, suggest that remodeling is hard to resolve once established and may explain the difficulties in improving symptoms and the prognosis in PAH-patients. Furthermore, some patients with severe disease have shown to display a lack of sensitivity towards the drugs most commonly used, and this resistance has recently been ascribed to remodeling of the veins and microvessels (80). This is in accordance with results presented in this thesis, which clearly show that remodeling affects the entire pulmonary circulation, both pre- and post-capillary vessels as well as microvessels.

*In summary* the results presented and summarized in this thesis highlight the inflammatory component of vascular remodeling, and in combination with other studies, suggest a pathological mechanism behind and / or in combination with at least some cases of PAH.

### **Vascular remodeling in different models of allergic airway inflammation?**

Interestingly nearly all studies investigating vascular remodeling in asthma, with the exception of studies investigating bronchial microcirculation (13, 124-126, 130), have been performed on autopsy material, highlighting the limitations of bronchial biopsies. As bronchial biopsies cannot be used and autopsy material is difficult to obtain, animal models offer an attractive solution.

The vascular remodeling found in animals subjected to an OVA-induced allergic airway inflammation was similar to the changes induced by HDM (figures 7 and 9), although the mechanisms behind the remodeling most likely differ significantly. Similar features were observed, for example increased proliferation, fibrosis and increased smooth muscle mass. The time needed for remodeling to develop differed somewhat between the two models; 7 days of OVA-exposure versus 7 or 20 weeks of HDM exposure

## *General discussion*

(Figures 8 and 10). 7 weeks of HDM induced alterations; however compared to 7 days of OVA they were usually very modest (Figure 10). This is most likely due to the different models, as no adjuvant was used in the HDM model in contrast to the OVA model. This further highlights the chronic nature of the HDM model and the acute nature of the OVA model. Interestingly, remodeling not only followed different time lines depending on the allergen, but the different allergens also appeared to induce slightly different histological alterations. In the HDM model the number of procollagen I-producing cells actually peaked at 7 weeks of exposure and declined at 20 weeks, and the same is true for the number of myofibroblasts (Figure 10). The total collagen content however, peaked at 7 and was sustained out to 20 weeks (Figure 10), suggesting that fibrosis builds up during the first weeks, but later on the production of new collagen is decreased (but existing collagen accumulates in the tissue). Smooth muscle mass is interesting, as it follows a different pattern (Figure 10), with this parameter increasing until 20 weeks of exposure: at this time, the smooth muscle mass is very similar to the amount found in OVA-exposed animals after 7 days of exposure. This disparity is most likely due to the differences in sensitization and challenges, in combination with differences in the mechanisms behind the inflammatory response.

Vascular remodeling of pulmonary arteries following long term OVA exposure was recently described by Daley and colleagues (70), who found increased vascular smooth muscle density in small- to medium-sized pulmonary arteries, as well as thickening of the arterial walls and increased proliferation. Interestingly, similar results were found using a model utilizing *Aspergillus fumigatus* as allergen. The authors found no physiological effect of OVA-induced vascular remodeling; however they did note an increased sensitivity to hypoxia in OVA-exposed animals. Unfortunately the study by Daley did not investigate any other parameters except smooth muscle. As described in the four papers included in this thesis, smooth muscle hypertrophy / hyperplasia is only one of several

remodeling changes that occur, and of these changes, which is most important is impossible to say today. It is of great interest however that an increased right ventricle pressure was noted in response to hypoxia (70), as this is very much in agreement with the findings of increased reactivity to several vascular stimuli described by Witzernath (127), and the findings of vascular remodeling in these two recent studies (70, 127) are in concurrence with our findings of vascular remodeling.

*In summary* this suggests that airway inflammation, irrespective of allergen, administration route, sensitization / challenge protocol etc., induces vascular remodeling. The remodeling changes appear histologically similar, although the mechanisms behind them, time frames of development etc. differ between the models.



## CONCLUSIONS

Based on the findings in papers I-IV, it is concluded that:

- Allergic airway inflammation affects all vasculature associated with the inflamed airways; both systemic tracheal microvessels as well as all parts of the pulmonary circulation (large bronchial-associated vessels, mid-sized and small solitary vessels) are affected.
- Pulmonary vascular remodeling includes similar histopathological features as seen in airway remodeling, although the magnitude, time frame and resolution capacity differed somewhat between the two compartments.
- Similar features of vascular remodeling are present in both the OVA and the HDM models, although the magnitude, time frame and resolution capacity differed.
- Vascular remodeling is only partially reversible, as some structural alterations seem to remain even when allergic inflammation is resolved. The seemingly irreversible alterations were primarily smooth muscle alterations and endothelial proliferation following OVA exposure and collagen deposition following HDM exposure.



## SUMMARY IN SWEDISH

### Populärvetenskaplig sammanfattning

Remodeling av den pulmonella cirkulationen –  
En ny svarsmekanism vid allergisk luftvägsinflammation

#### **Bakgrund**

Astma är en vanlig luftvägssjukdom som drabbar en stor del av Sveriges befolkning. Astma kan orsakas av olika faktorer, som kall luft, starka dofter, luftvägsinfektioner, fysisk ansträngning m.fl. men vanligast är allergisk astma. Uppskattningsvis har 8-10 % av Sveriges befolkning av astma, och det är vanligare bland barn och vuxna än bland äldre.

#### **Astmaanfallet**

När man drabbas av ett astmaanfall reagerar luftvägarna (bronkerna) på ett stimuli och en inflammation initieras. Inflammationen orsakar en akut respons som karaktäriseras av svullna slemhinnor i luftvägarna, kontraktion av musklerna runt luftvägarna i kombination med ökad slemproduktion och ödem, vilket ger svårighet att andas. I vävnaden orsakar inflammationen vävnadsförändringar i lungorna, s.k. remodeling, förändringar som kan göra nästa anfall värre. Genom att muskelmassan runt bronkerna ökar, ökar också den kontraherande kraften vid framtida anfall, och genom ökad deponering av bl.a. kollagen runt luftvägarna blir dessa mer rigida. Eftersom dessa förändringar har effekt för individen är det också av intresse att försöka förstå uppkomst, bakomliggande orsaker och hur man kan minska dessa.

#### **Modell**

De flesta studier av astma sker på vävnad från bronkiella biopsier, en metod vars nackdel är att endast de största luftvägarna undersöks på detta sätt.

## ***Summary in Swedish***

Nya studier har visat att det inte endast är dessa som är inblandade i astma, utan även övriga delar av lungan tycks vara inblandade. För att kunna studera hela lungor har två djurmodeller av allergisk luftvägsinflammation använts i projektet; 1) möss görs allergiska mot ett protein från ägg och sedan får inhalera detta protein, varpå en inflammation startar i lungorna, 2) mössen får inandas en lösning av kvalsterproteiner, en metod som ger ett liknande svar som 1) men administrationen pågår under en längre tid. Analyser av lungorna har gjorts genom immunohistokemi, vilket innebär att man lokaliserar olika proteiner med hjälp av antikroppar som binder till specifika antigen, och sedan studerar utbredning och inmärkt area.

## **Syfte med avhandlingen**

Målet med avhandlingens har varit att undersöka hur lungans blodkärl påverkas av allergisk luftvägsinflammation genom att använda två något olika djurmodeller. Jag har också studerat vilka förändringar som sker, vilka kärl som påverkas, och om påverkan är reversibel.

## **Resultat**

Studierna har visat att små kärl (mikrokärl) i väggen på luftstrupen påverkas av den allergiska luftvägsinflammationen, liksom de stora kärlen (pulmonella artärer) som löper längs bronkerna i lungorna och mindre kärl som ligger ute i lungvävnaden. I paper I beskrivs de strukturella förändringarna i mikrokärlen och de pulmonella artärerna – förändringar som tidigare varit i stort sett okända. De pulmonella artärerna uppvisade samma typ av förändringar som man tidigare beskrivit hos bronkerna; ökad mängd glatt muskel, ökad inlagring av kollagen m.fl. I paper II utökades intresseområdet och även kärl som låg längre från bronkerna studerades. Även dessa kärl reagerade på inflammation genom strukturell förändring på samma sätt som de pulmonella artärerna. I paper III studerades frågan om reversibilitet; kunde de strukturella förändringarna gå tillbaka när allergenet slutade administreras? Svaret blev att vissa förändringar gick tillbaka, medan andra stannade kvar. I paper IV studerades samma parametrar som i paper I, II och III, men en annan modell (mer lik situationen i en människa) användes. Även i denna modell hittades strukturella förändringar

i kärlen och inte heller här gick alla dessa förändringar bort efter att man slutat administrera allergenet.

### **Praktisk betydelse**

Det första arbetet gav förutom ökad kännedom om remodeling processen i kärl också en insikt i att kärlförändringar förekom i allergisk luftvägsinflammation. Alla arbetena har utökat kännedomen om inflammatoriska processer och bidragit till insikt om att allergisk luftvägsinflammation inte bara har effekt på de stora luftvägarna där allergen-exponeringen sker, utan påverkar hela lungan.



## ACKNOWLEDGEMENTS

This thesis would not have been accomplished without the support and encouragement from numerous people. I will particularly like to thank the following;

**Jonas Erjefält**, my main supervisor, for introducing and guiding me into the world of lungs and inflammation. Your efforts have made me an independent, strong and organized scientist.

**Gunilla Westergren-Thorsson**, my secondary supervisor. You jumped on the rollercoaster half way into the ride; I hope you have liked it. Your support and always enthusiastic approach to projects and results has been most appreciated.

**Lena Uller**, my research colleague and friend. Your enthusiasm and support over the years have been invaluable; I sincerely have no idea where I would be without you. Let's keep up a good work ☺.

**Cecilia Andersson** and **Michiko Mori** – My colleagues and friends, it would have been empty and dull without you. Thank you for all help and encouragement. Good luck with your projects!

**Jill Johnson**, a colleague and great friend. My sincerest thanks for everything, from housing me in Hamilton to correcting my thesis. I wish you the best of luck in the future, let's keep in touch!

**Karin Jansner**, for your technical support and expert knowledge; **Britt-Marie Nilsson**, for technical expertise and assistance.

My present and former colleagues in the LURN Journal Club: **Amelie, Annika, David, Gediminas, Kristian, Kristoffer, Lisa, Lizbet, Monika, Oskar, Pernilla**.

## *Acknowledgements*

Professor **Manel Jordana** and his group at McMaster University, Hamilton, Canada, for letting me visit your lab, and presenting my research.

My former PhD-student colleagues at F10; **Nils, Elin, Malin, Mikael, Katarina, Lina.**

Everyone at D12; the groups of **Per Hellstrand, Roland Andersson, Ingemar Carlstedt, Anders Malmström** and **David Erlinge** as well as the ones who used to be at F10; the groups of **Frank Sundler** and **Eva Ekblad.**

All **members of Lund Respiratory Network (LURN)**, for interesting discussions and presentations on our meetings and retreats.

**Katarina Danielsson, Martin Nyström, Agneta Persson** and **Annette Jönsson** for technical and administrative assistance.

**Anna, Pia** and **Sussie**, my close friends throughout these years. It has been invaluable to have someone who understands and to share experiences with. Thanks for your support and never-ending friendship, which I value highly.

**Mamma, pappa** och **Sofia**, tack för all uppmuntran, stöd och att ni alltid har trott på mig. För att ni har försökt förstå vad jag gör och vad min forskning betyder.

**Markus**, för att du är du och **Albin** för att du förde in en ny dimension i mitt liv.

**Övrig familj och alla vänner** – tack för att ni finns

And to all other not mentioned here – sincerest thanks

This work was supported by grants from the Swedish Heart and Lung Foundation, the Swedish Research Council, The Crafoord Foundation, the Asthma and Allergy Foundations, The Faculty of Medicine at Lund University, the Maggie Stephens Foundation and the Landshöding Per Westlings Foundation.

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