# NASOBRONCHIAL INTERACTION IN ALLERGIC RHINITIS AND ASTHMA

NEUS-LONG INTERACTIE
IN ALLERGISCHE RHINITIS EN ASTMA

Cover illustration: Nasal mucosa section of an allergic rhinitis patient after nasal allergen provocation. Immunohistochemically stained for E-selectin. Background staining with hematoxylin. Magnification x 100.

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# NASOBRONCHIAL INTERACTION IN ALLERGIC RHINITIS AND ASTHMA

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## Part II



## Part III

# 1

# General Introduction

## 1.1 Clinical aspects of nasobronchial cross-talk

The key to the diagnosis lies in taking a good medical history. This rule especially applies to allergic rhinitis and asthma. Both diseases have in common that they are often underdiagnosed[1] and lack proper treatment. Allergic rhinitis and asthma frequently occur together. Almost 40 % of the allergic rhinitis patients have lower airways involvement, whereas more than 80 % of the allergic asthma patients have concomitant rhinitis symptoms[2,3]. The latter percentage gets close to 95 % when a careful nasal history is taken and physical examination is performed[1].

Allergic asthma is characterized by a history of episodes of cough, dyspnea, shortness of breath, chest tightness and wheezing, in combination with variable bronchoconstriction and/or bronchial hyperresponsiveness (BHR)[4,5]. Mucosal inflammation is also considered an important hallmark of asthma[6]. However, the associations between mucosal inflammation and clinical parameters, such as variable airflow obstruction and BHR, are still controversial[7-9].

Allergic rhinitis is primarily based on a typical history of sneezing, rhinorrhoea, eye symptoms and nasal obstruction. As in allergic asthma, the atopic status needs to be confirmed[4,10-12]. In perennial rhinitis, chronic nasal obstruction can sometimes be the only symptom, which makes it difficult to confirm the diagnosis. The diagnosis of seasonal allergic rhinitis is less disputable, the symptomatology and seasonal occurrence are characteristic and have been well established for many years. The distinction between allergic asthma and rhinitis is sometimes difficult to make since symptom perception is widely variable; lung function can be normal in mild asthmatics and, although BHR is a constant feature of asthma, it is also frequently present in allergic rhinitis [13].

## 1.2 Historical background

Although anecdotal descriptions of asthma and allergic rhinitis date from earlier times[14], it was not until the 19th century that a pathophysiological association between these two allergic airways diseases was recognized. In 1872, Weber observed that nasal and bronchial mucosa shared many histological features[15]. One

year later, Charles Blackley established a relationship between grass-pollen exposure and the subsequent development of nasal and bronchial complaints[16]. Curschmann suggested that hypersecretion was responsible for airway obstruction in both asthma and rhinitis[17]. On the other hand, Sluder stated that a nasobronchial reflex was the most likely pathophysiological mechanism in asthma[18]. In the early 20th century, von Pirquet found that repeated vaccination with horse streptococcal antitoxin serum induced increased reactivity in susceptible persons[19]. He proposed the term "allergy" for the concept of changed reactivity. The term "atopy" was introduced several years later (in 1923) by Coca and Cooke for clinical forms of allergy, such as hay fever and asthma[20]. It was not until 1967 that IgE was identified as the major immunoglobulin involved in atopic disease[21,22].

## 1.3 Epidemiology

Prevalence rates of atopic diseases, such as asthma, rhinitis and eczema, vary all over the world[23]. In Europe, the prevalence of asthma has increased more than twofold in the last two decades and now affects between 10% and 15% of the adult population[24]. The prevalence of rhinitis closely follows that of asthma, but is up to three times greater[23].

A family history of atopic disease is recognized as a major risk factor for asthma and rhinitis[25,26]. Also, environmental factors have an unmistakable effect on the development of atopic disorders. An increase in the prevalence of rhinitis and atopic sensitization was observed among East German children between 1991 and 1996. However, no increase in the prevalence of asthma could be noticed in this population[27]. Therefore, genetic predisposition and early life influences - indoor allergen exposure[28-31], breast feeding[32] and maternal smoking[33] - may be more important for the development of childhood asthma, whereas allergic rhinitis may also be affected by environmental factors occurring later in life.

On the other hand, allergic rhinitis is regarded as a risk factor for the development of asthma, especially in the presence of BHR[34,35]. BHR and atopy are presumably coinherited and associated with asthma[36].


## 1.4 Treatment

Although accumulating evidence underlines the importance of allergic rhinitis in the control of asthma, both diseases are still treated as separate disorders. Few studies have investigated the effect of nasal treatment on asthma. Topical treatment with intranasal corticosteroids reduces lower airway symptoms and decreases BHR in allergic rhinitis patients with seasonal asthma[37-40]. In one study, delivery of beclomethasone to the nose even had a greater effect on BHR than delivery of the same dose via the oral route[41]. Only one report was found that demonstrated a beneficial effect of bronchial treatment with inhalation corticosteroids on nasal symptoms and inflammation[42]. Avoidance of inhalant allergens[43] and immunotherapy[44] may be effective for both asthma and rhinitis on a long term basis. Despite the benefit of antihistamines and cromoglycates in seasonal allergic rhinitis, the effect on lower airways is still doubtful[45]. On the other hand, systemic agents, such as leukotriene receptor antagonists, proved to have an additional therapeutic effect in asthma[46,47]. Whether it will also be applicable in the treatment of allergic rhinitis remains to be proven.

## 1.5 Mucosal inflammation

Allergic asthma and allergic rhinitis are characterized by a similar inflammatory process in which mast cells and eosinophils appear to be the major effector cells[48,49]. The migration of eosinophils from the blood into the tissue is dependent on the expression of cytokines and adhesion molecules. Eosinophilia in bronchial mucosa is regarded as one of the hallmarks of allergic asthma[6]. However, signs of allergic inflammation have also been found in induced sputum[50,51], broncho-alveolar lavage (BAL) fluid[52] and bronchial biopsy tissue[53] of allergic rhinitis patients without asthma. Although it is clear that the condition of the upper airways influences the lower airways, the mechanisms underlying this relationship are far from being understood.

As yet, the immunopathological aspects of cross-talk between nasal and bronchial mucosa have not been subject of investigation.

## 1.6 Conclusion

The insight, that allergic rhinitis and asthma are related, originates from the late 19th century when Blackley described his own personal experiences after nasal allergen provocation. Many years later, epidemiological studies showed that the diseases often co-exist and that they share a common genetic background. In addition, medicinal treatment of allergic rhinitis demonstrated an improvement of concomitant asthma and vice versa. To date, allergic rhinitis is regarded as an important risk factor in the development of asthma.

Until now, most studies have looked at the connection between nose and lung from the pathophysiological point of view. Studies investigating the immunopathological interaction between upper and lower airways have not been performed yet. Since allergic rhinitis and asthma are characterized by a similar inflammatory pattern, and bronchial inflammation has been demonstrated in nonasthmatic allergic rhinitis patients, there is reason to believe that immunopathological mechanisms may contribute to the cross-talk between nose and lungs.

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# 2

# Interaction mechanisms in allergic airways disease

## 2.1 Introduction

Allergic asthma and rhinitis are both manifestations of the atopic syndrome. Although both diseases commonly occur together, it is still unclear why some allergic patients develop only asthma and others rhinitis. The reason for the variety in clinical expression of allergic disease is not known. The specific structure and function of the airway involved could be contributing factors to this phenomenon. On the other hand, local and systemic inflammatory pathways seem to play an important discriminative role, as well. Allergen provocation studies are a good model to investigate local inflammatory processes. The possibility of cross-talk between nasal and bronchial mucosa after allergen provocation has not yet been examined. In this chapter, we will discuss the specific local features and possible interaction mechanisms in allergic airways disease. Furthermore, we will briefly review the use of allergen provocation models in practice.

## 2.2 Airway structure

### *2.2.1 Anatomy*

The nose and lungs are anatomically and physiologically closely related organs. Nevertheless, differences in anatomy and function between the nose and lungs have led to the division of the respiratory tract into upper and lower airways. The upper airways, from the nostrils to the vocal cords, have been the domain of the E.N.T. surgeon for many years, while the airways underneath the vocal cords have been the work area of the chest physician. This arbitrary division contributes to the fact that asthma and allergic rhinitis are still considered two different entities and are treated accordingly.

In order to understand the distinct manifestations of allergic airway disease, it is essential to have knowledge of the anatomy (Figure 1) and physiology (Figure 2) of the respiratory tract.

The nose is predominantly a rigid structure, consisting of the nostrils and nasal cavities, which are separated by the internal ostium (nasal valve). The internal nose is deeper than suggested by the visible nose, the length being 10-12 cm from the tip of the nose to the nasopharynx. The septum divides the nasal cavity into two halves. Due

to the prominence of the turbinates (inferior, middle, superior), each of the two nasal cavities is a narrow slit, only 2-4 mm wide[1].

The pharynx and larynx connect the upper with the lower airways and diseases involving this part of the airways can also influence airway function.

The lower airways comprise the tracheo-bronchial tree, the respiratory bronchioles, alveolar ducts and alveolar sacs. The large lower airways have a layer of cartilage and, as the airways progressively branch, the cartilage gradually disappears, so that the smallest airways are only supported by the elastic fibers of the surrounding parenchyma[2]. The total surface area of both nasal cavities is about 150 $cm^2$, whereas the total surface of the lower airways is close to 100 $m^2$.

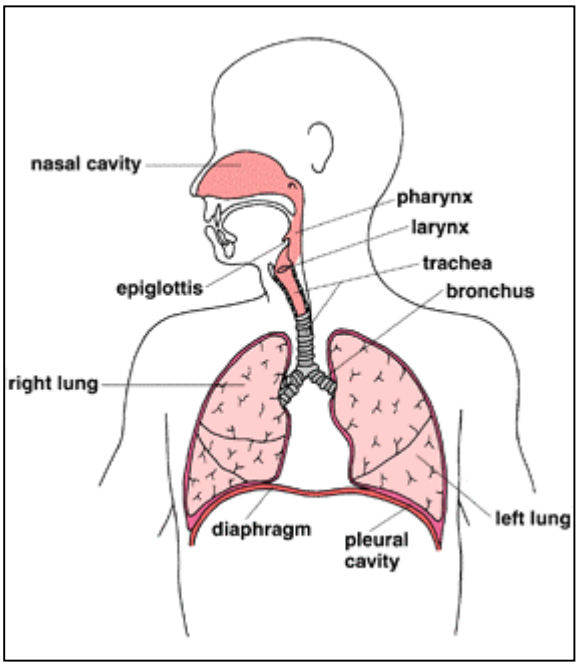


**Figure 1.** Anatomy of the respiratory tract

### *2.2.2 Function*

The anatomy of the nose is important for its functions: The control of airway size, filtration, air conditioning and smell.

Nasal airway resistance accounts for 40% of total airway resistance and is regulated by flow velocity and the volume of the nasal cavities in which the turbinates play an important role. The size of the nasal cavities are most susceptible to changes and can

be modified by several factors: the nasal cycle, exercise, emotions, vasomotor response to hormones, environment, pharmacotherapy, etc[1,3].

One of the major roles of an anatomically and functionally normal nose is the filtering function. The efficacy of the nose filter depends on the size of the particles. During normal breathing, only a few particles larger than 10 μm (pollen grains) can enter the lower airways after nasal filtration, while most particles smaller than 2 μm (mould spores) can easily bypass the nose without being trapped in the nasal mucous blanket[4].

As cold and dry air are known to cause bronchoconstriction and BHR in sensitive asthmatic patients[5-8], conditioning (heating and humidification) of the inhaled air is of major importance. Through the adjustment of the mucosal blood flow, the nose can either warm and humidify or extract heat and water from the passing air[9].

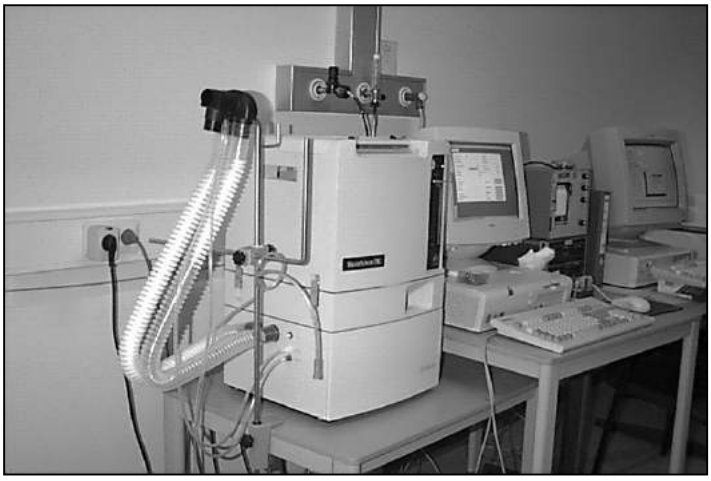

**Figure 2.** Spirometer setting used for analyzing lung function in our lab

Smell can also protect the lower airways as it warns subjects of several irritants and induces conscious and non-conscious defense responses[10].

The main function of the respiratory system is gas exchange. The most distal part of the lower airways provides an enormous surface area where the exchange of carbon dioxide for oxygen takes place depending on ventilation, diffusion and perfusion of the lungs[11]. Other functions include acid-base balance, phonation, pulmonary defense and metabolism[12,13].

### 2.2.3 Histology

The airways are lined with mucosa consisting of epithelium, basement membrane and lamina propria (Figure 3). Although there is no fundamental difference between the structure of the ciliated epithelium in upper and lower airways, epithelial fragility and increased basement membrane thickening is found in bronchial mucosa of asthmatic patients[14], but not in nasal mucosa of allergic rhinitis patients[15]. The nasal lamina propria has a subepithelial network of capillaries, arterio-venous shunts and venous sinusoids which can change in dimension and modulate upper airway resistance[1-3,16]. In bronchial lamina propria, there is a lower degree of vascularization than in the nose. The bronchial mucosa is surrounded by a smooth muscle layer regulating the lower airway caliber[17]. Apart from these differences in histology, allergic rhinitis and asthma have similar inflammatory findings in epithelium and lamina propria.

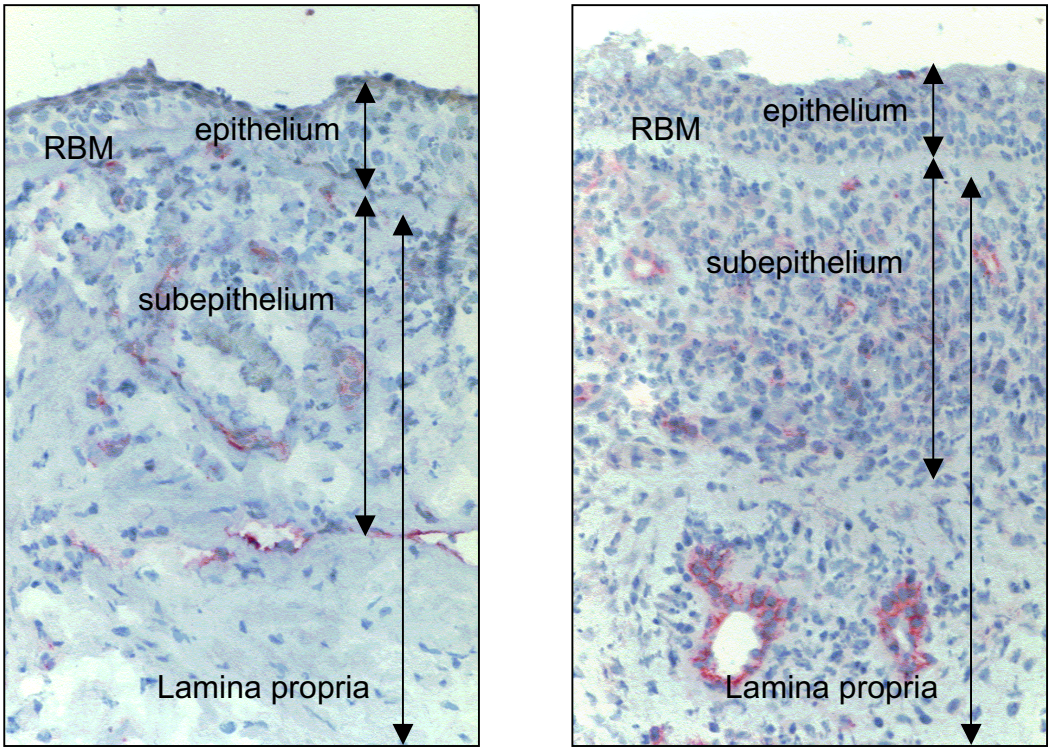


**Figure 3. A.** Bronchial mucosa section. **B.** Nasal mucosa section.
The airway mucosa can be divided into epithelium, subepithelium (a cell-rich area 100 µm deep in the lamina propria, along the length of the reticular basement membrane[RBM]) and lamina propria (total subepithelial mucosa).

## 2.3 Allergic inflammation

It is well established that allergic rhinitis and asthma are characterized by a similar inflammatory process (Figure 4) in which dendritic cells, mast cells, T-lymphocytes, eosinophils and basophils are important effector cells[15,18-21]. Allergic mucosal inflammation is the result of several sequential events:

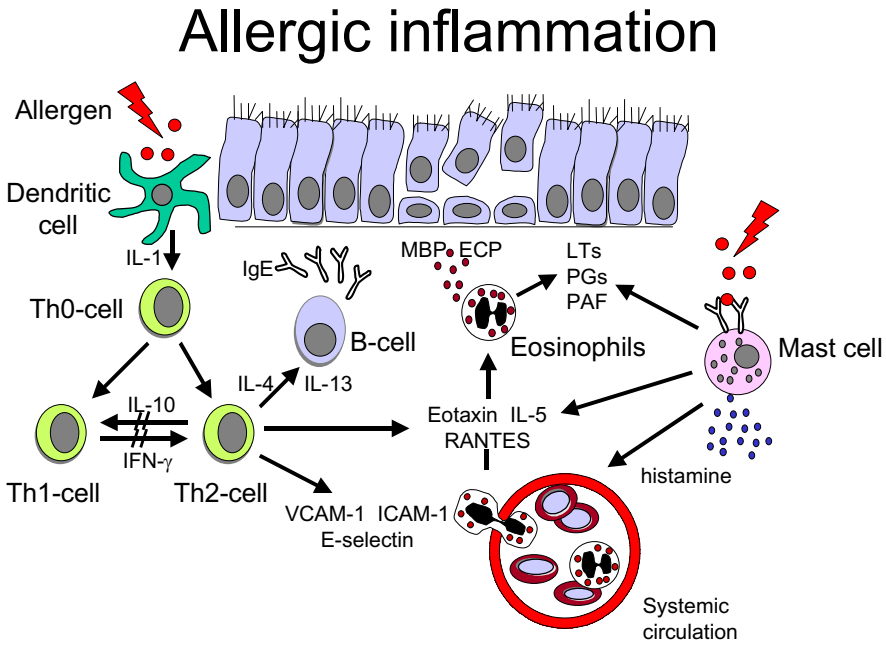


**Figure 4.** Cellular interaction in allergic inflammation

### *2.3.1 The early phase response*

The allergic response starts with the uptake of the antigen by antigen presenting cells (APC's), in particular dendritic cells, which are present in the superficial layers of skin and airway mucosa[22,23]. Dendritic cells can migrate to the regional lymph nodes where they present the antigen to naive T lymphocytes[24,25]. Effective mucosal immunity depends on antigen-priming of both T and B lymphocytes. B cells directly recognize antigen through their surface immunoglobulin (sIg) receptors. The T cell receptor recognizes fragments of antigens bound to the MHC molecules expressed on the surface of APCs. Activation of the T cell receptor in addition to the effect of co-stimulatory molecules[26,27] stimulates the naïve T helper cell, a Th0 cell, to differentiate to either a Th1 or a Th2 subset, characterized by their own cytokine

profile[28]. Activation of Th1 cells causes the release of IL-2 and INF-γ; Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Both cell types produce IL-3 and GM-CSF. Th2 cytokines are involved in IgE synthesis (IL-4) and triggering and maintenance of allergic inflammation in the tissue (IL-5)[28,29].

The binding of IgE and the subsequent cross-linking of the high affinity receptor for IgE (FcεRI) lead to degranulation of mast cells and release of histamine and proteases (chymase, tryptase) among others, characteristic of the early phase of the allergic immune response. Both mast cells and T-lymphocytes orchestrate the late phase reaction in which eosinophils and basophils migrate from the circulation towards the mucosa.

### *2.3.2 The late phase response*

Tissue eosinophilia is a characteristic feature of the late phase response of the allergic inflammation. The eosinophil is a source of leukotriens (LTs), prostaglandins (PGs), platelet activating factor (PAF), cytokines and cytotoxic proteins, which can be released upon stimulation and are toxic to the airway epithelium.

Although basophils comprise a minor component of the inflammatory cell influx, their cell number correlates with the severity of allergic airways disease[30]. Moreover, it has been demonstrated that basophils produce more IL-4 and IL-13 per cell than any other cell type and contribute to the modulation of the allergic immune response[31].

Leukocyte migration is dependent on the expression of cytokines, chemokines and adhesion molecules[32,33]. Several studies have stressed the importance of IL-5 and eotaxin for the chemotaxis of eosinophils in mucosal tissue[34-36] and blood[37,38]. Upregulation of VCAM-1, an adhesion molecule with a certain eosinophil specificity, results in firm leukocyte-endothelial adherence and trans-endothelial migration of leukocytes along a chemotactic gradient[32]. Eosinophilia in bronchial mucosa is regarded as one of the hallmarks of allergic asthma[39]. However, the presence of the eosinophil in the lower airways is not a very specific finding. Also in allergic rhinitis patients without asthma, signs of allergic inflammation have been found in induced sputum[40], broncho-alveolar lavage (BAL) fluid[41,42] and bronchial biopsies[43].

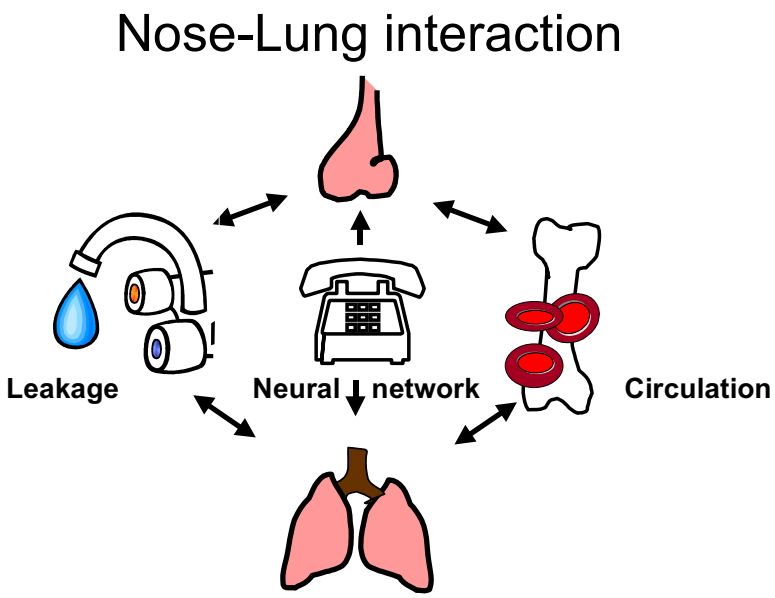


**Figure 5.** Pathways of interaction between nose and lungs

## 2.4 Nose-lung interaction: possible mechanisms

Several mechanisms (Figure 5) have been suggested to play a role in lower airway dysfunction among patients with rhinitis: These include impaired nasal function, pulmonary aspiration of nasal contents, the nasal-bronchial reflex and increased absorption of inflammatory mediators in the blood stream[44].

### *2.4.1 Impaired nasal function*

In allergic rhinitis, the nose can be blocked and mouth breathing is favoured. The reduced filter function of the nose leads to increased exposure of the lower airways to allergens. In susceptible persons, this may lead to inflammatory changes and an increase in airway hyperresponsiveness. According to Dunlop *et al.* surgical treatment of chronic rhino-sinusitis and nasal polyposis in asthmatic patients improved their asthmatic status[45], suggesting that improvement of nasal function results in better asthma control. In cat allergic patients, no enhanced asthmatic response to cat allergen was demonstrated after blocking the nose with a nose-clip[46]. Corren *et al.* observed that, after nasal allergen provocation, the degree of nasal blockage was not related to changes in bronchial hyperresponsiveness[47].

### *2.4.2 Aspiration of nasal contents*

The "mucociliary escalator" is another important mechanism in modulating airway condition through the removal of inhaled particles: all cilia in the nose, Eustachian tube, paranasal sinuses and the tracheo-bronchial tree, beat towards the pharynx. Particles trapped in the mucus layer are transported to the pharynx by the mucus escalator. In allergic rhinitis, nasal secretions, containing inflammatory cells and mediators, may be aspirated and could, therefore, be responsible for lower airways involvement[44].

However, aspiration occurs only in patients with decreased consciousness and cough reflexes, and it is not likely to play a role in normally functioning human airways[48]. Nasal secretions are mainly swallowed and neutralized in the stomach. Moreover, inhalation studies with radio-labeled allergen have shown no deposition of allergen in the lungs after nasal allergen application[47]. Therefore, this mechanism is not very likely to have a contributory effect on nasobronchial interaction.

### *2.4.3 Nasobronchial reflex*

Afferent sensory innervation in the nose is carried by the trigeminal nerve and efferent parasympathetic fibers are part of the vidian nerve in the nose. The lower airways receive afferent and efferent innervation through the vagal nerve. In the bronchi, the parasympathetic nerves regulate smooth muscle tone[49]. Mechanical or chemical stimulation of receptors in the nose, trachea, larynx or elsewhere in the respiratory tract may produce sneezing, coughing or bronchoconstriction to prevent deeper penetration of allergens or irritants into the airways. Although exposure of the nasal mucosa to cold dry air can result in immediate bronchoconstriction and bronchial hyperresponsiveness in asthmatic patients[5-8], no direct effect on $FEV_1$ could be detected after nasal allergen challenge[47,50,51]. Corren et al. demonstrated increased bronchial hyperresponsiveness 30 minutes after nasal provocation suggesting a role for a nasobronchial reflex mechanism[47]. However, since increased BHR was still present after 4½ hours, other mechanisms are likely to contribute to the interaction.

### *2.4.4 Increased absorption of inflammatory mediators in the blood stream*

Blood eosinophilia is a frequent finding in allergic rhinitis[52,53] and asthma[20,52,54]. Nasal provocation with methacholine in asthmatic patients with rhinitis resulted in an increase in lower airway resistance that could be blocked by premedication of nasal mucosa with phenylephrine, suggesting a role for systemic mediators in the induction of lower airway resistance[55]. Furthermore, studies in animals[37,38,56] and patients with allergic rhinitis, asthma and other atopic diseases[32,57,58] have consequently shown a rise in circulating inflammatory cells and progenitors after allergen inhalation followed by recruitment to sites of allergic inflammation. Corren *et al.* speculated that prolonged bronchial hyperresponsiveness after nasal allergen provocation could be explained by a continuous influx of inflammatory mediators reaching the lower airways through the systemic circulation[47].

## 2.5 Allergen provocation

### *2.5.1 Animal versus human models*

Although provocation studies are only an approximation of the natural situation, they serve as a good model to study the mechanisms of the allergic response. Allergen provocation studies have been performed in mice[36,37,59,60], guinea-pigs[38,59], dogs[56], primates[61] and human[41,62-68].

Animal provocation models have several advantages compared to human provocation models: the research population is very homogeneous, they allow multiple sampling of various organ systems and take place in a controlled laboratory setting. Their disadvantage is that animals are generally not sensitized to the allergens that cause allergic asthma and rhinitis in humans. Moreover, airway geometry and immunopathological mechanisms may vary among species. Therefore, in spite of less controlled conditions, human provocation studies are essential for the understanding of the pathogenesis of allergic disease. Most allergen provocation studies in nose and lung have focused their attention on local airway responses.

### *2.5.2 Segmental allergen provocation*

Segmental bronchial provocation (SBP) has previously been used to study local inflammatory processes in allergic rhinitis[41,69] and asthma patients[66,70]. Some investigators chose a patient dependent dose based on the individual skin-prick test results[41], others used a fixed dose[70]. A dose of 500 BU led to a sufficient local allergic response in asthmatic patients without causing too much discomfort[70].

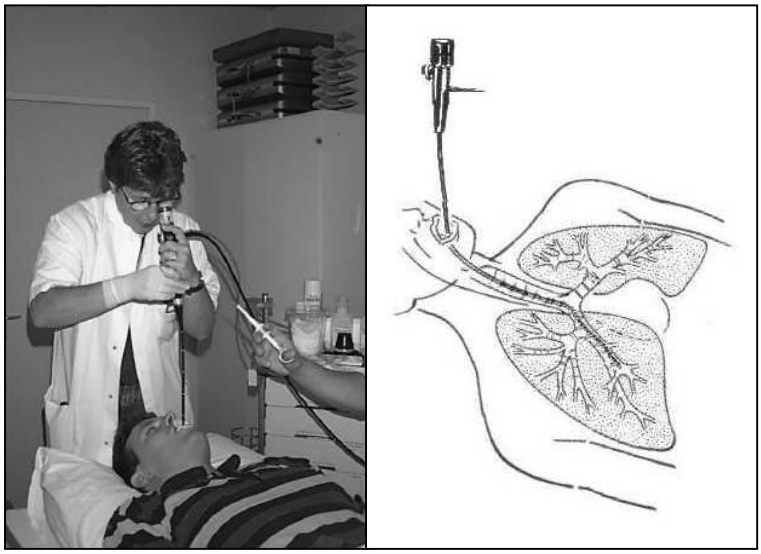

**Figure 6.** Segmental bronchial provocation according to Till.
The tip of the bronchoscope is wedged in the right middle lobe

According to a method by Till[70], the tip of the bronchoscope was wedged in the challenged segment and maintained in the occluded position, while the allergen solution was inserted (Figure 6). This approach makes intraluminal spread of antigen less likely. Although coughing and minor chest discomfort were reported, subjects generally tolerated the procedure well. The chances that minor coughing would result in an aerosol of grass pollen reaching the nose from the periphery of the lung are not very likely.

Increased numbers of eosinophils (EG2) were demonstrated in the lower airways of atopic, non-asthmatic subjects, along with decreased pulmonary function. Moreover, it has been shown that bronchial eosinophilia and clinical expression were dependent on the local dose of allergen[41].

### *2.5.3 Nasal allergen provocation*

Since the nose is more easy to access, the allergic response in the nose has been extensively studied after nasal allergen provocation (NP)[36,64,65,67,68,71]. Different methods of allergen provocation have been used, such as impregnated paper disks[51,72] and atomizers[33,47,63,65]. Paper disks have the advantage of reducing the risk of intraluminal spread. However, allergen spray more closely resembles the natural situation. Either method will result in some transportation of allergen to the oropharynx through the mucus blanket. Radio-labeled allergen provocation studies, however, did not reveal pulmonary aspiration after nasal application of the allergen[47,48].

A dose of 500 BU per nostril resulted in a good clinical response in seasonal rhinitis patients[64]. In order to minimize the risk of lower airway contamination, the allergen was delivered after deep inspiration during breath holding[47]. Nasal allergen provocation studies show that the inflammatory pattern in the nasal mucosa resembles that of the bronchial mucosa, although BHR seems to be more severe and persistant in the lower airways[16]. Only a few studies have investigated both nose and lungs simultaneously in an otherwise cross-sectional study design[15,73]. The possibility of cross-talk between nasal and bronchial mucosa after allergen provocation has not been examined.

## 2.6. Conclusion

The nose and lungs are anatomically and physiologically closely related organs. The nose plays an important role in the filtering and air-conditioning of the inhaled air. In allergic rhinitis these functions are impaired, which has consequences for the lower airways, as well.

Several mechanisms have been proposed to play a role in the interaction between upper and lower airways: i.e. mouth breathing, post-nasal drip, nasobronchial reflex or absorption of inflammatory mediators into the systemic circulation.

Allergen provocation studies have improved the insight into the local pathways of allergic inflammation. Segmental bronchial provocation and nasal allergen

provocation are procedures that are well tolerated by non-asthmatic, as well as asthmatic subjects. It is now well established that allergic asthma and allergic rhinitis are characterized by a similar inflammatory process. No attempt has thus far been made to investigate the effect of nasal allergen provocation on the lungs, or vice versa, to investigate the effect of segmental bronchial provocation on the nose.

## 2.7 References


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# 3

# Aim of the study

## 3 Aim of the study

Although it is clear from the literature that the condition of the upper airways influences the lower airways, the mechanisms underlying this relationship are far from being understood. In this thesis, the aspects of cross-talk between nasal and bronchial mucosa are investigated.

The following questions are addressed:

1. Is local allergen exposition essential for the induction of airway mucosal inflammation?
2. Does the systemic circulation contribute to the interaction between nose and lung?
3. Is mucosal inflammation associated with symptomatology and changes in airway calibre?

The first two questions are investigated in a two-way allergen provocation model in patients with allergic rhinitis without pre-existent asthma.

First, segmental bronchoprovocation (SBP) is performed and the allergic response is monitored in peripheral blood, bronchial and nasal mucosa. Chapter 4 describes the effect of SBP on cytokines and eosinophils. In the following chapter (Chapter 5), the dynamics of mast cells and basophils after SBP will be discussed.

Secondly, a nasal allergen provocation (NP) study is done and the effect on the lower airways is studied. In this second provocation study, we measure airway patency and symptomatology at 2 hour intervals after allergen challenge. The endothelial expression of adhesion molecules is assessed in nasal and bronchial mucosa and related to the influx of eosinophils (Chapter 6).

Finally, to address the last question, a cross-sectional study is performed to analyze both upper and lower airway mucosal inflammation in atopic patients with persistent perennial nasal and/or bronchial symptomatology. The relationship between allergic inflammation and clinical manifestation will be discussed in chapter 7. In chapter 8, tissue remodeling aspects are analyzed in nasal and bronchial mucosa.

# 4

# Segmental allergen provocation induces nasal inflammation in allergic rhinitis patients

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## 4.1 Summary

Allergic rhinitis and asthma often coexist and share a genetic background. Pathophysiologic connections between nose and lungs are still not entirely understood. This study was undertaken to compare allergic inflammation and clinical findings in the upper and lower airways after segmental bronchial provocation (SBP) in non-asthmatic allergic rhinitis patients. Eight nonasthmatic grass-pollen sensitive patients with allergic rhinitis and eight healthy controls were included. Bronchial biopsies and blood samples were taken before ($T_0$) and 24 hours ($T_{24}$) after SBP. Nasal biopsies were obtained at $T_0$, 1 hour after SBP ($T_1$), and $T_{24}$. Immunohistochemical staining was performed for eosinophils (BMK13), interleukin (IL)-5 and eotaxin. The number of eosinophils increased in the challenged and unchallenged bronchial mucosa ($p < 0.05$) and in the blood ($p = 0.03$) of atopics at $T_{24}$. We detected an increase of BMK13-positive and eotaxin-positive cells in the nasal lamina propria and enhanced expression of IL-5 in the nasal epithelium of atopics only at $T_{24}$ ($p < 0.05$). SBP induced nasal and bronchial symptoms, as well as reductions in pulmonary and nasal function in the allergic group. No significant changes could be observed in healthy controls. This study has shown that SBP in nonasthmatic allergic rhinitis patients results in peripheral blood eosinophilia and is able to induce allergic inflammation in the nose.

## 4.2 Introduction

Epidemiologic[1], pathophysiologic[2,3] and clinical studies[4,5] strongly suggest a link between rhinitis and asthma. Asthma and rhinitis, which are considered to be manifestations of the atopic syndrome, often coexist and share a common genetic background. Although several studies have demonstrated that asthma and rhinitis are characterized by a similar inflammatory process[6-10], pathophysiologic interactions between upper and lower airways are not entirely understood. It is clear that the condition of the upper airways definitely influences the lower airways. In allergic rhinitis patients without bronchial hyperreactivity (BHR), signs of allergic inflammation of the lower airways have been found in induced sputum, broncho-alveolar lavage (BAL) fluid and bronchial biopsies[11-14]. The nasal-bronchial reflex, an altered breathing pattern, pulmonary aspiration of nasal contents and increased

levels of inflammatory factors in the blood are possible mechanisms for lower airway dysfunction among patients with rhinitis[15]. To shed more light on the role of systemic induction in the allergic inflammatory response we designed a study in which blood samples and nasal and bronchial mucosal biopsies were taken from a group of nonasthmatic allergic rhinitis patients with an isolated grass pollen allergy after segmental bronchial provocation (SBP) at a time other than the grass pollen season. The aim of the study was to compare allergic mucosal inflammation and clinical findings in the upper and lower airways. Eosinophils, major effector cells in allergic inflammation, interleukin (IL)-5-positive and eotaxin-positive cells (necessary for eosinophil survival and chemotaxis) were chosen as markers of mucosal allergic inflammation.

## 4.3. Materials and methods

### *4.3.1 Subject groups*

Eight allergic rhinitis patients (two men and six women, age range 21 to 31 yr) and eight nonallergic healthy controls (four men and four women, age range 18 to 29 yr) were selected for the study. Subject characteristics are shown in Table 1. The rhinitis patients had a history of isolated grass pollen allergy for at least 2 yr, confirmed by a positive skin-prick test reaction to grass pollen extract alone (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands) and not to a panel of 13 other common allergens. The control subjects had no symptoms or signs of rhinitis and had negative skin-prick tests. None of the allergic rhinitis patients or controls had a clinical history of asthma. All had a normal forced expiratory volume in 1 s ($FEV_1$) and provocative concentration of methacholine causing a 20 % decrease in $FEV_1$ ($PC_{20}$ methacholine) > 8 mg/ml. Methacholine was administered according to a standardized tidal breathing method[16]. The response to methacholine was measured as change in $FEV_1$, expressed as a percent of the initial value. None of the subjects smoked or used any medication known to influence the results of this study. Biopsy specimens were obtained between February and April 1998, before the grass pollen season. None of the patients or control subjects had an infection of the respiratory tract or any nasal complaints during the 4 wk preceding the allergen challenges. All participants gave informed consent to

the study, which was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam, the Netherlands.

**TABLE 1 SUBJECT CHARACTERISTICS AT BASELINE**

| Patient | Age (yr) | Sex | $FEV_1$ (L) | $FEV_1$ (%) | IVC (L) | $FEV_1$/VC | BAR* (%) | $PC_{20}$ (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| **Allergic rhinitis** | | | | | | | | |
| 1 | 31 | F | 4.00 | 108 | 4.99 | 80 | 102 | 40 |
| 2 | 21 | M | 4.02 | 85 | 5.15 | 79 | 107 | 11.8 |
| 3 | 25 | F | 4.74 | 126 | 5.62 | 84 | 102 | 23.6 |
| 4 | 25 | M | 4.35 | 94 | 5.44 | 80 | 104 | 40 |
| 5 | 27 | F | 3.71 | 114 | 4.06 | 91 | 100 | 40 |
| 6 | 23 | F | 3.91 | 113 | 4.54 | 86 | 104 | 40 |
| 7 | 23 | F | 4,00 | 122 | 4.65 | 86 | 105 | 40 |
| 8 | 21 | F | 4.06 | 111 | 4.7 | 86 | 100 | 40 |
| **Controls** | | | | | | | | |
| 9 | 23 | F | 2.96 | 82 | 3.43 | 86 | 110 | 40 |
| 10 | 29 | M | 5.59 | 115 | 7.42 | 76 | 108 | 32.4 |
| 11 | 24 | M | 4.14 | 94 | 4.72 | 88 | 101 | 40 |
| 12 | 28 | F | 2.52 | 80 | 2.67 | 95 | 102 | 40 |
| 13 | 25 | F | 4.21 | 112 | 4.72 | 89 | 101 | 40 |
| 14 | 21 | M | 5.99 | 100 | 6.98 | 86 | 102 | 40 |
| 15 | 20 | M | 4.81 | 114 | 5.44 | 88 | 102 | 9 |
| 16 | 18 | F | 3.83 | 111 | 5.12 | 76 | 107 | 40 |

*Definition of abbreviations:* BAR = β-agonist response; IVC = inspiratory vital capacity; $PC_{20}$ = concentration of methacholine causing a 20 % decrease in $FEV_1$. BAR (terbutaline, 1.000 μg) data are presented as percentage improvement compared to initial value; a virtual value of 40 was assigned to subjects who did not reach a PC20 with 38 mg/ml methacholine.

### *4.3.2 Experimental design*

The study design is outlined in Table 2. Baseline nasal and bronchial biopsies were collected from patients and controls before SBP ($T_0$). Nasal biopsies were obtained 1 hour ($T_1$) and 24 hours ($T_{24}$) after SBP. Bronchoscopy and biopsy were repeated 24 h after SBP ($T_{24}$). Signs and symptoms of the patients and controls were recorded at the beginning of each visit (at $T_0$ and $T_{24}$) on a 10-cm visual analogue scale (VAS). Symptoms were divided into nasal complaints (rhinorrhea, watery eyes, nasal itching, sneezing, and nasal blockage) and pulmonary complaints (wheezing, coughing, shortness of breath and decreased exercise tolerance). Upper and lower airways obstruction was measured through peak nasal inspiratory flow (PNIF) and $FEV_1$. $FEV_1$

was determined at $T_0$, $T_1$ and $T_{24}$. PNIF was measured by a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL, USA) at $T_0$ and $T_{24}$. Blood samples were taken at $T_0$ and $T_{24}$. Blood eosinophils were counted by hemocytometry.

### *4.3.3 Bronchial Biopsies and Segmental Allergen Bronchoprovocation*

All bronchial biopsies were taken by the same pulmonary physician (S.E.O.). After intramuscular premedication of subjects with atropine (0.5 mg), oropharyngeal anesthesia was accomplished with topical 1% xylocaine spray. The vocal cords, trachea and bronchial tree were then anesthetized with oxybuprocaine. A fiberoptic bronchoscope was introduced into the airway via the oral route, and mucosal biopsies were taken from the carina of the left upper and lower lobe. Subsequently, segmental bronchial provocation was accomplished with a method described previously[17]. The tip of the bronchoscope (BF, type P20 D; Olympus Tokyo, Japan) was wedged in the anterior segment of the right upper lobe (RUL) and 10 ml. of 0.9% sterile saline was

**TABLE 2 STUDY DESIGN**

| | timepoint | VAS-score | $FEV_1$ | PNIF | Bronchial biopsy | Nasal biopsy | Blood samples |
|---|---|---|---|---|---|---|---|
| $T_0$ | Baseline | x | x | x | x | x | x |
| $T_1$ | 1 hr after SBP | | x | | | x | |
| $T_{24}$ | 24 hrs after SBP | x | x | x | x | x | x |

*List of abbreviations*: SBP = Segmental Bronchial Provocation; VAS = Visual Analogue Scale for scoring nasal and pulmonary symptoms; PNIF = Peak Nasal Inspiratory Flow

instilled as a control challenge. After the control challenge, the bronchoscope was wedged in a segmental bronchus of the right middle lobe (RML) and allergen challenge was performed by instilling 100 BU of grass pollen extract (Vivodiagnost, ALK Benelux BV, Groningen, The Netherlands) made up in 5 ml. of sterile saline.

The challenged site was observed for 5 min. In the absence of local bronchoconstriction, a further 400 BU of allergen in 5 ml of saline was administered, after which the bronchial segment was observed for 5 more minutes and the bronchoscope then quickly removed. After 24 h, each subject underwent a repeat bronchoscopy during which biopsies were taken from lobar segments of the unchallenged left side, the saline challenged RUL and the allergen-challenged RML. The biopsy specimens were embedded in Tissue-Tek II optimal cutting temperature (O.C.T.) compound (Sakura Finetek USA inc., Torrance, CA), frozen and stored at –150 $^{0}$C.

### *4.3.4 Nasal biopsies*

All biopsy specimens of nasal mucosa were taken by the same investigator (G.J.B.), according to the study design shown in Table 2. First, local anesthesia was induced by placing a cotton-wool carrier with 50 to 100 mg of cocaine and 3 drops of epinephrine (1:1,000) under the inferior turbinate, without touching the biopsy site. Second, mucosal biopsy samples were obtained from the lower edge of the inferior turbinate, about 2 cm posterior to the edge, by using a Gerritsma forceps with a cup diameter of 2.5 mm. The nasal biopsies were embedded in Tissue-Tek II O.C.T. compound, frozen and stored at –150 $^{0}$C[18].

### *4.3.5 Monoclonal Antibodies*

The monoclonal antibodies (mAbs) used in this study were BMK-13 ($IgG_1$, 0.2 μg/ml, Sanbio, Uden, the Netherlands) for total eosinophils, an antibody to human IL-5 MoAb ($IgG_1$, 50 μg/ml, clone nr. 5A5, a gift from Prof. Jan Tavernier, University of Ghent, Ghent, Belgium) and an anti-eotaxin antibody ($IgG_1$, 10 μg/ml, clone nr. 43911.11, R&D systems, Minneapolis, MN)

### *4.3.6 Immunohistochemical staining*

Staining for eosinophil with BMK13 was done with a modified alkaline phosphatase (AP) method[19]. IL-5 and eotaxin staining were done as follows: briefly, each tissue specimen was cut into serial, 6-μm-thick sections on a Reichert-Jung 2800e frigocut

cryostat (Leica, Wetzlar, Germany) and transferred onto poly-L-lysine-coated slides (Sigma Chemical Co., St. Louis, MO), dried and stored at -70$^0$C. The slides were stained within 3 months. They were heated to room temperature and subsequently dried and fixed in acetone for 10 min at room temperature. The slides were then rinsed in phosphate-buffered saline (PBS, pH 7.8) and placed in a semi-automatic stainer (Sequenza; Shandon, Sewickley, PA). Sections were incubated with 10% normal goat serum (CLB, Amsterdam, The Netherlands) for 10 min and were subsequently incubated for 60 min with the appropriate mAb (diluted in 10% normal human serum with 1% bovine serum albumin in PBS). They were then rinsed with PBS for 5 min, incubated with biotin-labeled goat-antimouse (Biogenex, San Ramon, CA), rinsed with PBS, incubated with AP-conjugated goat-antibiotin (Sigma) for 30 min, rinsed once more with PBS for 5 min, rinsed with Tris buffer (0.2 mol/L, pH 8.5) for 5 min, and incubated for 30 min with new fuchsin (Chroma, Köngen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity). The sections were then rinsed in distilled water, counterstained with Gill's hematoxylin, and mounted in glycerin gelatin. Control staining was done with an irrelevant mAb of the same subclass as the specific antibody.

### *4.3.7 Microscopic evaluation*

Biopsy specimens were coded, and two sections 120 μm apart were counted in a blinded fashion for each antibody. Bronchial sections were divided into epithelium and subepithelium (an area 100 μm deep in the lamina propria, along the length of the epithelial basement membrane) and counted as previously described[20]. Nasal biopsy sections were divided into epithelium, subepithelium, and lamina propria (total subepithelial mucosa). Positively stained epithelial and subepithelial cells were counted along the basement membrane, which had to be undamaged for a length of at least 1 mm before being accepted for evaluation. For lamina propria, a minimum area of 1 mm$^2$ was required for analysis. Cell numbers were determined as the number of positively stained cells per mm$^2$, using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of x 200.

### *4.3.8 Statistical analysis*

Statistical analysis was done with Friedman's and Wilcoxon's test for intragroup analysis and the Mann-Whitney U test for intergroup analysis. Data are presented as medians ± range. Correlations were evaluated with Spearman's rank correlation test. A value of $p < 0.05$ was considered significant.

## 4.4 Results

### *4.4.1 Clinical data*

The allergic rhinitis patients reported significantly more pulmonary symptoms after SBP than did the controls (Table 3), expressed by an increased total bronchial VAS score at $T_{24}$ ($p = 0.03$). A significant fall in $FEV_1$ of 9 % (range: -20 to +3 %), was measured at $T_1$ in allergic patients ($p = 0.03$), whereas in controls, $FEV_1$ barely changed (median: –1 %; range: –4 to +4 %). At $T_{24}$, $FEV_1$ was still 9 % (range: –11 to 0 %) lower than its baseline value in allergic patients, and only 2 % (range: –9 to 0 %) below its baseline value in healthy controls. Also, the nasal VAS score was significantly increased ($p = 0.02$) and PNIF was reduced ($p = 0.02$) at $T_{24}$, as compared with their baseline values in the allergic group. No effect on either nasal VAS score or on PNIF could be detected in control subjects.

### *4.4.2 Blood eosinophils*

No significant difference in baseline blood eosinophil count was observed in allergic patients (median: 175 x $10^6$/L; range 70 to 440 x10 $^6$/L) as compared with healthy

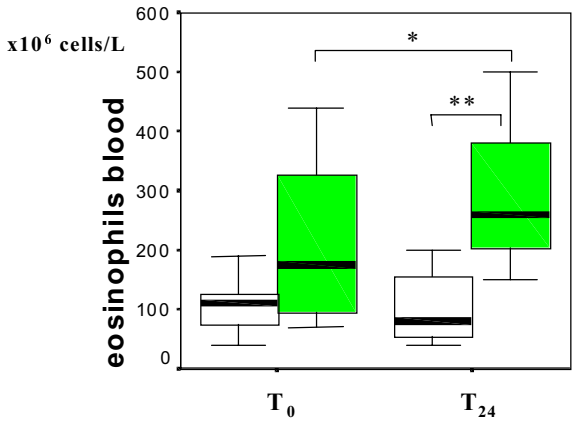


**Figure 1.** Number of peripheral blood eosinophils before ($T_0$) and 24 h ($T_{24}$) after SBP. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$, ** $p < 0.01$

controls (median: 95 x $10^6$/L; range: 40 to 190 x10 $^6$/L). Twenty-four hrs after SBP, total blood eosinophil counts were significantly elevated over baseline in allergic patients (p = 0.02) and as compared with those of controls (p = 0.001; Figure 1).

### *4.4.3 Immunostaining*

*General description.* Three nasal mucosa specimens and four bronchial biopsies were collected per patient. Of the 112 biopsy specimens, 106 met the criteria for evaluation. Neither bronchial epithelium (median length of evaluable basement membrane: 3.6 mm; range: 0 to 7.8 mm) nor subepithelium (median: 3.4 mm; range: 0 to 7.8 mm) could not be evaluated in five samples. Nasal epithelium (median: 5.2 mm; range: 1.1 to 12.5 mm), subepithelium (median: 5.4 mm; range: 1.1 to 12.5 mm) and

**TABLE 3 CLINICAL DATA**

| | FEV1 (L) | | PNIF (L) | | Lung symptoms | | Nose symptoms | |
|---|---|---|---|---|---|---|---|---|
| **Patient** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** |
| **Allergic rhinitis** | | | | | | | | |
| 1 | 4.00 | 3.82 | 235 | 190 | 6 | 37 | 7 | 56 |
| 2 | 4.02 | 4.05 | 265 | 250 | 26 | 68 | 52 | 128 |
| 3 | 4.74 | 4.34 | 200 | 180 | 0 | 123 | 0 | 35 |
| 4 | 4.35 | 4.21 | 270 | 260 | 36 | 16 | 35 | 35 |
| 5 | 3.71 | 3.35 | 230 | 220 | 24 | 31 | 46 | 54 |
| 6 | 3.91 | 3.94 | 220 | 215 | 13 | 34 | 0 | 47 |
| 7 | 4,00 | 3.77 | 250 | 230 | 10 | 27 | 36 | 49 |
| 8 | 4.06 | 3.64 | 220 | 220 | 8 | 50 | 12 | 28 |
| **Controls** | | | | | | | | |
| 9 | 2.96 | 2.97 | 120 | 115 | na | na | na | na |
| 10 | 5.59 | 5.13 | 290 | 185 | 28 | 15 | 36 | 53 |
| 11 | 4.14 | 4.1 | 160 | 240 | 4 | 9 | 7 | 6 |
| 12 | 2.52 | 2.46 | 230 | 240 | 31 | 68 | 49 | 203 |
| 13 | 4.21 | 4.08 | 235 | 205 | 7 | 6 | 19 | 63 |
| 14 | 5.99 | 4.69 | 300 | 265 | 8 | 30 | 11 | 43 |
| 15 | 4.81 | 5.68 | 180 | 195 | 21 | 24 | 56 | 53 |
| 16 | 3.83 | 3.62 | 230 | 210 | 3 | 10 | 10 | 17 |

*Definition of abbreviations:* na = not available; PNIF = peak nasal inspiratory flow; VAS = visual analogue scale. Symptoms were individually expressed in mm on a 10-cm visual analogue scale, and a composite score was obtained for nasal complaints (rhinorrhea, watery eyes, nasal itching, sneezing, and nasal obstruction) and pulmonary complaints (wheezing, coughing, shortness of breath, and decreased exercise tolerance). * = $p < 0.05$.

lamina propria (median: 4.63 $mm^2$; range: 0.88 to 8.31 $mm^2$) could not be evaluated in one case. These samples were excluded from the study. However, for all time points, a minimal number of seven subjects per subgroup could be included.

*Bronchial specimens.* In bronchial epithelium baseline $BMK13^+$ cell numbers were equal in allergic subjects and controls (Figure 2a). In the subepithelium, $BMK13^+$ cell numbers were higher in atopic than in control subjects ($p < 0.01$), but absolute cell numbers were very low at baseline (Figure 2b). At $T_{24}$, we found increased numbers

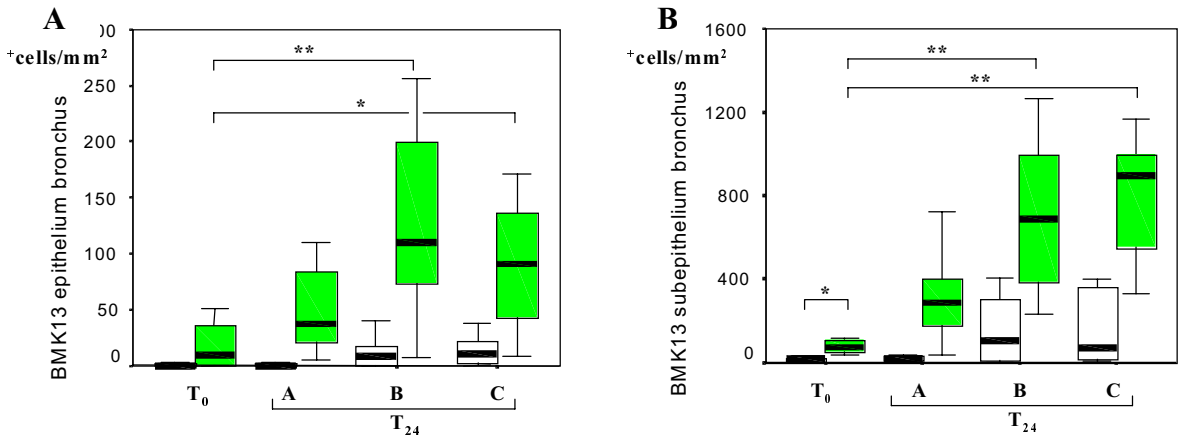


**Figure 2.** Number of $BMK13^+$ cells in bronchial epithelium (*A*) and subepithelial layer (*B*) before ($T_0$) and 24 hours ($T_{24}$) after SBP. A = unchallenged left lung; B = allergen-challenged right middle lobe; C = saline-challenged right upper lobe. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$, ** $p < 0.01$.

of $BMK13^+$ cells in the bronchial epithelium and subepithelium of allergic rhinitis patients, in the saline-challenged segment (epithelium $p = 0.02$; subepithelium $p = 0.03$) as well as in the allergen-challenged bronchial segment (epithelium $p = 0.01$; subepithelium $p = 0.02$). In the unchallenged segment, $BMK13^+$ cell numbers were not significantly affected by SBP ($p = 0.07$ for both epithelium and subepithelium). At $T_{24}$, we found a significant difference between allergic patients and controls in the number of eosinophils in the allergen-challenged segment (epithelium $p = 0.003$; subepithelium $p = 0.002$), saline-challenged segment (epithelium $p = 0.01$; subepithelium $p = 0.03$), and even in the unchallenged segment (epithelium $p = 0.001$; subepithelium $p = 0.001$). In none of the tissue areas of the control groups, were $BMK13^+$ cell numbers significantly altered at $T_{24}$.

After SBP, IL-5$^+$ cell numbers were markedly increased only in the allergen-challenged bronchial subepithelium in allergic rhinitis subjects (p = 0.07). No significant differences were found in the other investigated segments.

No significant changes were found in the number of eotaxin$^+$ cells in bronchial mucosa from before to after SBP.

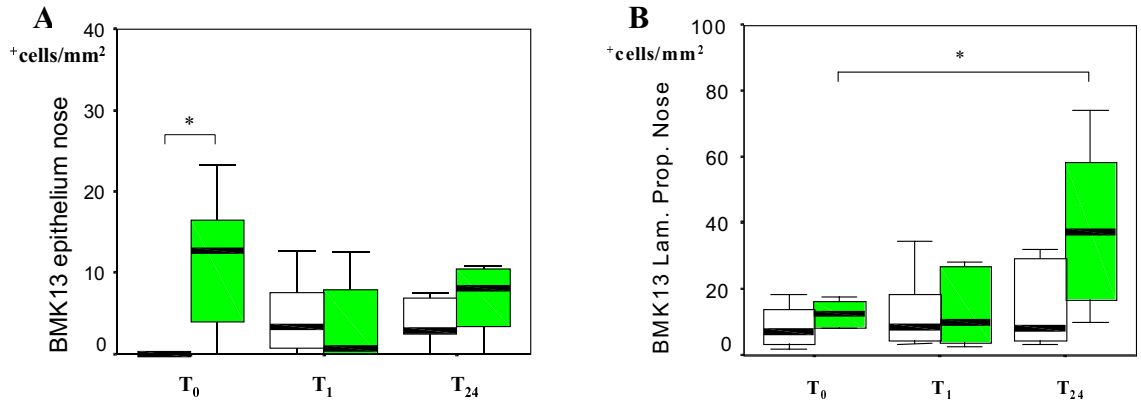


**Figure 3.** Number of BMK13$^+$ cells in nasal epithelium (*A*) and subepithelial layer (*B*) before ($T_0$), 1 hour ($T_1$) and 24 hours ($T_{24}$) after SBP. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$.

*Nasal specimens.* Although at $T_0$, BMK13$^+$ cell numbers in the nasal epithelium (Figure 3a) and subepithelium were slightly higher in the allergic group than in the controls, no differences were found at $T_1$ and $T_{24}$ as compared with baseline or

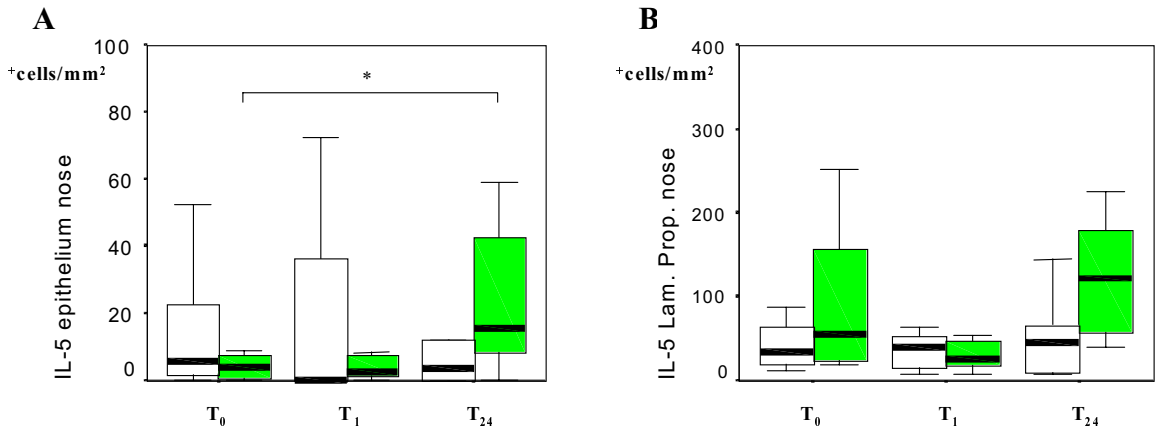


**Figure 4.** Number of IL-5$^+$ cells in nasal epithelium (*A*) and subepithelial layer (*B*) before ($T_0$), 1 hour ($T_1$) and 24 hours ($T_{24}$) after SBP. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$.

between the two groups. BMK13$^+$ cell numbers were not significantly different in the lamina propria of the allergic and control groups at $T_0$ (Figure 3b). At $T_{24}$ , however, the number of BMK13$^+$ cells in the nasal lamina propria was significantly greater in allergic patients than at baseline ($p = 0.04$).

A significant increase in the number of IL-5$^+$ cells was found in the nasal epithelium at $T_{24}$ ($p = 0.02$, Figure 4a). The numbers of IL-5$^+$ cells in the nasal subepithelium and lamina propria were increased (Figure 4b), but did not reach significance.

In the nasal epithelium, the eotaxin$^+$ cell number was significantly increased at $T_{24}$ as compared with that at $T_1$ ($p = 0.05$) in allergic patients, but not in controls. As compared with baseline, eotaxin$^+$ cells were significantly increased in the subepithelium ($p = 0.01$) and lamina propria ($p = 0.03$) of the nasal mucosa at $T_{24}$ in allergic patients, but not in controls. However, differences between allergic patients and controls did not reach statistical significance.

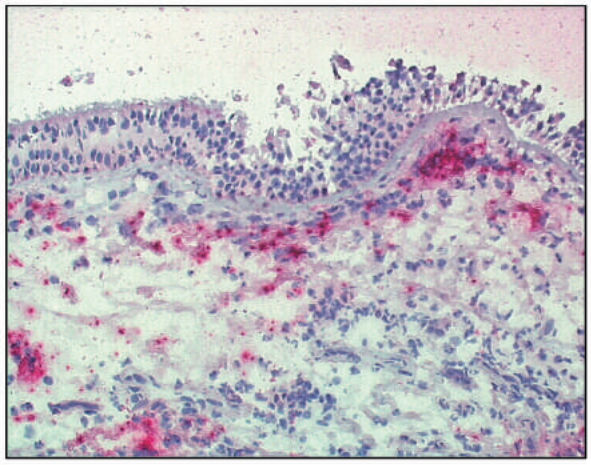

**Figure 5.** BMK13 immunoreactivity of a section of bronchial biopsy taken from an allergen-challenged segment of an allergic rhinitis patient 24 hours after SBP. Counterstained with hematoxylin. Original magnification: x 400.

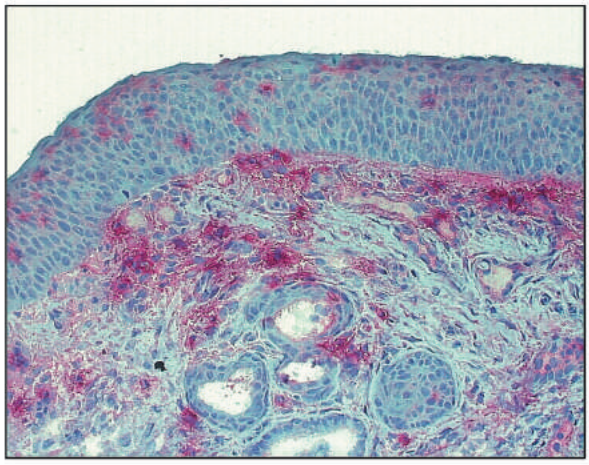

**Figure 6.** IL-5 immunohistochemical staining of a section of nasal biopsy taken from an allergic rhinitis patient 24 hours after SBP. Counterstained with hematoxylin. Original magnification: x 200.

*Comparison between nasal and bronchial inflammatory cell numbers.* At baseline, the numbers of eosinophils, IL-$5^+$ cells and eotaxin$^+$ cells in rhinitis patients and controls were low and not statistically different between bronchial and nasal biopsy specimens. The reason that only epithelial and subepithelial layers were compared is because of the difference in size between nasal and bronchial biopsy specimens. In atopic patients, the eosinophil number at $T_{24}$ was higher in the bronchial mucosa (RML, Figure 5) than in nasal mucosa for both epithelium ($p = 0.02$) and subepithelium ($p = 0.01$). In controls, no differences were found. In allergic patients, the number of IL-$5^+$ cells at $T_{24}$, however, was significantly greater in the nasal subepithelial layer (Figure 6) than in the bronchial subepithelium ($p = 0.01$), but this was not found in controls. In both atopic subjects and controls, the number of eotaxin$^+$ cells at $T_{24}$ was greater in the epithelium and subepithelium of the nasal mucosa than in the bronchial mucosa, but the difference did not reach statistical significance.

*Correlations between inflammatory markers, airway function and symptomatology.*

In the allergic subgroup, the number of bronchial eosinophils (RML) correlated with the number of blood eosinophils (epithelium $R_s = 0.56$, $p = 0.03$; subepithelium $R_s = 0.49$, $p = 0.05$). No correlation could be found between nasal inflammatory cell numbers, PNIF and nasal symptom score.

A correlation was found between the increase in bronchial eosinophils (subepithelium, RML) and the total nose symptom score in atopics ($R_s = 0.57$, $p = 0.02$), which was not found in the controls. In the allergic subgroup, total nose symptom score and total lung symptom score were highly correlated ($R_s = 0.57$, $p = 0.02$). No correlation was found between symptom scores and airway function ($FEV_1$ and PNIF).

## 4.5 Discussion

In this study, we were able to demonstrate allergic inflammation, characterized by eosinophil infiltration, in the bronchi of subjects with nonasthmatic allergic rhinitis at 24 h after SBP. This occurred not only in the allergen-challenged, but to a lesser extent also in the saline-challenged and control segments. These data suggest a more generalized effect on the lower airways after local allergen deposition, which is also reflected by sustained decrease in $FEV_1$ and increase of pulmonary symptoms after

SBP in allergic subjects. The increased nasal obstruction and symptom score at 24 h after SBP in allergic rhinitis patients indicate that the upper respiratory tract is also involved in the allergic response. Peripheral blood eosinophilia and increased eosinophil numbers deep in the nasal mucosa after SBP further support the idea that a more extended systemic inflammatory reaction takes place in these nonasthmatic patients. Although other authors have also reported activation and mobilization of blood eosinophils in nonasthmatic allergic subjects after SBP[13,21], we have found no published reports of signs of allergic inflammation in a remote and "upstream" organ after local allergen challenge. Generalized pulmonary inflammation cannot be explained by bronchoscopy and biopsy, per se[22]. To minimize intraluminal spread of antigen from the allergen-challenged segment to other segments, we held the bronchoscope in the wedged position for 5 min, as has been suggested by other authors[12]. It is also very unlikely that allergen spilled into the nose after SBP, since we performed bronchoscopy by the oral route, and it did not lead to excessive coughing. Other mechanisms that are more likely to explain the interaction between the nose and the lung are neural reflex mechanisms and systemic induction of inflammatory mediators and cells. Our results are most suggestive of general systemic activation of eosinophils, which leads to migration of these cells into both the upper and lower respiratory mucosa. The detection of increased expression of the pro-eosinophilic cytokines IL-5 and granulocyte-macrophage colony-stimulating factor in the serum of allergic rhinitis patients after nasal allergen provocation supports our hypothesis[23]. Nasal provocation with methacholine in asthmatic patients with rhinitis resulted in an increase in lower airway resistance that could be blocked by premedication of the nasal mucosa with phenylephrine, also suggesting a role for systemic absorption in the induction of lower airway resistance[24]. The contribution of a bronchonasal reflex mechanism to nasal allergic inflammation after SBP is currently under investigation.

Several studies have demonstrated the role of IL-5 and eotaxin in the influx of eosinophils in the mucosa[25-27]. Previous studies involving allergic rhinitis and asthma patients have shown a significant increase in IL-5 concentrations in the BALF

after SBP[12,28,29]. We could also demonstrate an increased expression of both IL-5 and eotaxin in the bronchial mucosa of allergic patients 24 h after SBP. The increase did not reach statistical significance, but this could have been due to the relatively late time point of sampling and the brief, transient presence of these cytokines in the bronchial tissue. A significant increase of IL-$5^+$ and eotaxin$^+$ cells, coinciding with the influx of eosinophils in the nasal lamina propria, was detected in the nasal mucosa at 24 h after SBP, illustrating a delay in activation of the inflammatory pathway in the nasal mucosa as compared with the bronchial mucosa.

Several investigators have shown that eosinophilic inflammation of the airways is correlated with the severity of asthma[7,8]. In our study, we also found a strong correlation of clinical data ($FEV_1$ and VAS score) with inflammatory parameters (eosinophils and IL-5 expression) in allergic lower airways, but were unable to demonstrate any correlation between clinical parameters and inflammatory cell numbers in the nose. Some authors[30,31] reported a correlation between the number of eosinophils or mediators released by eosinophils and symptoms after nasal provocation. Usually, no correlation was found[19,32,33].

It is not clear why allergic rhinitis patients without BHR do not have asthma in the natural situation. Our results have led us to hypothesize that allergic rhinitis patients do have asthma, but that the dose of allergen required to initiate an allergic response in the bronchi is probably higher in allergic rhinitics than in clinical asthma patients. Although the type and degree of inhalant allergy are known to play an important roles in the mechanism underlying nonspecific BHR[34], other factors, such as duration and severity of exposure, could also affect the clinical manifestation of the allergic response.

In conclusion, we have found an allergic inflammatory response, similar to asthma, in the lower airways of rhinitis patients without BHR after SBP. Interestingly, the allergic response was not restricted to the allergen-challenged segment, but was more widespread: eosinophilia also occurred in other parts of the lung, and was detectable in peripheral blood. Moreover, we found increased inflammatory cell numbers in the nasal mucosa after SBP, together with signs and symptoms of allergic rhinitis,

indicating that the nose is also involved in this systemic effect. We also observed a clear difference in the time course and the degree of clinical and immunopathological findings in the nose and bronchi of allergic subjects after local allergen challenge.

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# 5

# Segmental bronchoprovocation in allergic rhinitis patients affects mast cell and basophil numbers in nasal and bronchial mucosa

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## 5.1 Summary

Mast cells and basophils are cells that play an important role in the initiation and control of allergic inflammation in asthma and rhinitis. This study was undertaken to determine the presence and dynamics of mast cells and basophils in the nasal and bronchial mucosa of allergic rhinitis patients after segmental bronchial provocation (SBP). Eight nonasthmatic, grass pollen allergic rhinitis patients and eight healthy controls were included. Bronchial and nasal biopsies, as well as blood samples were taken before ($T_0$) and 24 hours ($T_{24}$) after SBP. Immunohistochemical staining was performed for mast cells (tryptase and chymase; phenotypes $MC_T$, $MC_{TC}$, $MC_C$) and basophils (BB1). In the bronchial mucosa, the number of $BB1^+$ cells significantly increased ($p < 0.05$) in allergic rhinitis patients after SBP. In the nasal mucosa, the numbers of $MC_C$ and $MC_{TC}$ significantly decreased, while $BB1^+$ numbers significantly increased in allergic rhinitis patients after SBP ($p < 0.05$). In blood, the number of basophils decreased ($p < 0.05$) and the level of interleukin (IL)-5 increased ($p < 0.05$) in atopic patients after SBP. No significant changes could be observed in healthy controls. This study shows that segmental bronchial provocation in nonasthmatic allergic rhinitis patients results in reduced numbers of mast cells in the nose as a result of enhanced de-granulation. At the same time, there is evidence for an influx of basophils from the blood into the nasal and bronchial mucosa.

## 5.2 Introduction

Asthma and rhinitis, which are considered to be manifestations of the atopic syndrome, often co-exist[1,2] and share a common genetic background[3]. Although several studies have demonstrated that asthma and rhinitis are characterized by a similar inflammatory process[4-7], patho-physiologic interactions between upper and lower airways are not entirely understood. It is clear that the condition of the upper airways influences the lower airways. In allergic rhinitis patients without bronchial hyper-reactivity (BHR), signs of allergic inflammation of the lower airways have been found in induced sputum, broncho-alveolar lavage fluid (BALF) and bronchial biopsies[8-10].

Mast cells and basophils are metachromatically staining cells, which are believed to play an important role in the initiation and control of upper and lower respiratory

allergy[11]. Both cell types express high affinity receptors for IgE (FcεRI). Binding of IgE and subsequent cross-linking of FcεRI lead to de-granulation of mast cells and release of histamine and proteases among others, characteristic of the early phase of the allergic immune response (EAR). Mast cells are also source of IL-4, IL-5, IL-6 and TNF-α[12]. Through the release of mediators, mast cells can clearly orchestrate the infiltration of leukocytes into sites of mast-cell activation[13]. Although basophils comprise a minor component of the inflammatory cell influx, they are capable of producing more IL-4 and IL-13 per cell than any other cell type[14,15]. Therefore, they are considered to be important modulators of the allergic immune response. Moreover, their total number and relative contribution increase during the late phase of the allergic response (LAR) and correlate with the severity of the disease[16]. The pattern of mediator release, particularly the lack of a secondary increase in PGD2 and the corticosteroid sensitivity of the LAR[17], indicates that the basophil and not the mast cell is the main source of histamine in the LAR. An increase in the number of basophil-like cells in the nasal mucosa has been reported after nasal allergen provocation[18,19]. In a previous article, we demonstrated that eosinophils, IL-5$^+$ and eotaxin$^+$ cells are increased in nasal mucosa of AR patients 24 hours after segmental bronchial provocation (SBP). This indicates that a simultaneous LAR may take place in other organs than those exposed to the allergen[20]. Here, we report on the contribution of metachromatic cells to the inflammatory process in both the challenged lung and in the nose, with special emphasis on early allergic events. Bronchial and nasal mucosal biopsy specimens and peripheral blood samples were taken from a group of non-asthmatic rhinitis patients with an isolated grass pollen allergy after SBP at a time other than the grass pollen season. The presence of mast cells was established after immunohistochemical double staining for tryptase and chymase (phenotypes $MC_T$, $MC_{TC}$, $MC_C$). The presence of basophils was assessed with a novel monoclonal antibody BB1, which recognizes a unique granule constituent of basophils[21].

## 5.3 Material and methods

### *5.3.1 Subject groups*

Eight non-asthmatic, allergic rhinitis patients (two men and six women, age range 21 to 31 years) and 8 non-allergic healthy controls (four men and four women, age range 18 to 29 years) were included into the study. Subject characteristics are shown in Table 1. The rhinitis patients had a history of isolated grass pollen allergy for at least

**TABLE 1 SUBJECT CHARACTERISTICS AT BASELINE**

| Patient | Age (yr) | Sex | $FEV_1$ (L) | $FEV_1$ (%) | IVC (L) | $FEV_1$/VC | BAR* (%) | $PC_{20}$ (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| **Allergic rhinitis** | | | | | | | | |
| 1 | 31 | F | 4.00 | 108 | 4.99 | 80 | 102 | 40 |
| 2 | 21 | M | 4.02 | 85 | 5.15 | 79 | 107 | 11.8 |
| 3 | 25 | F | 4.74 | 126 | 5.62 | 84 | 102 | 23.6 |
| 4 | 25 | M | 4.35 | 94 | 5.44 | 80 | 104 | 40 |
| 5 | 27 | F | 3.71 | 114 | 4.06 | 91 | 100 | 40 |
| 6 | 23 | F | 3.91 | 113 | 4.54 | 86 | 104 | 40 |
| 7 | 23 | F | 4,00 | 122 | 4.65 | 86 | 105 | 40 |
| 8 | 21 | F | 4.06 | 111 | 4.7 | 86 | 100 | 40 |
| **Controls** | | | | | | | | |
| 9 | 23 | F | 2.96 | 82 | 3.43 | 86 | 110 | 40 |
| 10 | 29 | M | 5.59 | 115 | 7.42 | 76 | 108 | 32.4 |
| 11 | 24 | M | 4.14 | 94 | 4.72 | 88 | 101 | 40 |
| 12 | 28 | F | 2.52 | 80 | 2.67 | 95 | 102 | 40 |
| 13 | 25 | F | 4.21 | 112 | 4.72 | 89 | 101 | 40 |
| 14 | 21 | M | 5.99 | 100 | 6.98 | 86 | 102 | 40 |
| 15 | 20 | M | 4.81 | 114 | 5.44 | 88 | 102 | 9 |
| 16 | 18 | F | 3.83 | 111 | 5.12 | 76 | 107 | 40 |

*Definition of abbreviations:* BAR = β-agonist response; IVC = inspiratory vital capacity; $PC_{20}$ = concentration of methacholine causing a 20 % decrease in $FEV_1$. BAR (terbutaline, 1.000 µg) data are presented as percentage improvement compared to initial value; a virtual value of 40 was assigned to subjects who did not reach a PC20 with 38 mg/ml methacholine.

two years, confirmed by a positive skin-prick test reaction to grass pollen extract alone (Vivodiagnost, ALK Benelux BV, Groningen, The Netherlands) and not to a panel of 13 other common allergens. The control subjects had no symptoms or signs of rhinitis and had negative skin-prick tests. None of the allergic rhinitis patients or controls had a clinical history of asthma. All had a normal $FEV_1$ and a provocative concentration of methacholine causing a 20% decrease in $FEV_1$ ($PC_{20}$ methacholine) > 8 mg/ml.

Methacholine was administered according to a standardized tidal breathing method[22]. None of the subjects smoked or used any medication known to influence the results of the study. Biopsy specimens were obtained between February and April 1998, before the grass pollen season. None of the patients or control subjects had an infection of the respiratory tract or any nasal complaints during the four weeks preceding the allergen challenge. All participants gave informed consent to the study, which was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam, The Netherlands.

### *5.3.2 Experimental design*

The study design is outlined in Table 2. Baseline nasal and bronchial biopsies were collected from patients and controls just before SBP ($T_0$). Nasal biopsies were obtained 1 hour ($T_1$) and 24 hours ($T_{24}$) after SBP. Collection of bronchial biopsy specimens was repeated 24 hours after SBP ($T_{24}$). Signs and symptoms were scored at the beginning of each visit (at $T_0$ and $T_{24}$) on a 10 cm visual analogue scale (VAS).

**TABLE 2 STUDY DESIGN**

| | timepoint | VAS-score | $FEV_1$ | PNIF | Bronchial biopsy | Nasal biopsy | Blood samples |
|---|---|---|---|---|---|---|---|
| $T_0$ | Baseline | x | x | x | x | x | x |
| $T_1$ | 1 hr after SBP | | x | | | x | |
| $T_{24}$ | 24 hrs after SBP | x | x | x | x | x | x |

*List of abbreviations*: SBP = Segmental Bronchial Provocation; VAS = Visual Analogue Scale for scoring nasal and pulmonary symptoms; PNIF = Peak Nasal Inspiratory Flow

Symptoms were divided into nasal and ocular complaints (rhinorrhea, watery eyes, nasal itching, sneezing, and nasal blockage) and pulmonary complaints (wheezing, coughing, shortness of breath and decreased exercise tolerance). Upper and lower

airways obstruction were measured through peak nasal inspiratory flow (PNIF) and $FEV_1$. $FEV_1$ was determined at $T_0$, $T_1$ and $T_{24}$. PNIF was measured by a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL, USA) at $T_0$ and $T_{24}$. Blood samples were taken at $T_0$ and $T_{24}$. The basophil percentage of total white blood cells was determined by hemocytometric differential cell count (Sysmex NE 8000). IL-5 was measured in serum with a commercially available ELISA-kit, following the instructions of the manufacturer (Cytoscreen™, BioSource International Inc., Camarillo, CA). The minimal detectable dose of hIL-5 is < 4 pg/ml and no cross-reactivity was found with other cytokines.

### *5.3.3 Bronchial Biopsies and Segmental Allergen Bronchoprovocation*

All bronchial biopsies were taken by the same pulmonary physician (S.E.O.). After intramuscular premedication with atropine (0.5 mg), oropharyngeal anesthesia was accomplished with topical xylocaine spray 1%. The vocal cords, trachea and bronchial tree were then anesthetized with oxybuprocaine. The fiberoptic bronchoscope was introduced into the airway via the oral route, and mucosal biopsies were taken from the carina of the left upper and lower lobe. Subsequently, segmental bronchial provocation was accomplished with a method described previously[20]. The tip of the bronchoscope (Olympus BF, type P20 D, Tokyo, Japan) was wedged in the anterior segment of the right upper lobe (RUL) and 10 ml. of 0.9% sterile saline was instilled as a control challenge. Following the control challenge, the bronchoscope was wedged in a segmental bronchus of the right middle lobe (RML) and allergen challenge was achieved by instilling 100 BU of grass-pollen extract (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands) made up in 5 ml. of sterile saline. The challenged site was observed for 5 min. In the absence of local bronchoconstriction, a further 400 BU of allergen in 5 ml. of saline was administered, after which the bronchial segment was observed for 5 more minutes. The bronchoscope was then quickly removed. After 24 h, each subject underwent a repeat bronchoscopy, during which biopsies were taken from lobar segments of the unchallenged left side, the saline-challenged RUL and the allergen-challenged RML. The bronchial biopsies were embedded in Tissue-Tek II

Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA), frozen and stored at –150 $^0$C.

### *5.3.4 Nasal biopsies*

All biopsy specimens of nasal mucosa were taken by the same investigator (G.J.B.). First, local anesthesia was induced by placing a cotton-wool carrier with 50 to 100 mg of cocaine and 3 drops of epinephrine (1:1000) under the inferior turbinate, without touching the biopsy site. Second, mucosal biopsy samples were obtained from the lower edge of the inferior turbinate, about 2 cm posterior to the edge, by using a Gerritsma forceps with a cup diameter of 2.5 mm. Nasal biopsies were embedded in Tissue-Tek II OCT compound, frozen and stored at –150 $^0$C[23].

### *5.3.5 Monoclonal Antibodies*

Monoclonal antibodies (mAbs) used in this study were anti-IgE ($IgG_1$, 0.2 μg/ml, HM25M, CLB, the Netherlands), mouse-anti-human-chymase ($IgG_1$, 1 μg/ml, B7, Chemicon, Brunschwig, the Netherlands), mouse-anti-human-tryptase ($IgG_1$, 0.7 μg/ml, G3, Chemicon, Brunschwig, the Netherlands) and anti-basophilic granules ($IgG_{2a}$, <1 μg/ml, BB1, Immunopharmacology Group, Southampton General Hospital, Southampton, U.K.). The monoclonal antibody BB1 recognizes a unique granule constituent of basophils[21].

### *5.3.6 Double staining tryptase and chymase*

Staining was done using a modified method as previously described[24]. Briefly, each tissue specimen was cut into serial 6-$\mu$m-thick sections on a Reichert-Jung 2800e Frigocut cryostat (Leica, Wetzlar, Germany) and transferred onto poly-L-lysine-coated slides (Sigma Chemical Co. St Louis , MO), dried and stored at -80$^0$C. Before staining, slides were heated to room temperature (RT) and subsequently dried and fixed in acetone for 10 min at RT. Slides were then rinsed in phosphate-buffered saline (PBS, pH 7.8) and placed in a semiautomatic stainer (Sequenza; Shandon, Sewickley, PA). Endogenous peroxidase was blocked with 0.1% sodium azide and 0.01%

hydrogen peroxide in PBS for 30 min. Sections were then rinsed in PBS for 10 min, incubated with 10% normal goat serum (CLB, Amsterdam, The Netherlands) and 10% normal rabbit serum (CLB, Amsterdam, The Netherlands). Subsequently, slides were incubated with mouse-anti-human tryptase for 60 min at RT. Sections were rinsed with PBS for 5 min, incubated with peroxidase (PO)-conjugated-rabbit-anti-mouse (1:200 Sigma) immunoglobulin serum, rinsed with PBS, incubated with PO-conjugated-mouse-anti-PO (1:200 Sigma) for 30 minutes. Slides were incubated with 10% normal mouse serum (CLB, Amsterdam, The Netherlands) for 10 min and were incubated with biotinylated mouse-anti-human-chymase (B7) for 60 min. They were rinsed once more with PBS for 5 min and incubated with AP-conjugated-goat-anti-biotin (1:50 Sigma) for 30 min. Next, slides were rinsed with PBS for 5 min and with TRIS buffer (0.1 M, pH 8.5) for 5 min and incubated for 30 min in Fast Blue substrate containing levamisole to block endogenous AP enzyme activity. Finally, sections were rinsed with sodium acetate (0.1 M, pH 4.6) for 5 minutes and incubated with AEC (amino-ethyl-carbazole 0.05 % in sodium acetate 0.1M pH 4.6 and 0.01 % peroxide) substrate for 30 minutes. The slides were then rinsed with distilled water and mounted in glycerin gelatin. Control staining was done with an irrelevant mAb of the same subclass at the same protein concentration as the specific antibody.

### *5.3.7 Single staining and Tyramide Signal Amplification*

The mAb stainings were developed with the super sensitive immuno-alkaline phosphatase (ss-AP) method as previously described[24]. The basophil staining method differed from the staining method described above due to the inclusion of Tyramide Signal Amplification (TSA) (NEN Inc., Boston, MA, USA) step to enhance the BB1 signal. Briefly, tissue fixation was carried out as described above followed by blocking of endogenous avidin and biotin with Vector Blocking Kit, according to the specifications of the manufacturer (Vector Lab, Burlingame, CA). Subsequently, slides were rinsed in PBS (5 min.) and incubated with BB1 antibody for 1 hr. After PBS rinsing, endogenous peroxidase was blocked using azide (0.2 %) and peroxidase (0.02 %) and methanol (50 %) in PBS. TSA was done by subsequent incubation with streptavidin PBS/BSA solution (1:100) (NEN Inc., Boston, MA, USA) for 10 min,

rinsing with PBS and incubation with alkaline-phophatase-conjugated-goat-anti-biotin (1:50 Sigma) for 30 min. This was followed by incubation in new fuchsin (Chroma, Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for a maximum of 30 min. The sections were then rinsed in distilled water, counterstained with Gill's hematoxylin, and mounted in glycerin gelatin. Control staining was done with an irrelevant mAb of the same subclass at the same protein concentration as the specific antibody.

**TABLE 3 CLINICAL DAT**

| | FEV1 (L) | | PNIF (L) | | Lung symptoms | | Nose symptoms | |
|---|---|---|---|---|---|---|---|---|
| **Patient** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** | **$T_0$** | **$T_{24}$** |
| **Allergic rhinitis** | | | | | | | | |
| 1 | 4.00 | 3.82 | 235 | 190 | 6 | 37 | 7 | 56 |
| 2 | 4.02 | 4.05 | 265 | 250 | 26 | 68 | 52 | 128 |
| 3 | 4.74 | 4.34 | 200 | 180 | 0 | 123 | 0 | 35 |
| 4 | 4.35 | 4.21 | 270 | 260 | 36 | 16 | 35 | 35 |
| 5 | 3.71 | 3.35 | 230 | 220 | 24 | 31 | 46 | 54 |
| 6 | 3.91 | 3.94 | 220 | 215 | 13 | 34 | 0 | 47 |
| 7 | 4,00 | 3.77 | 250 | 230 | 10 | 27 | 36 | 49 |
| 8 | 4.06 | 3.64 | 220 | 220 | 8 | 50 | 12 | 28 |
| **Controls** | | | | | | | | |
| 9 | 2.96 | 2.97 | 120 | 115 | na | na | na | na |
| 10 | 5.59 | 5.13 | 290 | 185 | 28 | 15 | 36 | 53 |
| 11 | 4.14 | 4.1 | 160 | 240 | 4 | 9 | 7 | 6 |
| 12 | 2.52 | 2.46 | 230 | 240 | 31 | 68 | 49 | 203 |
| 13 | 4.21 | 4.08 | 235 | 205 | 7 | 6 | 19 | 63 |
| 14 | 5.99 | 4.69 | 300 | 265 | 8 | 30 | 11 | 43 |
| 15 | 4.81 | 5.68 | 180 | 195 | 21 | 24 | 56 | 53 |
| 16 | 3.83 | 3.62 | 230 | 210 | 3 | 10 | 10 | 17 |

*Definition of abbreviations*: na = not available; PNIF = peak nasal inspiratory flow; VAS = visual analogue scale. Symptoms were individually expressed in mm on a 10-cm visual analogue scale, and a composite score was obtained for nasal complaints (rhinorrhea, watery eyes, nasal itching, sneezing, and nasal obstruction) and pulmonary complaints (wheezing, coughing, shortness of breath, and decreased exercise tolerance). * = $p < 0.05$.

### *5.3.8 Microscopic evaluation*

Biopsies were coded and two sections 120 μm apart were counted blind for each antibody. Bronchial sections were divided into epithelium and sub-epithelium (an area 100 μm deep into the lamina propria, along the length of the epithelial basement

membrane) and were counted as previously described[20]. Nasal biopsy sections were divided into epithelium, sub-epithelium and lamina propria (total sub-epithelial mucosa). Positively stained epithelial and sub-epithelial cells were counted along the basement membrane, which had to be undamaged for a length of at least 1 mm before being accepted for evaluation. For lamina propria a minimum of 1 $mm^2$ was required for analysis. Cell numbers in the sub-epithelial mucosa were determined as the number of immuno-stained cells per $mm^2$ and in the epithelium as cells per mm reticular basement membrane (RBM), using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of x 200.

### *5.3.9 Statistical analysis*

Data were first analyzed with Friedman's test for several related samples. In case of significance, further analysis was done with Wilcoxon's test for intra-group analysis and Mann-Whitney U test for inter-group analysis. Data are presented as medians ± range. Correlations were evaluated with Spearman's rank correlation test. A value of $p < 0.05$ was considered significant.

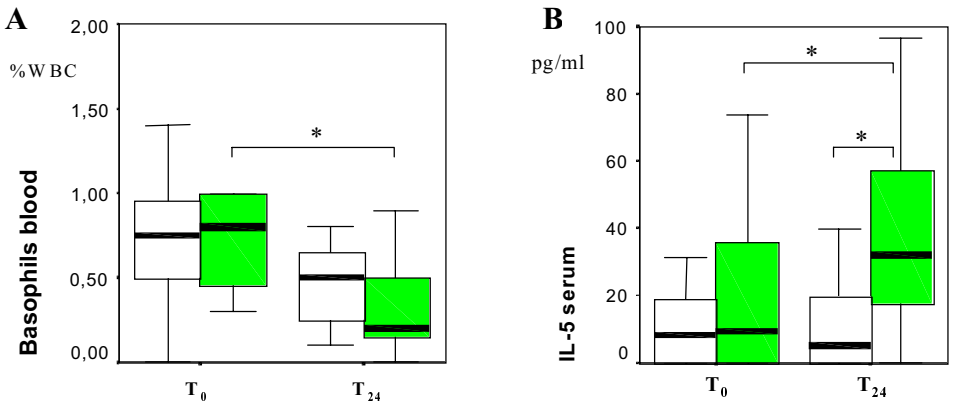


**Figure 1.** *A*. The percentage peripheral blood basophils of white blood count (WBC) and *B*. serum interleukin (IL)-5 before ($T_0$) and 24 hours ($T_{24}$) after SBP. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as median ± range. * = $p<0.05$

## 5.4 Results

### *5.4.1 Clinical data*

All control subjects and allergic rhinitis patients received a total dose of 500 BU grass pollen solution, without any macroscopic bronchoconstriction. An overview of the clinical data is given in Table 3 (chapter 4). A significant decrease in $FEV_1$ of 9 % (range: - 20 to + 3 %) was

measured at $T_1$ in the allergic rhinitis group ($p = 0.03$), whereas in controls, $FEV_1$ barely changed (median: - 1 %; range: - 4 to + 4 %). At $T_{24}$, $FEV_1$ was still 9 % (range: - 11 to 0 %) lower than its baseline value in allergic patients, and only 2 % (range - 9 to 0 %) below its baseline value in healthy controls. Although the overall increase in nasal and pulmonary symptoms was higher in the allergic subgroup at $T_{24}$, no significant differences were found in total nasal and pulmonary VAS scores between allergic rhinitis patients and controls.

### *5.4.2 Peripheral blood parameters*

No significant difference in baseline blood basophil percentage was observed in allergic patients (median 0.8, range 0.3 to 2.0 %) compared to healthy controls (median 0.75, range 0 to 1.4 %). At $T_{24}$, the percentage of blood basophils was significantly decreased in allergic patients when compared to baseline ($p = 0.02$, Figure1a). This was accompanied by a smaller reduction in the control group ($p = 0.2$). No significant difference was found between allergic rhinitis patients and controls at $T_{24}$.

At $T_0$, the concentration of IL-5 in the serum of allergic rhinitis patients (median 9.6 pg/ml, range 0 to 73.6 pg/ml) was comparable to controls (median 8.3 pg/ml, range 0 to 31.3 pg/ml). At $T_{24}$, however, the median concentration of IL-5 in allergic rhinitis patients was increased three fold compared to baseline concentration ($p = 0.02$, Figure 1b). The concentration of serum IL-5 in the control group did not change after SBP and was significantly lower than the serum IL-5 of the allergic rhinitis group ($p = 0.05$).

### *5.4.3 Immunostaining*

*General description.* Three nasal mucosa specimens and 4 bronchial biopsies were collected per patient. Out of the 112 biopsy specimens, 106 reached criteria for evaluation. In bronchial biopsy specimens, the median length of evaluable basement membrane was 3.7 mm (range 1.1 to 7.8 mm). The median surface area of sub-epithelium was 0.37 $mm^2$ (range 0.11 to 0.78 $mm^2$). Bronchial epithelium and sub-epithelium could not be evaluated in five samples. In nasal biopsy specimens, the

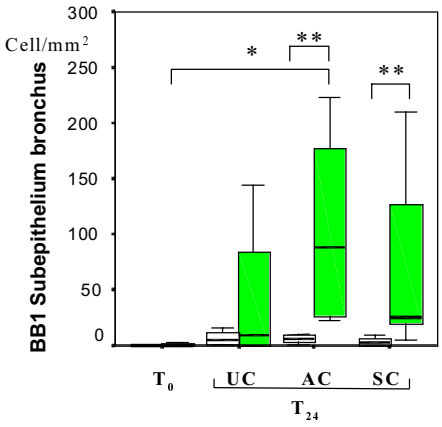


**Figure 2.** Number of $BB1^+$ cells in the bronchial subepithelial layer before ($T_0$) and 24 hours ($T_{24}$) after SBP. UC = unchallenged left lung; AC = allergen-challenged right middle lobe; SC = saline-challenged right upper lobe. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$, ** $p < 0.01$

median length of evaluable basement membrane was 5.2 mm (range 1.1 to 12.5 mm), the median surface area of nasal sub-epithelium was 0.54 $mm^2$ (range 0.11 to 1.25 $mm^2$) and of lamina propria was 4.69 $mm^2$ (range 1.88 to 8.31 $mm^2$). The specified areas could not be evaluated in one case. A minimal number of seven subjects per subgroup could be included for every time point.

*Bronchial specimens.* At $T_0$, the numbers of $IgE^+$, $BB1^+$, $tryptase^+$ and $chymase^+$ cells (phenotypes $MC_T$, $MC_{TC}$ and $MC_C$) in bronchial epithelium and subepithelium were low and not significantly different between allergic rhinitis patients and controls (Table 4). At both $T_0$ and $T_{24}$, no changes in $IgE^+$, $MC_T$, $MC_{TC}$, $MC_C$ and $BB1^+$ cells could be detected in control subjects in all tissues studied. In bronchial epithelium of allergic rhinitis patients, only few mast cells and $BB1^+$ cells could be detected before and after SBP. At $T_{24}$, the epithelial $IgE^+$ cell number tended to be higher in the unchallenged ($p = 0.05$) and saline challenged ($p = 0.1$) segments and was significantly higher in the allergen challenged ($p = 0.04$) segment of allergic rhinitis patients compared to controls. At $T_{24}$, the numbers of $IgE^+$ cells and mast cells in the sub-epithelium of allergic patients were not significantly affected. The number of $BB1^+$

cells in the bronchial sub-epithelium (Figure 2) of allergic patients, however, significantly increased in the allergen challenged segment ($p = 0.02$), saline control segment ($p = 0.02$) and even increased in the unchallenged segment ($p = 0.08$). At $T_{24}$, the number of $BB1^+$ cells in the bronchial sub-epithelium was significantly increased in allergic subjects compared to controls for the allergen challenged segment ($p = 0.002$) and saline challenged segment ($p = 0.01$), but not for the unchallenged segment ($p = 0.6$).

**TABLE 4 CELL NUMBERS IN BRONCHIAL SPECIMENS**

| | group | layer | $T_0$ | $T_{24}$ | | |
|---|---|---|---|---|---|---|
| | | | | UC | AC | SC |
| IgE | AR | epithelium | 0 (0-22) | 14 (0-48) | 2 (0-29) | 3 (0 - 58) |
| | | subepithelium | 21 (0-442) | 41 (21-264) | 76 (0-128) | 138 (32-943) |
| | C | epithelium | 0 (0-3) | 0 (0-2) | 0 | 0 (0-21) |
| | | subepithelium | 15 (0-35) | 4 (0-133) | 10 (0-245) | 10 (0-120) |
| BB1 | AR | epithelium | 0 | 0 (0-1) | 0 | 0 |
| | | subepithelium | 0 (0-4) | 9 (0-144) | 88 (22-223)* | 25 (5-350)* |
| | C | epithelium | 0 | 0 | 0 | 0 |
| | | subepithelium | 0 (0-4) | 5 (0-16) | 5 (0-88) | 2 (0-33) |
| $MC_C$ | AR | epithelium | 0 | 0 (0-3) | 0 | 0 |
| | | subepithelium | 2 (0-28) | 0 (0-43) | 0 (0-21) | 4 (0-143) |
| | C | epithelium | 0 | 0 | 0 (0-8) | 0 (0-3) |
| | | subepithelium | 0 (0-8) | 0 (0-3) | 0 (0-2) | 2 (0-5) |
| $MC_{TC}$ | AR | epithelium | 0 (0-33) | 0 (0-3) | 0 (0-5) | 0 |
| | | subepithelium | 36 (0-141) | 42 (0-126) | 31 (4-131) | 33 (0-81) |
| | C | epithelium | 0 (0-3) | 0 (0-3) | 0 (0-3) | 0 (0-8) |
| | | subepithelium | 14 (0-79) | 8 (0-184) | 15 (0-89) | 27 (0-265) |
| $MC_T$ | AR | epithelium | 1 (0-10) | 8 (0-55) | 0 (0-22) | 0 (0-24) |
| | | subepithelium | 111 (0-622) | 161 (28-351) | 139 (25-323) | 74 (0-326) |
| | C | epithelium | 0 (0-19) | 0 (0-13) | 8 (0-31) | 9 (0-36) |
| | | subepithelium | 127 (8-203) | 56 (10-140) | 151 (0-414) | 110 (0-300) |

*Definition of abbreviations:* AR = allergic rhinitis patients; C = control subjects; UC = unchallenged left lung; AC = allergen-challenged right middle lobe; SC = saline-challenged right upper lobe. Data are presented as median ± range. * = $p < 0.05$ (Wilcoxon signed rank test)

**TABLE 5 CELL NUMBERS IN NASAL SPECIMENS**

| | group | layer | $T_0$ | $T_1$ | $T_{24}$ |
|---|---|---|---|---|---|
| **IgE** | **AR** | epithelium | 34 (0-140) | 13 (0-215) | 6 (0-42) |
| | | subepithelium | 106 (9-1211) | 157 (19-794) | 39 (0-282) |
| | | lamina propria | 74 (6-255) | 71 (14-192) | 46 (5-88) |
| | **C** | epithelium | 4 (0-20) | 1 (0-11) | 1 (0-24) |
| | | subepithelium | 32 (10-55) | 29 (3-168) | 16 (0-34) |
| | | lamina propria | 14 (6-40) | 12 (8-34) | 10 (2-28) |
| **BB1** | **AR** | epithelium | 0 | 0 (0-1) | 0 |
| | | subepithelium | 1 (0-2) | 0 (0-1) | 4 (1-12)* |
| | | lamina propria | 1 (0-5) | 2 (0-3) | 7 (3-14)* |
| | **C** | epithelium | 0 | 0 (0-1) | 0 (0-1) |
| | | subepithelium | 1 (0-1) | 1 (0-1) | 1 (0-9) |
| | | lamina propria | 1 (0-5) | 1 (0-4) | 2 (0-10) |
| **$MC_C$** | **AR** | epithelium | 0 (0-16) | 0 (0-6) | 0 |
| | | subepithelium | 29 (8-86) | 22 (11-45) | 15 (0-179) |
| | | lamina propria | 18 (5-48) | 11 (3-21) | 3 (1-25)* |
| | **C** | epithelium | 0 | 0 | 0 |
| | | subepithelium | 9 (0-55) | 6 (2-59) | 5 (1-19) |
| | | lamina propria | 4 (1-27) | 5 (1-47) | 3 (0-15) |
| **$MC_{TC}$** | **AR** | epithelium | 0 (0-14) | 1 (0-27) | 0 (0-5) |
| | | subepithelium | 235 (54-694) | 449 (189-555) | 118 (0-451) |
| | | lamina propria | 92 (22-178) | 114 (77-137) | 42 (0-111)* |
| | **C** | epithelium | 0 (0-15) | 0 | 0 |
| | | subepithelium | 228 (46-540) | 223 (80-431) | 179 (0-324) |
| | | lamina propria | 105 (47-185)_ | 95 (56-127) | 89 (0-172) |
| **$MC_T$** | **AR** | epithelium | 0 (0-7) | 4 (0-9) | 0 (0-15) |
| | | subepithelium | 5 (0-40) | 11 (0-123) | 9 (0-26) |
| | | lamina propria | 2 (0-15) | 2 (0-11) | 2 (0-6) |
| | **C** | epithelium | 0 | 0 | 0 (0-3) |
| | | subepithelium | 6 (0-23) | 3 (0-78) | 3 (0-45) |
| | | lamina propria | 2 (1-6) | 4 (0-11) | 1 (0-13) |

*Definition of abbreviations:* AR = allergic rhinitis patients; C = control subjects; UC = unchallenged left lung; AC = allergen-challenged right middle lobe; SC = saline-challenged right upper lobe. Data are presented as median ± range. * = $p < 0.05$ (Wilcoxon signed rank test)

*Nasal specimens*. As in the bronchial mucosa, only few chymase$^+$, tryptase$^+$ (Figure 4) or BB1$^+$ cells were seen in the nasal epithelium of all subjects before and after SBP (Table 5). At $T_0$, IgE$^+$ cells could be detected in the nasal epithelium and cell numbers were higher in allergic patients than in controls ($p = 0.06$). At $T_0$, no significant differences were found between allergic patients and control subjects for the number of IgE$^+$ cells, $MC_{TC}$, $MC_T$ and BB1$^+$ cells in the nasal lamina propria. The number of $MC_C$, however, was higher in rhinitis patients than in controls at $T_0$ ($p = 0.03$). At $T_1$, no significant changes in cell numbers were found for mast cells, IgE$^+$ cells and BB1$^+$ cells in nasal epithelium and lamina propria of allergic patients, when compared to baseline. At $T_{24}$, however, the numbers of $MC_C$ ($p = 0.02$) and $MC_{TC}$ ($p = 0.04$) in the nasal lamina propria of allergic patients were significantly decreased (fig 4a and fig 4b). In contrast, $MC_T$ and IgE$^+$ cell numbers remained stable, while BB1$^+$ cell numbers were significantly increased ($p = 0.01$, fig 4c). At $T_{24}$, the BB1$^+$ cell number was significantly higher in allergic patients compared to controls in the nasal sub-epithelium ($p = 0.01$) and lamina propria ($p = 0.02$). No significant increases were seen for IgE$^+$, mast cell and BB1$^+$ cell numbers in healthy controls at $T_1$ and $T_{24}$, when compared to baseline.

*Comparison of cell counts in upper and lower airways.* At $T_0$, IgE$^+$ cell numbers were significantly higher in nasal epithelium (Table 5) than in bronchial epithelium (Table 4) in allergic rhinitis patients ($p = 0.02$). Also in controls , IgE$^+$ cells were predominantly present in nasal epithelium compared to bronchial epithelium ($p = 0.02$), but cell counts were lower than in allergic subjects. In the sub-epithelium, there

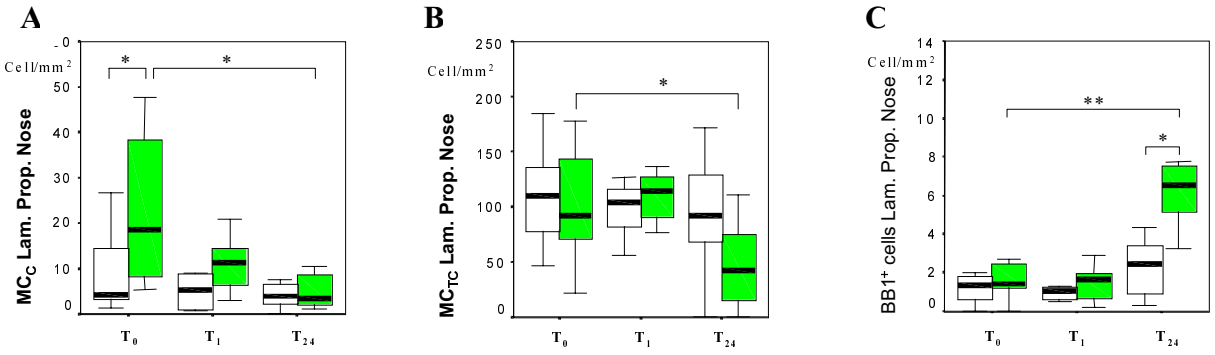


**Figure 4.** The number of $MC_C$ (A), $MC_{TC}$ (B) and BB1$^+$ (C) cells in the nasal lamina propria before ($T_0$), 1 hour ($T_1$) and 24 hours ($T_{24}$) after SBP. *Grey boxes* indicate allergic patients, *open boxes* indicate controls. Data are presented as medians ± range. * $p < 0.05$, ** $p < 0.01$.

was no difference between the number of $IgE^+$ cells in the nose and in the bronchi of both allergic patients and control subjects. At $T_0$, $BB1^+$ cells could hardly be detected in both nasal and bronchial mucosa. At $T_{24}$, however, the influx of $BB1^+$ cells was much higher in the bronchial subepithelium (Figure 5) than in nasal subepithelium ($p = 0.04$), while no difference was found in the control subjects. There was a clear difference in the relative distribution of $chymase^+$ and $tryptase^+$ cells between nasal

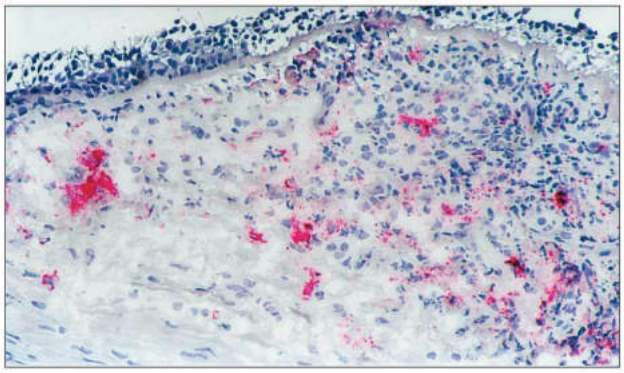

**Figure 5 ↑.** Bronchial biopsy specimens of an allergen-challenged allergic rhinitis patient stained immunohistochemically for $BB1^+$ cells. Counterstained with hematoxylin. Magnification: x 200.

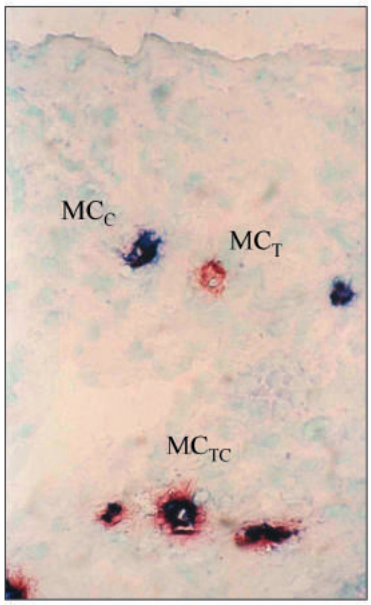


**Figure 6 →.** Nasal biopsy section of an allergic rhinitis patient showing immunoreactivity for mast cells (tryptase/chymase). Counterstained with methyl green. Magnification: x 400.

and bronchial mucosa. In the bronchi, $MC_T$ is the predominant cell type (74 % in atopics and 89 % in controls), followed by $MC_{TC}$ (23 % in atopics vs. 10 % in controls) with almost no $MC_C$ (3 % in atopics vs. 1 % in controls). In contrast, in the nose $MC_{TC}$ (Figure 6) are the most abundant (79 % in atopics and 89 % in controls), followed by $MC_C$ (19 % in atopics and 5 % in controls) with very few $MC_T$ (3 % in both groups). At $T_{24}$, the percentage of $MC_T$ significantly increased in the nasal subepithelium ($p = 0.04$) and to a lesser extent also in the bronchial subepithelium ($p = 0.2$) of allergic rhinitis patients, at the expense of $MC_{TC}$ and $MC_C$.

## 5.5 Discussion

In this study, we were able to demonstrate changes in nasal mast cell and basophil numbers in allergic rhinitis patients 24 hours after segmental bronchial provocation (SBP). Furthermore, we found an influx of basophils in the bronchi of the nonasthmatic allergic rhinitis patients 24 hours after SBP. This occurred not only in the challenged but also in the saline challenged and unchallenged segments albeit to a lesser extent. In addition to the inflammatory findings, a sustained decrease in $FEV_1$ was noticed after SBP in allergic rhinitis subjects.

In a previous article, we have demonstrated that SBP in allergic rhinitis patients induces blood eosinophilia and mucosal inflammation - characterized by increased numbers of eosinophils, IL-$5^+$ and eotaxin$^+$ cells - in both upper and lower airways[20]. These data suggest a more generalized effect of local allergen deposition.

Granule secretion of mucosal mast cells and the influx of basophils from the blood into the tissue has been associated with disease activity in the upper and lower airways of patients with allergic rhinitis and asthma[16,18,25-27]. Since these two cell types are important for the initiation and modulation of the allergic reaction[11,14,15], we studied the dynamics of mast cells and basophils in the nasal mucosa after allergen provocation in a distant and "downstream" organ.

In the nasal mucosa, the numbers of chymase$^+$ ($MC_C$) and tryptase$^+$/chymase$^+$ ($MC_{TC}$) mast cells significantly decreased, while BB1$^+$ numbers significantly increased in allergic rhinitis patients 24 hours after SBP. In contrast to the nose, the number of mast cells in the bronchial subepithelium, which are predominantly tryptase-positive[24,28,29], did not significantly change after SBP. A possible explanation for the relatively stable number of tryptase$^+$ cells in the nasal and bronchial mucosa could be the influx of tryptase$^+$ basophils from the peripheral blood into the bronchial mucosa[30].

Discrepancies in mast cell counts between different studies, probably due to different methods for detection of mast cells, have lead to various opinions about mast cell dynamics. Transmucosal migration of mast cells into the epithelium has been suggested as a possible mechanism to explain an increase in epithelial mast cells after allergen exposure[5,31]. Degranulation has been proposed and demonstrated by other

authors and could also contribute to changes in mast cell numbers in nasal and bronchial mucosa[10,26,32,33]. Immunohistochemical analysis of mast cells is hampered by the fact that there is no pan mast cell membrane associated marker. In our study, we have found no indication for the redistribution of mast cells in nasal and bronchial mucosa, at early ($T_1$) and late ($T_{24}$) time points. Therefore, degranulation is a more likely mechanism to explain the sudden decrease in $MC_C$ and $MC_{TC}$ in the nasal lamina propria of allergic rhinitis patients after SBP, while the number of $IgE^+$ cells remains stable. Degranulation of mast cells in the nose after SBP occurred at a relatively late time point compared to what has been demonstrated in experiments after nasal allergen challenge. KleinJan *et al.* demonstrated an increase in the number of basophils and a reduction in mast cell numbers in nasal mucosa of allergic rhinitis subjects within 1 hour after nasal allergen provocation[28]. In our study, no changes in the number of nasal mast cells and basophils were seen at 1 hour after SBP. At $T_{24}$, however, over 50% of $MC_{TC}$ in the nasal lamina propria of allergic rhinitis patients (compared to only 10% in controls) had lost their granular contents, which is in accordance with findings during natural exposure[26]. Lozewicz and Wagenmann have demonstrated that unilateral nasal allergen challenge results in an immediate reduction of the number of stainable mast cells[34] and in a delayed increase in basophil numbers and histamine production in the contralateral unstimulated nostril[35]. The authors speculated that a neural reflex mechanism was most likely to contribute to mast cell degranulation in the unchallenged nostril. The delayed effect on nasal mucosal mast cells that we see in our model, however, is more suggestive of a systemic allergic response.

In allergic rhinitis patients, we found a decreased number of basophils in the peripheral blood 24 hours after SBP. The decrease in circulating basophils at this time point could be the result of active migration and redistribution of these cells from the blood to the affected tissue[16]. Moreover, we detected increased expression of IL-5 in serum of allergic rhinitis patients after SBP, which suggests that a systemic allergic response is triggered. Cytokines such as IL-5, but also IL-1, IL-3, GM-CSF, stem-cell factor, nerve growth factor, histamine-releasing factor, and IFN-γ are capable of priming

basophils[36]. Both IL-3 and GM-CSF were undetectable in serum samples in our study (data not shown). Comparable serum IL-5 results have been demonstrated in patients with allergic asthma after bronchial allergen provocation[37] and natural exposure[38]. Furthermore, nasal provocation with methacholine in asthmatic patients with rhinitis resulted in an increase in lower airway resistance that could be blocked by premedication of the nasal mucosa with phenylephrine[39]. This is also suggestive of a systemic effect of inflammatory mediators in the induction of lower airway resistance. Another possible interaction mechanism could be the systemic allergen absorption, as has been suggested by Kontou-Karakitsos in the early seventies[40]. However, the allergen we used, grass pollen, has a much higher molecular weight than the antigen fragments used in the peanut extract in that study. Therefore, we find it less plausible that grass pollen particles cross the mucosa barrier and merge in the blood.

Eventually, it is highly unlikely that the methodological approach in our study would contribute to a systemic allergic response. Bronchoscopy and biopsy, *per se*, do not result in generalized bronchial inflammation[41]. To minimize the intraluminal spread of antigen from the allergen challenged segment to other segments, the bronchoscope was held in the wedged position. It is also very unlikely that allergen spilled into the nose after SBP, since bronchoscopy was performed via the oral route and did not lead to excessive coughing. This is supported by a small study which addressed the issue of cross-contamination of nose and lungs with a radioactive tracer. In a pilot-study, a radionuclide was placed bronchoscopically in the bronchial tree in four patients and was still clearly visible in the same position after 24 hours, suggesting that contamination of the nose through intraluminal spread is unlikely to take place[42].

From our results, we hypothesize that allergic rhinitis patients without bronchial hyperresponsiveness can express "asthma-like" symptoms and inflammation. However, the dose required to initiate an allergic response in the bronchi is probably higher in allergic rhinitis patients than in clinical asthma patients. Apparently, such a dose is not encountered during natural exposure to allergen. Moreover, other factors, such as the type of inhalant allergy and the duration and severity of exposure are important in this context[43]. In addition, we have demonstrated that segmental

bronchial provocation influences the number of stainable mast cells and basophils in the upper airways of allergic rhinitis patients. We speculate that enhanced production of inflammatory mediators at the site of allergen provocation leads to a more generalized effect on metachromatic cells throughout the respiratory system and the blood .

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# 6

# Nasal allergen provocation induces adhesion molecule expression and tissue eosinophilia in upper and lower airways

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## 6.1 Summary

Allergic rhinitis (AR) and asthma are characterized by means of a similar inflammatory process in which eosinophils are important effector cells. The migration of eosinophils from the blood into the tissues is dependent on adhesion molecules.

The objective was to analyze the aspects of nasobronchial cross-talk, we studied the expression of adhesion molecules in nasal and bronchial mucosa after nasal allergen provocation (NP).

Nine nonasthmatic subjects with seasonal AR and 9 healthy control subjects underwent NP out of season. Bronchial and nasal biopsies were taken before ($T_0$) and 24 hours after NP ($T_{24}$). Mucosal sections were analyzed for the presence of eosinophils (MBP), interleukin (IL)-5, eotaxin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and human endothelium (CD31).

At $T_{24}$, an influx of eosinophils was detected in nasal epithelium ($p = 0.01$) and lamina propria ($p < 0.01$), as well as in bronchial epithelium ($p = 0.05$) and lamina propria ($p < 0.05$) of patients with AR. At $T_{24}$, increased expression of ICAM-1 in the lamina propria as well as increased percentages of ICAM-$1^+$, VCAM-$1^+$ and E-selectin$^+$ vessels were seen in nasal and bronchial tissue of AR patients. The number of mucosal eosinophils correlated with the local expression of ICAM-1, E-selectin and VCAM-1 in patients with AR.

This study shows that nasal allergen provocation in allergic rhinitis patients results in generalized airway inflammation through upregulation of adhesion molecules.

## 6.2 Introduction

Allergic rhinitis (AR) and asthma are closely related diseases that share a common genetic background[1,2]. Accumulating evidence underlines the importance of AR in the development[3-5]and control of asthma[6-8]. However, pathophysiological interactions between upper and lower airways are not well described.

It has been well established that allergic mucosal inflammation in allergic asthma and rhinitis is characterized by tissue eosinophilia[9,10]. Tissue eosinophilia is the result of several sequential events. Eosinophils are released from the bone-marrow under

influence of serum-IL-5 and eotaxin, resulting in an increase of circulating eosinophils[11,12]. Eosinophil migration is dependent on the expression of cytokines, chemokines and adhesion molecules[13,14]. Upregulation of endothelial adhesion molecules, through the release of IL-1β, IL-4 and TNFα from resident cells (e.g., mast cells and T lymphocytes)[15-17], results in firmer leukocyte-endothelial adherence and trans-endothelial migration of leukocytes along a chemotactic gradient. ICAM-1, VCAM-1 and E-selectin are known endothelial adhesion molecules. ICAM-1 and E-selectin mediate the attachment of all classes of leukocytes to endothelial cells, while VCAM-1 interacts with VLA-4, which is expressed on lymphocytes, monocytes, eosinophils and basophils, but not on neutrophils[13]. Increased expression of ICAM-1, VCAM-1 and E-selectin has been demonstrated locally after experimental nasal and bronchial allergen challenge[14,18].

The possibility of cross-talk between nasal and bronchial mucosa after nasal allergen provocation (NP) leading to endothelial activation has not been examined. The aim of the current study was to determine allergic mucosal inflammation and adhesion molecule expression simultaneously in the upper and lower airways after nasal allergen provocation. Therefore, we designed a study in which nasal and bronchial mucosal biopsies, as well as blood samples were taken from a group of nonasthmatic AR patients with an isolated grass pollen allergy after NP. Eosinophils, major effector cells in allergic inflammation, IL-$5^+$ and eotaxin$^+$ cells – necessary for eosinophil survival and chemotaxis - were chosen as markers of mucosal allergic inflammation. Because IL-5 is vital for the differentiation of progenitor cells into mature eosinophils, we determined serum IL-5 concentrations before and after NP.

## 6.3 Materials and methods

### *6.3.1 Subject groups*

Nine patients with AR (4 men and 5 women, age range, 22 to 34 years) and 9 non-allergic healthy control subjects (7 men and 2 women, age range, 19 to 32 years) were selected for the study. Subject characteristics are shown in Table 1. The patients with

**TABLE 1 Subject characteristics**

| Patient | Age (yr) | Sex | $FEV_1$ (L) | $FEV_1$ (%) | IVC (L) | $FEV_1$/VC | BAR* (%) | $PC_{20}$ (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| **Allergic rhinitis patients** | | | | | | | | |
| 1 | 22 | F | 3.93 | 108 | 4.72 | 88 | 103 | 40 |
| 2 | 34 | M | 3.2 | 87 | 3.65 | 88 | 98 | 40 |
| 3 | 26 | M | 4.37 | 95 | 5.67 | 77 | 104 | 40 |
| 4 | 25 | M | 4.51 | 101 | 4.91 | 92 | 105 | 40 |
| 5 | 22 | F | 3.82 | 112 | 4.06 | 94 | 100 | 40 |
| 6 | 26 | F | 3.28 | 93 | 4.28 | 77 | 104 | 38.4 |
| 7 | 22 | M | 4.26 | 90 | 5.19 | 82 | 107 | 19.6 |
| 8 | 26 | F | 4.72 | 126 | 5.52 | 86 | 102 | 20 |
| 9 | 27 | F | 3.34 | 102 | 3.8 | 88 | 105 | 40 |
| **Control subjects** | | | | | | | | |
| 10 | 30 | M | 5.3 | 110 | 7.49 | 76 | 108 | 32.4 |
| 11 | 21 | F | 4.34 | 114 | 5.41 | 80 | 104 | 32.7 |
| 12 | 22 | M | 5.75 | 109 | 6.64 | 87 | 102 | 33.5 |
| 13 | 19 | F | 3.79 | 104 | 4.79 | 79 | 107 | 40 |
| 14 | 22 | M | 5.75 | 118 | 6.38 | 90 | 100 | 40 |
| 15 | 25 | M | 4.14 | 94 | 4.75 | 87 | 101 | 40 |
| 16 | 21 | M | 4.68 | 97 | 5.55 | 84 | 102 | 40 |
| 17 | 24 | M | 4.09 | 86 | 5.35 | 76 | 103 | 40 |
| 18 | 32 | M | 4.96 | 113 | 6.39 | 78 | 102 | 40 |

*Abbreviations used:* IVC = inspiratory vital capacity; BAR = β-agonist response.

*β-agonist response (terbutaline, 1000 μg) data are presented as percentage improvement compared with initial value; a virtual value of 40 was assigned to subjects who did not reach a $PC_{20}$ with 38 mg/mL methacholine.

AR had at least a 2-year history of isolated grass pollen allergy confirmed by a positive skin prick test reaction to grass pollen extract alone (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands) and not to 13 other common allergens. None of the study subjects had a clinical history of asthma. All had a normal $FEV_1$ and a methacholine $PC_{20}$ > 8 mg/ml. Methacholine was administered according to a standardized tidal breathing method[19]. None of the subjects smoked or used medication that could influence the results of this study. Biopsy specimens were obtained out of the grass pollen season. None of the subjects had experienced a respiratory tract infection in the four weeks prior to allergen challenge. All participants provided informed written consent to the study, which was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam, the Netherlands.

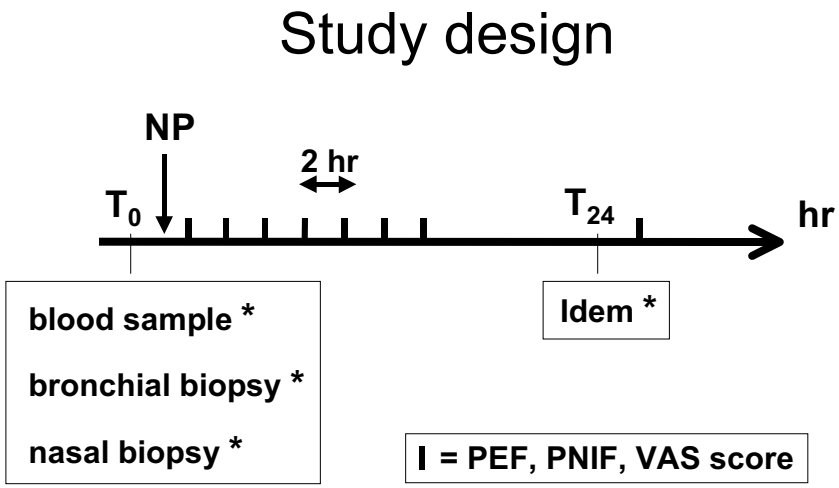


**Figure 1.** Nasal and bronchial biopsy specimens, as well as blood samples were collected from patients and control subjects before ($T_0$) and 24 hours ($T_{24}$) after NP. Symptom scores were recorded at the beginning of each visit ($T_0$ and $T_{24}$), one half hour after NP and bronchoscopy ($T_{24}$ and $T_{24½}$), and at 2-hour intervals after NP ($T_2$ - $T_{12}$).

### *6.3.2 Experimental design*

The study design is outlined in Figure 1. Bronchial and nasal biopsies, as well as blood samples, were collected before ($T_0$) and 24 hours ($T_{24}$) after NP. Nasal and bronchial symptoms (Visual Analogue Scale [VAS] scores), peak nasal inspiratory flow (PNIF) and peak expiratory flow (PEF) were recorded at baseline ($T_0$), at ½ h after NP ($T_{½}$), at 2-hour intervals after NP ($T_2$ - $T_{12}$) and at day 2 ($T_{24}$ and $T_{24½}$). PNIF was measured with a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL) and PEF was measured with a Personal Best peak expiratory flow meter (Respironics Inc., Cedar Grove, NJ). $FEV_1$ was determined by standard spirometry at $T_0$ and $T_{24}$.

### 6.3.3 Blood samples

The total number of blood eosinophils was determined by hemocytometry (Sysmex NE 8000). IL-5 was measured in serum with a commercially available ELISA-kit, according to the instructions of the manufacturer (Cytoscreen™, BioSource International Inc., Camarillo, CA). The sensitivity of the ELISA kit was 11,7 pg/ml.

### 6.3.4 Bronchial Biopsies

All bronchial biopsies were taken by the same pulmonary physician (S.E.O.), according to a previously described method[20]. During the first bronchoscopy, mucosal biopsies were taken from the carinae of the left upper and lower lobes. After 24 hours, each subject underwent a repeat bronchoscopy, during which biopsies were taken from lobar segments of the right lung. The bronchial biopsies were embedded in Tissue-Tek II Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA), frozen and stored at –150 $^0$C.

### 6.3.5 Nasal biopsy specimens and NP

Biopsy specimens of nasal mucosa were taken by the same investigator (G.J.B.), according to a previously described method[20]. Subsequently, patients and control subjects were challenged with 10,000 BU grass pollen extract ( Vivodiagnost ALK Benelux BV, Groningen, the Netherlands) by means of a pump spray delivering a fixed dose of 50 $\mu$L into each nostril. This concentration results in a good clinical response in seasonal rhinitis patients[21]. The allergen was delivered after deep inspiration during breath holding. After 24 h, a second mucosal biopsy specimen was taken from the contralateral nostril. The nasal biopsies were embedded in Tissue-Tek II OCT compound, frozen and stored at – 150 $^0$C.

### 6.3.6 Monoclonal Antibodies

The mAbs used in this study were anti-major basic protein (MBP) antibody ($IgG_1$, 0.2 µg/ml, clone nr. BMK13, Sanbio, Uden, the Netherlands) for identifying eosinophils, anti-human IL-5 mAb ($IgG_1$, 50 µg/ml, clone nr. 5A5, a gift from Prof. Jan Tavernier,

University of Ghent, Ghent, Belgium), anti-eotaxin antibody ($IgG_1$, 10 μg/ml, clone nr. 43911.11, R&D systems, MN), anti-E-selectin antibody (IgG1, 2 μg/ml, clone nr. ENA1, Sanbio), anti-VCAM-1 antibody (IgG1, 2 μg/ml, clone nr. 1G11B1, Sanbio), anti-ICAM-1 antibody (IgG1, 0.2 μg/ml, clone nr. MEM-112, Sanbio) and anti-human endothelium (CD31) antibody ( IgG1, 1 μg/ml, clone nr. EN4, Sanbio)

### *6.3.7 Immunohistochemical staining*

Each tissue specimen was cut into serial 6 $\mu$m thick sections on a Reichert-Jung 2800e frigocut cryostat (Leica, Wetzlar, Germany) and transferred onto poly-L-lysine-coated slides (Sigma Chemical Co., St. Louis, MO), dried and stored at - 80 $^0$C. The mAb stainings were developed with the super sensitive immuno-alkaline phosphatase (ss-AP) method as previously described[20]. Isotype-specific control staining was done with Mouse IgG1 antibody to *Aspergillus niger* glucose oxidase (Dako catalog No. X 0931) at the same protein concentration as the specific antibody.

### *6.3.8 Microscopic assessment of immunohistochemical staining*

Biopsies were coded and 2 sections 120 μm apart were counted blind for each antibody as has been described previously[20]. Cell numbers were taken to be the number of positively stained cells per square millimeter by using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of x 200.

Expression of adhesion molecules was assessed by 2 different methods of quantification at 100x magnification. First, intensity and extent of adhesion molecule expression was determined by using a computer-assisted image analysis system(Leica, Rijswijk, the Netherlands). This method is based on densitometric differences between positively stained tissue and background after setting thresholds for color, saturation and intensity.

Secondly, with the use of an eye graticule, the total lamina propria of the nasal and bronchial specimens was examined for endothelial adhesion molecules. Only stained vessels with a distinct lumen were counted and extravascular staining was excluded,

according to a method described by Lee *et al*[14]. The percentage of endothelial adhesion molecule expression was calculated from the ratio of vessels positive for a specific adhesion molecule and the total number of vessels staining positive for CD31. The numbers of vessels stained with a specific adhesion molecule and with CD31 were obtained from consecutively cut sections.

### *6.3.9 Statistical analysis*

Wilcoxon's signed-rank test for within-group analysis and the Mann-Whitney U test for between-group analysis were performed if there were 2 samples. In case of repeated measurements, a mixed model ANOVA (analysis of variance) with compound symmetry was done with the SAS software package (SAS Institute Inc., Cary, NC). Data are presented as medians and ranges or mean ± SE, as indicated. Correlations were evaluated with Spearman's rank correlation test. A p-value less than 0.05 was considered significant.

## 6.4 Results

### *6.4.1 Clinical data*

At baseline, AR patients and control subjects were comparable for clinical parameters. At the consecutive 2-hour intervals after NP, the AR patients had increased total nasal VAS score ($p = 0.0002$, repeated measurement ANOVA), and lower PNIF values ($p = 0.0001$) than control subjects. The shape of the nasal VAS score (Figure 3a) and PNIF (Figure 3b) curves in AR patients was bimodal with peaks at $T_{½}$ and $T_{12}$. At $T_{24}$, PNIF and nasal symptom score returned to baseline values. The same bimodal pattern was seen for bronchial VAS score (Figure 3c) and PEF (Figure 3d) measurements in patients with AR. These findings differed significantly from those of control subjects ($p = 0.01$; $p = 0.04$, respectively). The $FEV_1$ did not significantly change after NP. No effect of NP could be detected on nasal VAS score, PNIF, bronchial VAS score and PEF in control subjects.

### *6.4.2 Blood cells and mediators*

At baseline, blood eosinophil numbers were comparable in patients with AR (median 140, range 10 to 360 x10 $^{6}$/L) and control subjects (median 110, range 30 to 180 x10 $^{6}$/L). At $T_{24}$, the number of blood eosinophils significantly increased in AR patients (median 300, range 140 to 550 x10 $^{6}$/L) compared with baseline ($p = 0.008$), and

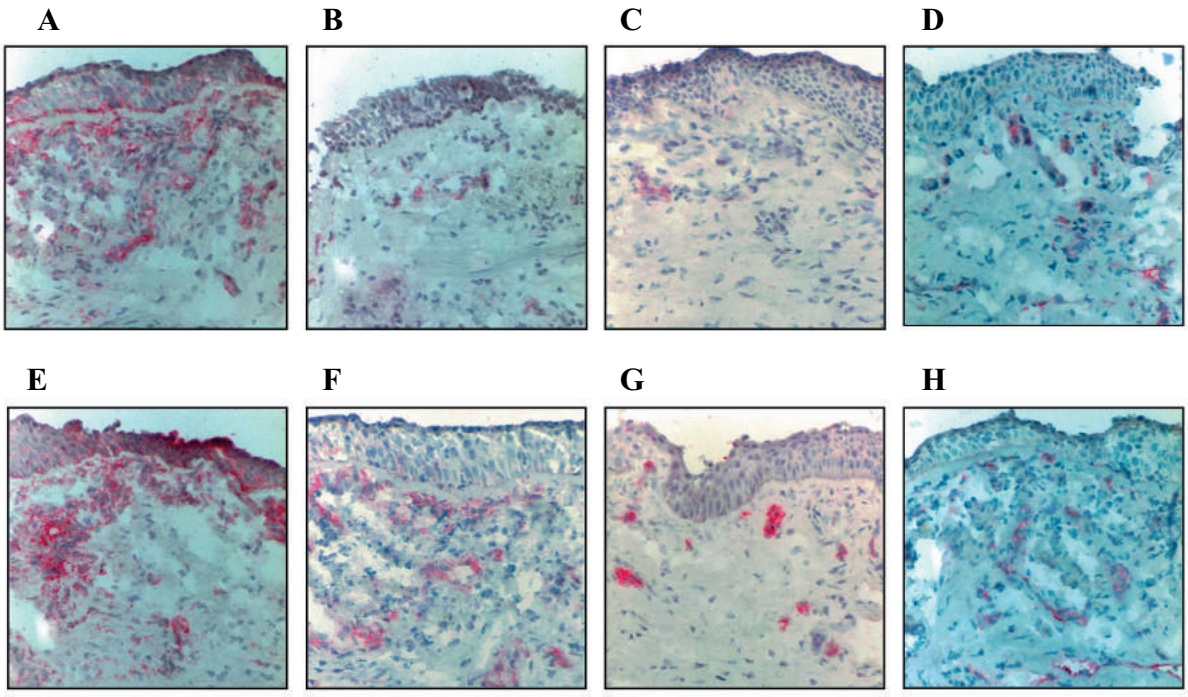


**Figure 2.** Nasal mucosal sections taken from patients with AR before and 24 hours after nasal provocation. Immunohistochemical staining for ICAM-1 (**A** and **E**), VCAM-1 (**B** and **F**), E-selectin (**C** and **G**), and CD31 (**D** and **H**). Counterstained with hematoxylin (original magnification 100 x)

compared with control subjects (median 140, range 40 to 180 x10 $^{6}$/L; $p < 0.001$).

At $T_0$ and $T_{24}$, the concentrations of serum IL-5 were significantly higher ($p = 0.01$) in patients with AR (median: 27.7 pg/ml; range: 13.0 to 165.1 pg/ml) than in control subjects (median: 14.6 pg/ml; range: < 11.7 to 81 pg/ml).

### *6.4.3 Immunostaining of mucosal biopsies*

*General description.* Out of the 72 biopsy specimens, 69 reached criteria for evaluation. Two bronchial and one nasal biopsy specimen could not be evaluated. These samples were excluded from the study. In bronchial biopsy specimens, the

median length of evaluable basement membrane was 3.8 mm (range 1.3 to 6.8 mm); in nasal biopsy specimens 4.0 mm (range 1.2 to 8.1 mm). The median surface area of bronchial subepithelium was 0.38 $mm^2$ (range 0.13 to 0.68 $mm^2$) and nasal subepithelium 0.42 $mm^2$ (range 0.21 to 0.81 $mm^2$). The median surface area of nasal lamina propria was 3.0 $mm^2$ (range 1.0 to 6.1 $mm^2$). Staining with isotype-specific control antibodies was negative in all cases.

*Nasal specimens*. At $T_0$, the numbers of eosinophils, IL-$5^+$ and eotaxin$^+$ cells were comparable in nasal epithelium, subepithelium and lamina propria of patients with AR and control subjects. At $T_{24}$ , the number of eosinophils was significantly increased in nasal epithelium ($p = 0.01$), subepithelium ($p = 0.01$) and lamina propria ($p < 0.01$) of patients with AR. At $T_{24}$, the number of IL-$5^+$ cells was significantly higher in nasal epithelium ($p = 0.04$), whereas the number of eotaxin$^+$ cells was significantly increased in the lamina propria ($p = 0.05$) of the nasal mucosa.

At baseline, there were no differences in ICAM-1 (Figure 2a), VCAM-1 (Figure 2b) and E-selectin (Figure 2c) expression in nasal mucosa between AR patients and control subjects. At $T_{24}$, ICAM-1 ($p = 0.02$), VCAM-1 ($p = 0.008$) and E-selectin ($p = 0.02$) expression was significantly increased in the nasal lamina propria of the AR patients compared to baseline (Table 2). However, no difference was found between patients with AR and control subjects. At $T_{24}$, the percent of ICAM-$1^+$ ($p = 0.01$, Figure 2e ), VCAM-$1^+$ ($p = 0.008$, Figure 2f) and E-selectin$^+$ ($p = 0.008$, Figure 2g) vessels was significantly increased in the allergic group compared with baseline (Table 3) and compared with control subjects ($p = 0.004$, $p = 0.05$, $p = 0.01$ respectively). No significant difference in cell numbers and adhesion molecule expression was found in the nasal mucosa of control subjects after NP. There was no difference between patients and control subjects in the total number of vessels in the nasal mucosa at $T_0$ (Figure 2d) and $T_{24}$ (Figure 2h).

*Bronchial specimens.* At $T_0$, the numbers of eosinophils , IL-$5^+$, and eotaxin$^+$ cells were comparable in allergic subjects and control subjects in bronchial epithelium and subepithelium. At $T_{24}$, we found increased numbers of eosinophils in the bronchial epithelium ($p = 0.05$) and subepithelium ($p = 0.02$) of AR patients. No significant

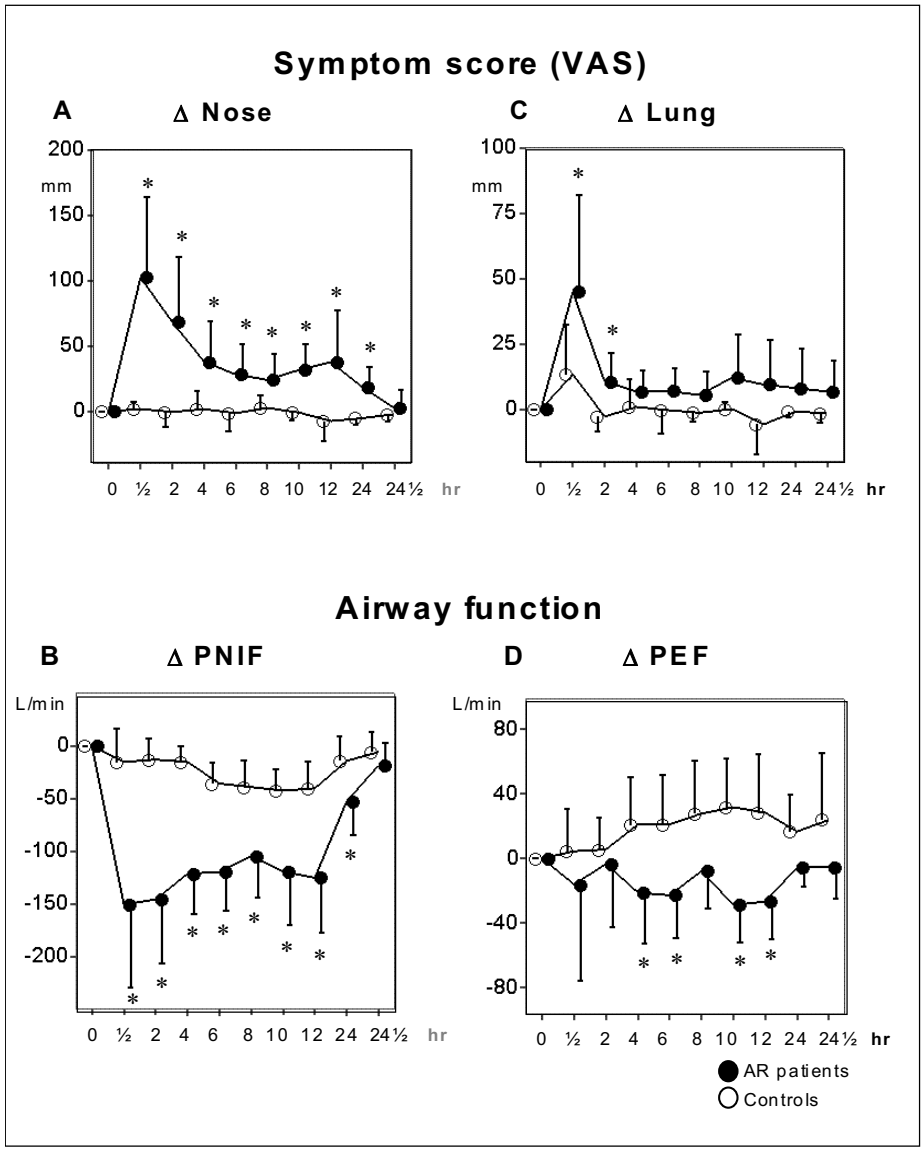


**Figure 3.** Symptoms were individually expressed in millimeters on a 10-cm VAS, and a composite score was obtained for nasal complaints (**A**, rhinorrhea, watery eyes, nasal itching, sneezing, and nasal blockage) and pulmonary complaints (**C**, wheezing, coughing, shortness of breath, and decreased exercise intolerance). PNIF (**B**) and PEF (**D**) were expressed in liters per minute. Baseline values were taken to be 0. Consecutive measurements were plotted as the difference from baseline (mean ± SEM). *$p < 0.05$ (Mann-Whitney U test).

changes were found in the number of IL-$5^+$ and eotaxin$^+$ cells in bronchial mucosa before and after NP.

At baseline, there were no differences in ICAM-1 (Figure 4a), VCAM-1 (Figure 4b) and E-selectin (Figure 4c) expression in bronchial epithelium, lamina propria and endothelium between patients with AR and control subjects. In bronchial lamina

propria of AR patients, ICAM-1 ($p = 0.05$) expression was significantly increased at $T_{24}$ compared to baseline (Table 2). However, no difference was found between AR

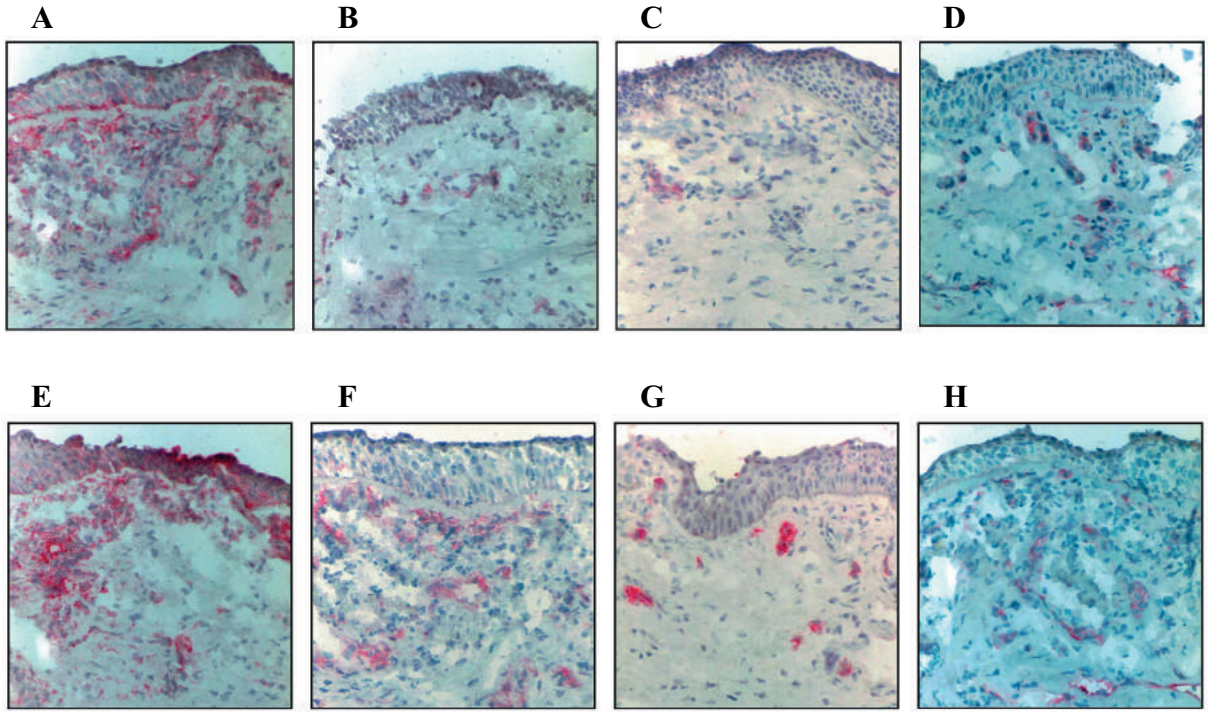


**Figure 4.** Bronchial mucosal sections taken from patients with AR before and 24 hours after nasal provocation. Immunohistochemical staining for ICAM-1 (**A** and **E**), VCAM-1 (**B** and **F**), E-selectin (**C** and **G**), and CD31 (**D** and **H**). Counterstained with hematoxylin (original magnification 100 x)

patients and control subjects. At $T_{24}$, the percentage of ICAM-$1^+$ ($p = 0.01$, Figure 4e), VCAM-$1^+$ ($p = 0.04$, Figure 4f) and E-selectin$^+$ ($p = 0.02$, Figure 4g) vessels in the bronchial mucosa was significantly increased in patients with AR compared with baseline (Table 3) and control subjects ($p = 0.008$, $p = 0.05$, $p = 0.06$, respectively).

No significant difference in cell numbers and adhesion molecule expression was found in the bronchial mucosa of control subjects after NP. There was no difference between patients and control subjects in the total number of vessels in the bronchial mucosa at $T_0$ (Figure 4d) and $T_{24}$ (Figure 4h).

*Correlations between inflammatory markers and symptomatology.* In the bronchial subepithelium, the number of eosinophils correlated with the percentage of VCAM-$1^+$ vessels ($r = 0.65$, $p = 0.005$) and E-selectin$^+$ vessels ($r = 0.58$, $p = 0.01$) as well as with the total bronchial symptom score ($r = 0.50$, $p = 0.03$).

**TABLE 2 Image analysis results**

| | ICAM-1 | | VCAM-1 | | ELAM-1 | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_{24}$ | $T_0$ | $T_{24}$ | $T_0$ | $T_{24}$ |
| **Patients with AR** | | | | | | |
| *Nose* | | | | | | |
| epithelium | 6 (1-14) | 8 (1-26) | 1 (0-4) | 1 (0-13) | | |
| lamina propria | 4 (1-12) | 12 (1-34)* | 2 (0-6) | 4 (1-21)* | 0.3 (0.2-1.1) | 1.0 (0.2-2.4)* |
| *Bronchus* | | | | | | |
| epithelium | 1 (0-11) | 4 (1-14) | 4 (0-17) | 2 (0-5) | | |
| lamina propria | 2 (0-5) | 3 (1-10)* | 2 (0-8) | 1 (0-5) | 0.0 (0-1.7) | 0.1 (0-1.1) |
| **Control subjects** | | | | | | |
| *Nose* | | | | | | |
| epithelium | 12 (3-25) | 10 (0-24) | 1 (0-7) | 0 (0-3) | | |
| lamina propria | 7 (2-25) | 6 (1-14) | 2 (1-8) | 4 (1-6) | 0.4(0.1-1.0) | 0.4 (0.1-3.8) |
| *Bronchus* | | | | | | |
| epithelium | 2 (0-4) | 6 (0-11) | 1 (0-9) | 1 (0-4) | | |
| lamina propria | 2 (0-4) | 3 (2-8) | 1 (0-3) | 1 (0-2) | 0.1 (0-0.3) | 0.0 (0-0.2) |

Data are expressed as medians (range). Values represent percentage of area staining positive for the particular adhesion molecule. ELAM = endothelial leukocyte adhesion molecule. *$p < 0.05$.

## 6.5 Discussion

In the current study, we were able to show increased expression of endothelial adhesion molecules and eosinophilic allergic inflammation in the nasal and bronchial mucosa of nonasthmatic patients with AR after NP. Recently, we have demonstrated that segmental bronchial provocation in patients with AR induces blood eosinophilia and mucosal inflammation characterized by increased numbers of eosinophils, IL-$5^+$ and eotaxin$^+$ cells in both upper and lower airways[20]. The current study further supports the hypothesis that local allergen exposure results in generalized airway inflammation.

Several studies have stressed the importance of IL-5 and eotaxin in the influx of eosinophils from the blood stream into the mucosa[22-24]. Analysis of bronchoalveolar lavage fluid in AR and asthma patients has shown enhanced local IL-5 expression after segmental bronchial provocation[25-27]. In the present study, the expression of IL-5 and eotaxin was elevated in the nasal mucosa and blood of patients with AR. In bronchial

mucosa these signaling molecules were also elevated, albeit not significantly when compared with levels found in control subjects after NP.

**TABLE 3 Endothelium results**

| | ICAM-1/CD31 | | VCAM-1/CD31 | | ELAM-1/CD31 | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_{24}$ | $T_0$ | $T_{24}$ | $T_0$ | $T_{24}$ |
| **Patients with AR** | | | | | | |
| *Nose* | | | | | | |
| subepithelium | 13 (0-33) | 44 (19-95)* | 13 (0-51) | 65 (5-94)* | 42 (0-60) | 54 (42-82)* |
| lamina propria | 28 (7-58) | 61 (30-94)* | 28 (0-43) | 67 (16-85)* | 38 (17-59) | 69 (48-82)* |
| *Bronchus* | | | | | | |
| subepithelium | 50 (0-57) | 82 (29-100)* | 0 (0-50) | 45 (0-66)* | 0 (0-25) | 35 (0-86)* |
| **Control subjects** | | | | | | |
| *Nose* | | | | | | |
| subepithelium | 28 (18-43) | 19 (11-50) | 19 (4-41) | 19 (4-56) | 44 (13-67) | 39 (21-73) |
| lamina propria | 31 (16-78) | 28 (12-51) | 25 (15-67) | 33 (10-56) | 44 (18-69) | 38 (13-86) |
| *Bronchus* | | | | | | |
| subepithelium | 30 (0-50) | 38 (0-75) | 6 (0-48) | 11 (0-69) | 0 (0-14) | 0 (0-62) |

Data are expressed as medians (range). Values represent percentage of area staining positive for the particular adhesion molecule. ELAM = endothelial leukocyte adhesion molecule. *$p < 0.05$.

In the process of leukocyte arrest and migration, adhesion molecules have an important role to play. Increased expression of endothelial ICAM-1, VCAM-1 and E-selectin has been demonstrated in nasal and bronchial mucosa after local allergen exposure[14,28,29]. Others did not detect differences in adhesion molecule expression[30,31]. These conflicting results are based on different methodological approaches to assess adhesion molecule expression. By its nature, semi-quantitative scoring methods used in previous work[30,31] may well be less suitable to detect small changes in adhesion molecule expression. Moreover, attention should be focused on the expression of adhesion molecules on the endothelium because this is the site where cells are recruited from the circulation. In our study, we used two methods to quantify adhesion molecule expression: computer-assisted image analysis and microscopic evaluation of endothelium.

A significant increase was found in the ratio of ICAM-$1^+$, VCAM-$1^+$ and E-selectin$^+$ vessels versus the total number of vessels in both the nose and bronchi in the AR

group compared with control subjects. We used CD31 as a marker for vascular endothelium because it produced a more stringent signal in bronchial mucosa than Factor VIII (Von Willebrand) applied to nasal mucosa by Lee *et al*[14] in a similar study. In contrast, the image analysis method showed increased expression of ICAM-1, VCAM-1 and E-selectin in both nasal and bronchial mucosa in the AR group. However, this was not significantly different from control tissue. Therefore we propose that analyzing expression of adhesion molecules by the endothelium may be more relevant with regard to eosinophil recruitment than determining the extent of adhesion molecule expression throughout the tissue.

It is very unlikely that our results are influenced by allergen spill from the nose into the lower airways. Inhalation studies with radiolabeled allergen have shown no deposition of allergen in the lungs after nasal application, according to the technique we used[32]. Although postnasal drainage of inflammatory mediators into the lower airways can not be excluded, it is not likely to play a role in vivo[33]. Also, bronchoscopy and taking of biopsy specimens per se do not lead to pulmonary inflammation[34].

Pathophysiological mechanisms, that could explain the interaction between the nose and the lung, are neural reflex mechanisms and systemic induction of inflammatory mediators and cells. Although exposure of the nasal mucosa to cold dry air results in immediate bronchoconstriction in asthmatic patients[35], no direct effect on $FEV_1$ could be detected after nasal allergen challenge[32,36,37]. Nasal provocation with methacholine in asthmatic patients with rhinitis resulted in an increase in lower airway resistance that could be blocked by premedication of nasal mucosa with phenylephrine, suggesting a role for systemic mediators in the induction of lower airway resistance[38]. Our results are most likely to be the result of a systemic inflammatory response, which is IL-5 mediated, involving eosinophil release from the bone marrow and migration into airway mucosa.

In this study we have demonstrated that the expression of VCAM-1 and E-selectin on the bronchial microvasculature is upregulated after NP and is associated with bronchial eosinophilia. Do these findings indicate that the AR patients in our study

actually have asthma? Although significant changes in pulmonary symptom score and PEF were found in the AR subgroup, no significant effect on $FEV_1$ was detected 24 h after NP. Therefore the clinical implications of single nasal provocation, with respect to the lower airways, remain disputable in AR patients without asthma. It is, however, very well possible that repetitive allergen stimulation of the nose, inducing a continuous bombardment of the lower airways with systemic inflammatory mediators, may result in the development of asthma eventually. Moreover, in AR patients with preexistent asthma, nasal allergen exposure could have an important contributory effect on bronchial inflammation, lung function and pulmonary symptoms. Future studies need to address these questions.

In conclusion, we have found an allergic inflammatory response similar to asthma in the lower airways of nonasthmatic patients with AR after NP. We speculate that the absorption of inflammatory mediators at the site of allergen challenge results in release of eosinophils from the bone marrow into the blood and subsequently in their recruitment to the nasal and bronchial mucosa through upregulation of adhesion molecule expression by the endothelium.

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# 7

# Inflammation in upper and lower airways does not discriminate between allergic rhinitis and asthma

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*Submitted*

## 7.1 Summary

Local airway inflammation is considered important in the clinical expression of both asthma and rhinitis.

We analyzed nasal and bronchial inflammation in atopic patients with persistent perennial nasal and/or bronchial symptomatology.

Five experimental groups were formed: allergic asthma and rhinitis ($A^+R^+$; n = 19); allergic rhinitis, no asthma ($A^-R^+$; n = 18); allergic asthma, no rhinitis ($A^+R^-$; n = 3); atopic subjects, no asthma, no rhinitis ($A^-R^-$; n = 8) and nonallergic healthy control subjects (HC; n = 16). Nasal and bronchial biopsy specimens were collected in one session during stable disease. Immunohistochemistry was performed for eosinophils (MBP), basophils (BB1), mast cells (CD117), dendritic cells (CD1a) and macrophages (CD68). The relationships between the clinical phenotype (atopy, rhinitis and/or asthma) and the numbers of different inflammatory cells were assessed by multiple regression analysis.

Atopy was related with increased numbers of blood eosinophils ($p = 0.03$), $IgE^+$ cells in nasal and bronchial mucosa and subepithelial eosinophilia in the bronchial mucosa ($p = 0.006$). The presence of rhinitis was associated with increased numbers of eosinophils in nasal mucosa ($p = 0.03$). In bronchial epithelium, numbers of eosinophils were higher in asthmatic as well as in rhinitis patients compared to HC subjects ($p = 0.01$; $p = 0.02$, respectively). Moreover, there were more dendritic cells present in bronchial mucosa of allergic rhinitis patients than in HC subjects ($p = 0.03$). No difference was found between asthma and rhinitis with regard to inflammatory cells in nasal and bronchial mucosa. In the investigated groups, no correlation could be established between inflammatory cell numbers and airway function parameters or clinical symptom scores.

In conclusion, allergic inflammation is present in the upper and lower airways of atopic patients, irrespective of their clinical manifestations. Our results illustrate that factors other than local inflammatory cell infiltrate contribute to the clinical expression of atopic disease.

## 7.2 Introduction

Allergic rhinitis and asthma commonly occur together and are manifestations of the atopic syndrome[1]. Almost 40% of allergic rhinitis patients have asthma symptoms, whereas more than 80% of allergic asthma patients have concomitant rhinitis[2,3]. Allergic rhinitis is regarded as a risk factor for the development of asthma, especially in the presence of bronchial hyperresponsiveness (BHR)[4,5].

Allergic rhinitis and asthma can be discriminated on the basis of clinical history, variable bronchoconstriction and BHR. This distinction is sometimes difficult to make since symptom perception is widely variable; lung function can be normal in mild asthmatics and, although BHR is a constant feature of asthma, it is also frequently present in allergic rhinitis[6].

Allergic rhinitis and asthma are characterized by a similar inflammatory process in which dendritic cells, mast cells, eosinophils and basophils are important effector cells[7-11]. In the early phase reaction, dendritic cells activate Th2-lymphocytes resulting in the production of several pro-inflammatory cytokines. Binding of IgE and subsequent cross-linking of FcεRI lead to degranulation of mast cells. Both mast cells and T-lymphocytes orchestrate the late phase reaction, in which eosinophils and basophils migrate from the circulation towards the mucosa.

Bronchial inflammation, and eosinophilia in particular, is still considered to be one of the hallmarks of allergic asthma[12]. However, inflammatory cells and mediators have been found in broncho-alveolar lavage (BAL) fluid, mucosal biopsies and induced sputum of nonasthmatic allergic rhinitis patients, as well[13-15]. Also, eosinophil infiltration has been demonstrated in the upper airways of asthmatic patients, even in the absence of rhinitis[16]. This illustrates that the relationship between cellular aspects of inflammation and clinical manifestation is not straightforward.

Few studies have investigated upper and lower airways simultaneously in atopic patients. One study compared nasal and bronchial inflammation in asthmatic patients with perennial rhinitis and found more eosinophilia and airway remodeling in bronchial than in nasal mucosa[10]. Nonasthmatic allergic subjects were not included in this study. We have previously demonstrated that generalized airway inflammation is

present in nonasthmatic allergic rhinitis patients after segmental bronchial provocation (SBP) and after nasal provocation (NP)[17,18].

The present study is aimed at analyzing both upper and lower airway mucosal inflammation in atopic patients with persistent perennial nasal and/or bronchial symptomatology. Multiple regression analysis was used to analyze the relationship between clinical manifestations of allergic disease and numbers of inflammatory cells in nasal and mucosal biopsy specimens. Eosinophils, mast cells, basophils, dendritic cells and macrophages were chosen as markers of mucosal allergic inflammation.

## 7.3 Materials and methods

### *7.3.1 Subject groups and study design*

Inclusion of subjects started in June 1998 and was completed in February 2000. Patients were selected on the basis of persistent perennial nasal and/or bronchial symptomatology. Five experimental groups were formed: allergic rhinitis and asthma ($A^+R^+$; n = 19); allergic rhinitis, no asthma ($A^-R^+$; n = 18); allergic asthma, no rhinitis ($A^+R^-$; n = 3); atopic subjects, no asthma, no rhinitis ($A^-R^-$; n = 8) and nonallergic healthy control subjects (HC; n = 16). Diagnosis of asthma was made according to ATS criteria[19]. Diagnosis of allergic rhinitis was based on a positive skin-prick test for house dust mite and a history of perennial rhinitis symptoms for at least two years[20]. Allergy was assessed by skin-prick test. All subjects were tested for a panel of 14 common inhalant allergens (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands). A skin-prick test was considered positive when a wheal diameter > 3 mm was recorded after 15 minutes. Other allergens, such as dander or pollen, were allowed. Controls had a negative skin prick test. The atopy score[21] was determined as follows: 0 = no different from saline control, + = 1 - 2 mm larger than control, ++ = 3 - 5 mm larger than control, +++ = 6 - 8 mm larger than control, ++++ = > 8 mm larger than control. For each subject, a composite score was obtained.

All subjects were non-smokers. Treatment with short-acting bronchodilators was allowed up to 8 hours and long-acting bronchodilators and antihistamines up to 48 hours prior to investigation. Treatment with sodium cromoglycate, astemizole, inhaled or intranasal corticosteroids was stopped at least 6 weeks before entering the study.

None of the study subjects had used oral or systemic corticosteroids 6 months prior to the study.

All patients and control subjects were in stable clinical condition, according to the GINA guidelines[22]: no subject had experienced an infection of the respiratory tract, an acute asthmatic attack or hospital admission 6 weeks prior to investigation. All participants gave informed written consent to the study, which was approved by the medical ethics committee of the Erasmus university Medical Center Rotterdam, the Netherlands.

### *7.3.2 Clinical parameters*

Signs and symptoms were scored at the beginning of the visit on a 10-cm visual analogue scale (VAS). Symptoms were divided into nasal and ocular complaints (rhinorrhea, watery eyes, nasal itching, sneezing, and nasal blockage) and pulmonary complaints (wheezing, coughing, shortness of breath and exercise intolerance).

Upper and lower airways obstruction was determined with peak nasal inspiratory flow (PNIF) and forced expiratory volume in 1 s ($FEV_1$) measurements. PNIF was measured by a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL). $FEV_1$ was determined by standard spirometry. All study subjects had a $FEV_1 > 60$ % of predicted. The provocative concentration of methacholine causing a 20 % decrease in $FEV_1$ ($PC_{20}$ methacholine) was ≤ 8 mg/ml in asthmatic patients. All nonasthmatic patients had values > 8 mg/ml. Methacholine was administered according to a standardized tidal breathing method[23].

### *7.3.3 Blood samples*

Blood sampling and collection of bronchial and nasal biopsy specimens were performed on the same day. The total number of blood eosinophils was determined by hemocytometric differential cell count (Sysmex NE 8000).

### *7.3.4 Bronchial biopsies*

All bronchial biopsies were taken by the same pulmonary physician (S.E.O.). After intramuscular premedication with atropine dinitrate (0.5 mg), oropharyngeal

anaesthesia was accomplished with topical xylocaine spray 1%. Next, the vocal cords, trachea and bronchial tree were anaesthetized with oxybuprocaine. The fiber-optic bronchoscope (BF, type P20 D; Olympus Tokyo, Japan) was introduced into the airway via the oral route and mucosal biopsies were taken from the segmental carinae of the left and right lung. Bronchial biopsies were embedded in Tissue-Tek II Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA), frozen and stored at –150 $^0$C.

### *7.3.5 Nasal biopsies*

All biopsy specimens of the nasal mucosa were taken by the same investigator (G.J.B.). First, local anesthesia was induced by placing a cotton-wool carrier with 50 to 100 mg of cocaine and 3 drops of epinephrine (1:1,000) under the inferior turbinate, without touching the biopsy site. Secondly, a mucosal biopsy sample was obtained from the lower edge of the inferior turbinate about 2 cm posterior to the edge, using a Gerritsma forceps with a cup diameter of 2.5 mm[24]. The nasal biopsies were embedded in Tissue-Tek II OCT compound, frozen and stored at – 150 $^0$C.

### *7.3.6 Monoclonal Antibodies*

The monoclonal antibodies (mAbs) used in this study were anti-major basic protein (MBP) antibody ($IgG_1$, 0.2 μg/ml, clone nr. BMK13, Sanbio, Uden, the Netherlands) for identifying eosinophils, anti-basophil antibody ($IgG_{2a}$, <1 μg/ml, clone nr. BB1, gift of A.F. Walls, Immunopharmacology Group, Southampton General Hospital, Southampton, U.K.), anti-CD1a antibody ($IgG_1$, 4 μg/ml, clone nr. OKT6) for dendritic cells, anti-CD68 antibody ($IgG_1$, 3 μg/ml, clone nr. EBM11, Dako) for macrophages, anti-CD117 antibody ($IgG_1$, 1 μg/ml, clone nr. YB5.B8, Pharmingen) for mast cells and anti-IgE antibody ($IgG_1$, 10 μg/ml, clone nr. MH25, CLB, the Netherlands).

### *7.3.7 Immunohistochemical staining*

Each tissue specimen was cut into serial 6 $\mu$m thick sections. The mAb stainings were developed with the supersensitive immuno-alkaline phosphatase (ss-AP) method as

previously described[17]. Isotype-specific control staining was done with Mouse $IgG_1$ antibody to *Aspergillus niger* glucose oxidase (Dako cat. nr. X 0931) at the same protein concentration as the specific antibody.

### *7.3.8 Microscopic assessment of immunohistochemical staining*

Biopsies were coded and two sections, at least 120 μm apart, were counted blind for each antibody as has been described previously[17]. Bronchial and nasal sections were divided into epithelium and subepithelium (area 100 μm deep into the lamina propria along the length of the epithelial basement membrane). Positively stained inflammatory cells localized in epithelium and subepithelium were counted along the basement membrane, which had to be undamaged for a length of at least 1 mm before being accepted for evaluation. Cell numbers in epithelium were expressed as positively stained cells per mm basement membrane (BM); subepithelial cell numbers were expressed as the number of cells per $mm^2$, using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of x 200.

### *7.3.9 Statistical analysis*

Data were analyzed using multiple linear regression analysis with stepwise selection of the independent variables. When outcome variables were positively skewed, they were ln-transformed prior to the analysis. The independent variables were presence of atopy (Y/N), asthma (Y/N) and rhinitis (Y/N). As the variables asthma and rhinitis were not mutually exclusive, also their interaction was taken into account. All three explanatory variables being equal to “N” defined the healthy control group. If the interaction between asthma and rhinitis appeared not to be significant at the 5% level, it was eliminated from the model. For each outcome variable a regression model was estimated. The outcome variables were the absolute numbers of each inflammatory cell in the blood, nasal or bronchial mucosa. As there were many outcome variables dealt with, models were considered significant if their overall p-value was less than 0.02. Correlations were evaluated with Spearman's rank correlation test. Data are presented as medians ± range or mean ± SEM.

## 7.4 Results

### *7.4.1 Subject characteristics*

The subject groups were comparable for sex, age, vital capacity (VC) and $FEV_1$ (Table 1). By definition, asthmatic patients had a significantly decreased $PC_{20}$ (methacholine), lower $FEV_1$/VC ratio and more pulmonary symptoms compared to nonasthmatic subjects. Also, rhinitis patients had more nasal symptoms than non-rhinitic subjects. No difference in PNIF and total nasal symptom score was detectable between patients with and without asthma.

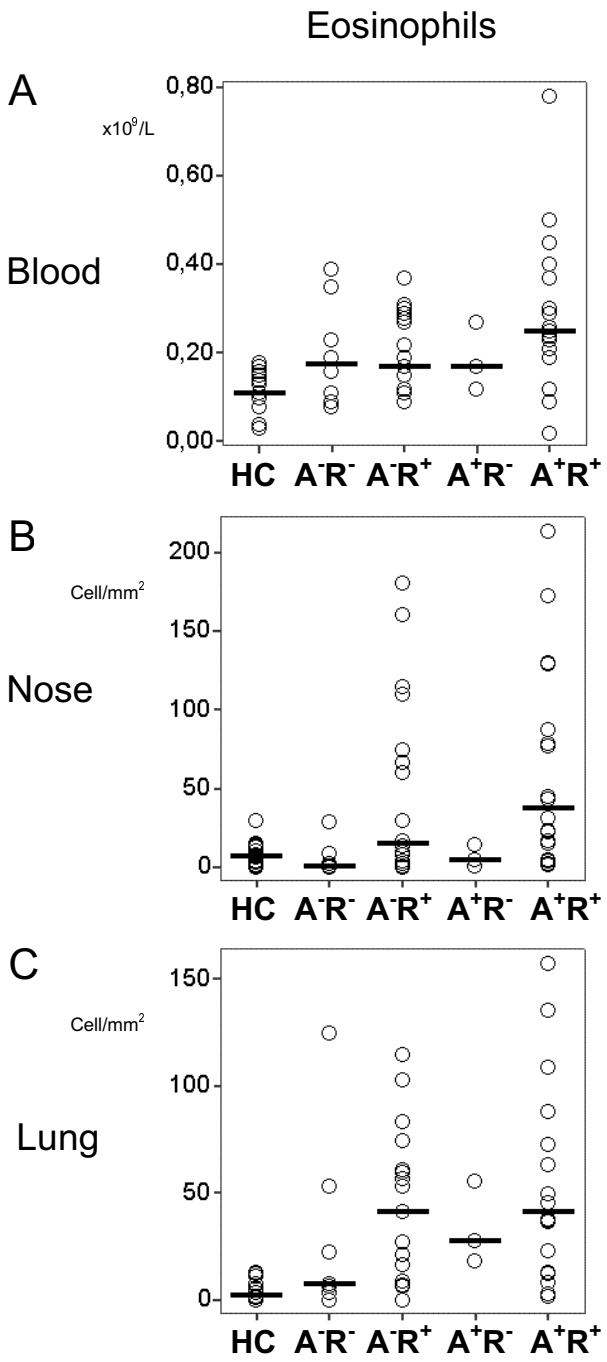


**Figure 1:**
Numbers of eosinophils in three compartments.
**A**: peripheral blood,
**B**: subepithelium nose and
**C**: subepithelium bronchus.

*Abbreviations used:*
HC = healthy control subjects;
$A^-R^-$ = atopic subjects, no asthma, no rhinitis;
$A^-R^+$ = allergic rhinitis, no asthma;
$A^+R^-$ = allergic asthma, no rhinitis;
$A^+R^+$ = allergic asthma and rhinitis.

Horizontal bars represent medians and open circles represent individual values.

By definition, HC subjects had an atopy score of 0. Patients with asthma and rhinitis had a higher atopy score than patients with rhinitis only. A positive family history for allergy was found more frequently in rhinitis patients with and without asthma than in control subjects.

**TABEL 1 PATIENT CHARACTERISTICS**

| | HC | $A^-R^-$ | $A^-R^+$ | $A^+R^-$ | $A^+R^+$ |
|---|---|---|---|---|---|
| **Number** | 16 | 8 | 18 | 3 | 19 |
| **Sex male** | 8 | 3 | 9 | 2 | 6 |
| **female** | 8 | 5 | 9 | 1 | 13 |
| **Age (yr)** | 23.5 (18 - 46) | 23.5 (23 - 31) | 29 (18 - 59) | 24 (21 - 25) | 27 (19 - 57) |
| **Atopy score (N+)** | 0 | 3 (2 - 9) | 8 (3 - 20) | 5 (4 - 10) | 14 (7 - 27) |
| **Positive family history (%)** | 37.5 | 37.5 | 81 | 100 | 79 |
| **$PC_{20}$ (mg/ml)** | 40 (32.4 - 40) | 40 (16.9 - 40) | 40 (9.3 - 40) | 0.4 (0.1 - 4.7) | 1.9 (0.2 - 6.1) |
| **VC(%)** | 104 (73 - 123) | 100 (85 - 122) | 100 (86 - 120) | 104 (98 - 112) | 108 (88 - 126) |
| **$FEV_1$(%)** | 106 (75 - 118) | 106 (83 - 122) | 103 (78 - 129) | 93 (87 - 102) | 101 (62 - 128) |
| **$FEV_1$/VC (%)** | 101 (89 - 114) | 102 (94 - 110) | 102 (92 - 118) | 91 (75 - 100) | 94 (70 - 104) |
| **PNIF (L/min)** | 232 (100 - 320) | 187 (155 - 230) | 180 (120 - 290) | 205 (200 - 350) | 180 (80 - 250) |
| **VAS nose (mm)** | 9 (0 - 38) | 19 (0 - 46) | 53 (5 - 229) | 20 (3 - 59) | 74 (10 - 371) |
| **VAS lung (mm)** | 0 (0 - 31) | 9 (0 - 41) | 8 (0 - 46) | 17 (4 - 206) | 33 (0 - 259) |

*Abbreviations used:* HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. $PC_{20}$ = The provocative concentration of methacholine causing a 20 % decrease in $FEV_1$. VC = vital capacity. $FEV_1$ = forced expiratory volume in 1 s. PNIF = peak nasal inspiratory flow. VAS = visual analogue scale. Data are expressed as medians and range.

### *7.4.2 Blood cells*

Figure 1A shows the results of the hemocytometric measurement of peripheral blood eosinophils. The number of eosinophils in the blood was related to the presence of atopy ($\beta = 0.33$, $p = 0.03$). The number of blood eosinophils was higher in patients with asthma and rhinitis than in patients with rhinitis only, although asthma and rhinitis were no explanatory variables of influence ($p > 0.05$).

### *7.4.3 Immunostaining of mucosal biopsies*

*General description.* We collected one nasal and several bronchial mucosa biopsy specimens per patient. Hundred and twenty-one specimens reached criteria for evaluation. The mean length of evaluable basement membrane was 5.3 mm ± 0.20 (SEM) in bronchial mucosa and 6.6 mm ± 0.30 in nasal mucosa. Epithelium and/or subepithelium could not be evaluated in six bronchial and one nasal sample. These samples were excluded from the study.

Since the $A^-R^-$ (n = 8) and $A^+R^-$ (n = 3) group were to small in number to be statistically analyzed as separate groups, we studied the relationship between the clinical phenotypes (atopy, asthma and rhinitis) and inflammation parameters.

**TABEL 2 Inflammatory cells nose**

| Cell type | compartment | HC (n=16) | $A^-R^-$ (n=8) | $A^-R^+$(n=18) | $A^+R^-$(n=3) | $A^+R^+$(n=19) |
|---|---|---|---|---|---|---|
| $MBP^+$ | epithelium | 0 (0 - 1) | 0 (0 - 1) | 0 (0 - 12) | 0 | 0 (0 - 11) |
| | subepithelium | 7 (0 - 30) | 1 (0 - 29) | 16 (0 - 181) | 5 (1 - 15) | 38 (2 - 214) |
| $BB1^+$ | epithelium | 0 | 0 | 0 (0 - 2) | 0 | 0 (0 - 5) |
| | subepithelium | 3 (0 - 33) | 4 (0 - 5) | 0 (0 - 259) | 4 (0 - 6) | 3 (0 - 80) |
| $CD117^+$ | epithelium | 1 (0 - 3) | 1 (0 - 1) | 0 (0 - 8) | 1 (0 - 4) | 2 (0 - 20) |
| | subepithelium | 103 (43 - 188) | 96 (55 - 162) | 78 (0 - 167) | 104 (74 - 106) | 108 (11 - 193) |
| $CD1a^+$ | epithelium | 4 (0 - 10) | 6 (1 - 10) | 8 (1 - 20) | 8 (4 - 15) | 8 (1 - 34) |
| | subepithelium | 16 (1 - 64) | 12 (2 - 48) | 45 (0 - 88) | 18 (15 - 55) | 36 (8 - 200) |
| $CD68^+$ | epithelium | 13 (3 - 30) | 13 (5 - 18) | 10 (2 - 25) | 11 (2 -21) | 10 (1 -40) |
| | subepithelium | 285 (90 - 518) | 242 (169 - 463) | 198 (13 - 665) | 144 (70 - 203) | 202 (30 - 385) |
| $IgE^+$ | epithelium | 0 (0 - 14) | 7 (0 - 15) | 7 (0 - 24) | 8 (6 - 11) | 8 (2 - 25) |
| | subepithelium | 47 (0 - 192) | 108 (26 - 212) | 120 (23 - 300) | 206 (42 - 216) | 160 (14 - 340) |

*Abbreviations used*: HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. Cell numbers were expressed as positively stained cells per mm basement membrane; subepithelial cell numbers were expressed as the number of cells per $mm^2$ subepithelium (layer 100 μm deep under the basement membrane). Data are expressed as medians and range.

*Nasal specimens*. In both layers of the nasal mucosa (Table 2 and 4), we found increased numbers of $IgE^+$ cells in atopic patients compared to the control group (p = 0.001).

In nasal epithelium, there were more $CD117^+$ cells present in asthmatic patients than in HC subjects ($p = 0.004$).

In subepithelium, we found significantly more $MBP^+$ (Figure 1B and 2A) cells in rhinitis patients compared to HC subjects ($p = 0.002$). No difference could be detected between asthma and rhinitis patients for all investigated cell types. The numbers of $BB1^+$ and $CD68^+$ cells were not different in the investigated groups.

**TABEL 3 Inflammatory cells lung**

| Cell type | compartment | HC (n=16) | $A^-R^-$ (n=8) | $A^-R^+$(n=18) | $A^+R^-$(n=3) | $A^+R^+$(n=19) |
|---|---|---|---|---|---|---|
| $MBP^+$ | epithelium | 0 (0 - 1) | 0 (0 - 1) | 1 (0 - 5) | 1 (0 - 1) | 1 (0 - 5) |
| | subepithelium | 3 (0 - 13) | 8 (0 - 25) | 41 (0 - 115) | 28 (19 - 56) | 42 (2 - 158) |
| $BB1^+$ | epithelium | 0 | 0 | 0 (0 - 2) | 0 (0 - 1) | 0 (0 - 2) |
| | subepithelium | 1 (0 - 10) | 0 (0 - 11) | 1 (0 - 16) | 1 (0 - 7) | 2 (0 - 14) |
| $CD117^+$ | epithelium | 0 | 0 (0 - 1) | 0 (0 - 4) | 1 (0 - 1) | 0 (0 -6) |
| | subepithelium | 32 (5 - 110) | 20 (8 - 125) | 58 (0 - 143) | 40 (10 - 79) | 45 (0 - 195) |
| $CD1a^+$ | epithelium | 0 (0 - 2) | 0 (0 - 3) | 1 (0 - 7) | 1 (0 - 5) | 1 (0 - 6) |
| | subepithelium | 5 (0 - 17) | 7 (0 - 23) | 20 (0 - 123) | 15 (0 -50) | 19 (0 - 98) |
| $CD68^+$ | epithelium | 2 (1 - 8) | 2 (0 - 6) | 3 (0 - 14) | 3 (0 - 4) | 2 (0 - 5) |
| | subepithelium | 131 (64 - 261) | 95 (47 - 415) | 148 (9 - 396) | 102 (44 - 107) | 107 (30 - 372) |
| $IgE^+$ | epithelium | 0 | 0 (0 - 2) | 1 (0 - 7) | 2 (0 - 8) | 3 (0 -9) |
| | subepithelium | 8 (0 - 83) | 40 (10 - 96) | 82 (20 - 180) | 67 (10 - 126) | 95 (15 - 226) |

*Abbreviations used:* HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. Cell numbers were expressed as positively stained cells per mm basement membrane; subepithelial cell numbers were expressed as the number of cells per $mm^2$ subepithelium (layer 100 μm deep under the basement membrane). Data are expressed as medians and range.

*Bronchial specimens.* In both layers of the bronchial mucosa (Table 3 and 5), higher numbers of $IgE^+$ cells were detected in all atopic subgroups compared to the control group ($p < 0.001$).

In bronchial epithelium, numbers of $MBP^+$ cells were significantly higher in asthmatic as well as in rhinitis patients compared to HC subjects ($p = 0.01$; $p = 0.02$, respectively). Moreover, there were more $CD1a^+$ cells present in rhinitis patients than

in HC subjects (p = 0.03). No difference was found between asthma and rhinitis with regard to inflammatory cells in the bronchial mucosa. In atopic subjects, few $CD117^+$ cells were found in the epithelium, while no $CD117^+$ cells could be detected in the epithelium of HC subjects.

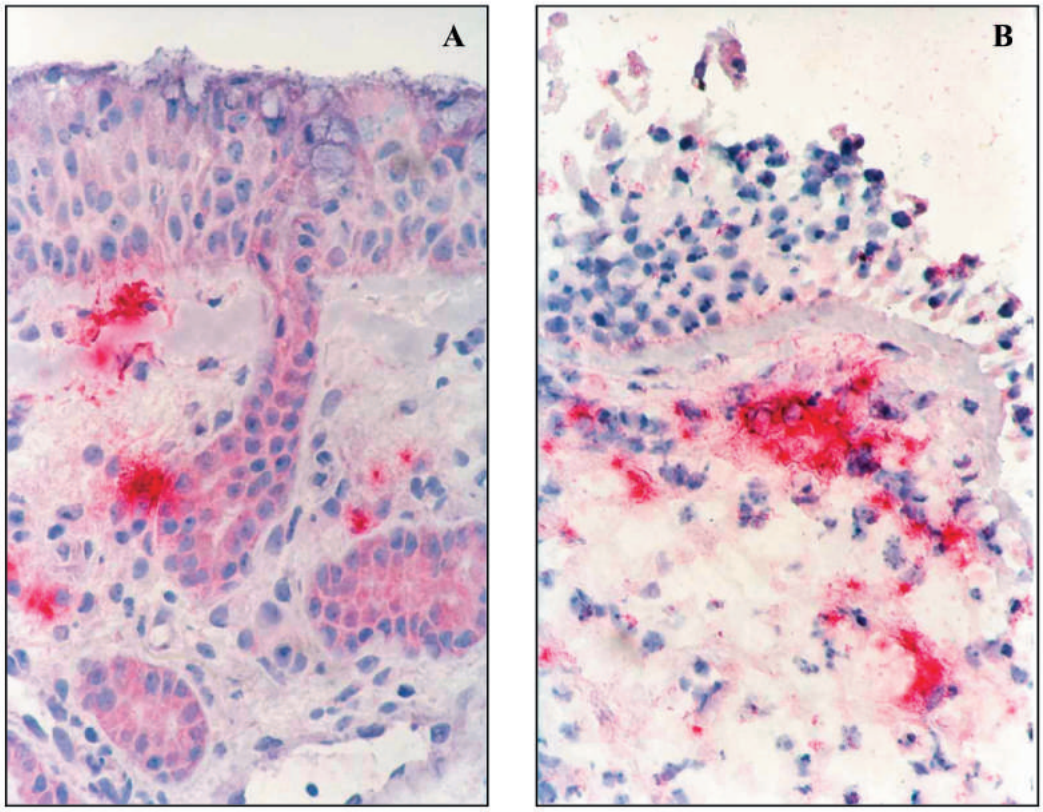


**Figure 2.** Immunohistochemical staining of eosinophils (Major Basic Protein (MBP). Nasal mucosa (**A**) and bronchial mucosa (**B**) of allergic rhinitis patient without asthma. Original magnification 400 x.

In subepithelium, we found a significantly higher number of $MBP^+$ cells (Figure 1C and 2B) in atopic patients compared to HC subjects (p = 0.006). The number of $CD1a^+$ cells was elevated in rhinitis patients compared to HC subjects (p = 0.04).

In the bronchial mucosa, inflammatory cell numbers were similar in the symptomatic atopic patient groups. No differences could be found between the investigated groups for the number of $BB1^+$ and $CD68^+$ cells.

**TABEL 4 Results of multiple regression analysis in the nose**

| | | partial correlation coefficients | | | | |
|---|---|---|---|---|---|---|
| Cell type | compartment | ATOPY | ASTHMA | RHINITIS | $r^2$ | p-value† |
| $MBP^+$ | epithelium | 0.17 | -0.02 | 0.36 | 0.14 | ns |
| | subepithelium | -0.21 | 0.21 | 0.53 | 0.23 | <0.001 |
| $BB1^+$ | epithelium | -0.19 | 0.12 | 0.24 | 0.05 | ns |
| | subepithelium | -0.14 | 0.13 | 0.09 | 0.22 | ns |
| $CD1a^+$ | epithelium | 0.16 | 0.17 | 0.15 | 0.15 | 0.02 |
| | subepithelium | -0.09 | 0.27 | 0.24 | 0.15 | 0.02 |
| $CD68^+$ | epithelium | -0.11 | -0.02 | -0.08 | 0.04 | ns |
| | subepithelium | -0.16 | -0.14 | -0.05 | 0.09 | ns |
| $CD117^+$ | epithelium | -0.08 | 0.41 | 0.04 | 0.16 | 0.02 |
| | subepithelium | -0.04 | 0.21 | -0.2 | 0.06 | ns |
| $IgE^+$ | epithelium | 0.49 | 0.2 | 0.07 | 0.44 | <0.001 |
| | subepithelium | 0.53 | 0.09 | 0.01 | 0.34 | <0.001 |

**TABEL 5 Results of multiple regression analysis in the bronchus**

| | | partial correlation coefficients | | | | |
|---|---|---|---|---|---|---|
| Cell type | compartment | ATOPY | ASTHMA | RHINITIS | $r^2$ | p-value† |
| $MBP^+$ | epithelium | 0.006 | 0.73 | 0.47 | 0.29 | 0.001 |
| | subepithelium | 0.42 | 0.11 | 0.19 | 0.38 | <0.001 |
| $BB1^+$ | epithelium | 0.05 | 0.16 | 0.08 | 0.05 | ns |
| | subepithelium | 0.03 | 0.2 | 0.11 | 0.08 | ns |
| $CD1a^+$ | epithelium | 0.05 | 0.04 | 0.36 | 0.17 | 0.02 |
| | subepithelium | -0.002 | 0.07 | 0.36 | 0.16 | ns |
| $CD68^+$ | epithelium | 0.02 | -0.2 | 0.1 | 0.03 | ns |
| | subepithelium | -0.13 | -0.15 | 0.04 | 0.05 | ns |
| $CD117^+$ | epithelium | 0.4 | -0.09 | 0.006 | 0.14 | ns |
| | subepithelium | -0.07 | 0.06 | 0.08 | 0.07 | ns |
| $IgE^+$ | epithelium | 0.43 | 0.16 | 0.22 | 0.48 | <0.001 |
| | subepithelium | 0.56 | 0.04 | 0.19 | 0.51 | <0.001 |

Independent variables: Atopy, asthma, rhinitis

Dependent variables: inflammatory cell numbers in epithelium and subepithelium

† = Analysis of variance (ANOVA)

*Correlations between inflammatory markers, airway function and symptomatology*

In methacholine responsive subjects ($PC_{20}$ < 38 mg/ml; n = 34), $PC_{20}$ correlated with atopy score (Figure 3A; r = -0.40, p = 0.018). The number of eosinophils in peripheral blood of all allergic subjects (n = 48) correlated with the $FEV_1$/VC ratio (Figure 3B; r = -0.40, p = 0.005) and total nasal symptom score (r = 0.38, p = 0.008). In allergic subjects, the atopy score correlated with $MBP^+$ cells in nasal (r = 0.46, p = 0.001) and bronchial subepithelium (r = 0.41, p = 0.005). In the subepithelial layer, nasal inflammatory cell numbers significantly correlated with bronchial cell numbers in atopic patients ($MBP^+$: r = 0.33, p = 0.03; $BB1^+$: r = 0.37, p = 0.01; $CD1a^+$: r = 0.42, p = 0.004 and $CD68^+$: r = 0.65, p < 0.001). In HC subjects, no such correlations were found.

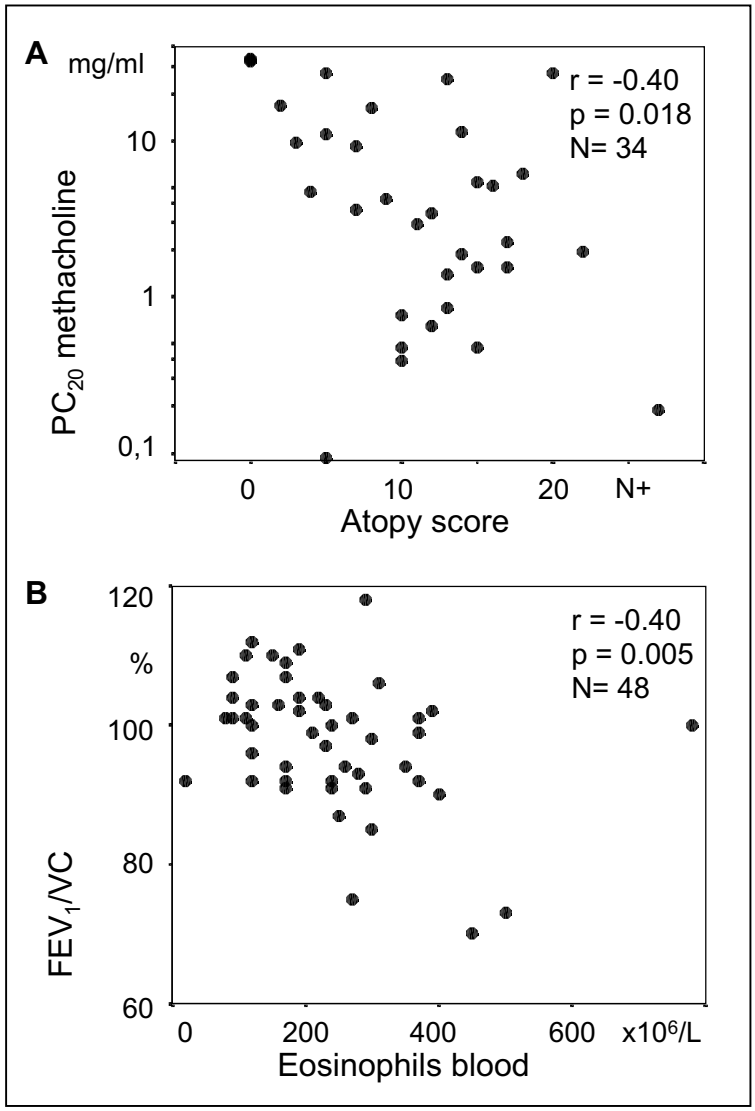


**Figure 3.**

**A**. Correlation between $PC_{20}$ methacholine and atopy score in methacholine responsive subjects ($PC_{20}$ < 38 mg/ml, N = 34).

**B**. Correlation between $FEV_1$/FVC and number of blood eosinophils in atopic patients (N = 48). Correlations were evaluated with a Spearman's Rank test.

## 7.5 Discussion

We were able to collect nasal and bronchial biopsy specimens from subjects representing the total range of the atopic spectrum. Asthmatic and nonasthmatic subjects were discriminated on the basis of frequent pulmonary symptoms and presence of BHR. This study is unique, in that inflammatory and clinical findings in upper and lower airways are investigated simultaneously in these patient groups. Mucosal inflammation was determined by quantification of several inflammatory cell types, which are known to play an effector role in the subsequent stages of the allergic response[25-27]. A possible relationship between inflammatory cell numbers and clinical phenotype was examined by using multiple regression analysis.

An interesting observation in our study was that we could enroll only 3 patients (out of a pool of 98 potential subjects) with asthma and without (clinical) rhinitis, suggesting that rhinitis is ubiquitous in allergic asthma. Extended airway disease – i.e. upper and lower airways involvement - was associated with a higher atopy score and increased numbers of blood eosinophils, but not with mucosal inflammation in general. Mucosal eosinophilia is the factor most related to the clinical expression of allergic airways disease. However, it did not discriminate between rhinitis and asthma in both upper and lower airways. In fact, the number of subepithelial eosinophils in the bronchial mucosa was dependent on atopy and not on asthma. Therefore, factors other than the local inflammatory cell infiltrate are likely to be more important in the clinical expression of allergic airway disease[28,29].

In the nose, most studies have been performed in seasonal allergic rhinitis patients. Higher numbers of eosinophils, basophils, mast cells and dendritic cells were present in the nasal subepithelium after NP[7,30] and natural exposure[31]. However, changes in eosinophils and mast cells were related to nasal symptoms in the acute stage of allergic inflammation only[27,32]. Few studies have investigated the allergic response in perennial allergic rhinitis patients. They show that mucosal eosinophilia is less marked in perennial than in seasonal disease and usually not related to symptom expression[28].

In our study, we found a good correlation between inflammatory cell numbers in nasal and those in bronchial mucosa, which suggests that these cells are recruited into the tissue via a common or at least similar mechanism. These results are in accordance

with our previous work, where we demonstrated that local allergen challenge in either upper or lower airways induces an inflammatory response in the entire airways through a systemic mechanism involving bone-marrow and circulation[17,18].

BHR and chronic inflammation are considered important features of allergic asthma. Several studies have pointed towards the relationship between bronchial inflammation and BHR[14,25]. In these studies, only hyperresponsive subjects were included in the analysis. In our study, we could not find an association between the presence of inflammatory cells in bronchial mucosa and airway responsiveness in atopic (n = 48), in responsive ($PC_{20}$ methacholine < 38 mg/ml; n = 34) or in hyperresponsive subjects ($PC_{20}$ methacholine ≤ 8 mg/ml; n = 22). Also, the fact that increased bronchial inflammation was found in nonasthmatic atopic patients, even in the absence of airway symptoms, challenges the direct relationship between the presence of inflammatory cells, in particular eosinophils and airway responsiveness. The results of our study are supported by several other investigators, who also failed to detect a relationship between BHR and bronchial inflammation in allergic rhinitis[33] and asthma patients[33-37]. Crimi *et al.* analyzed sputum, BAL and bronchial biopsy specimens from 71 patients with chronic asthma. No correlation could be found between $PC_{20}$ methacholine and bronchial inflammation as assessed by each of the three study methods. Although some authors reported that treatment with inhaled steroids resulted in a decrease in BHR accompanied by a reduction in bronchial eosinophils and other inflammatory cells[8,36,38], others demonstrated a relative dissociation[39].

Djukanovic *et al.* demonstrated inflammatory changes, similar to our study, in bronchial mucosa of both atopic asthmatics and atopic nonasthmatics compared to control subjects. He found a gradual increase in the number of eosinophils going from controls to atopic nonasthmatics and atopic asthmatics. This increase was related to the clinical expression of asthma. In the present study, however, no significant differences were found in the quantity of the bronchial inflammatory cell infiltrate between allergic rhinitis patients with and without asthma. We speculate that this aspect of the inflammatory infiltrate in the lower airways of these perennial allergic patients reflects chronic rather than acute inflammation. This distinction is relevant since the quality of

the inflammatory infiltrate may discriminate the chronic from the acute phase of disease. This aspect of the inflammatory infiltrate encompasses mediators such as degranulation products, cytokines, chemokines, and growth factors. We speculate that inflammatory cells are in an "active" mode during acute inflammation and become "quiescent" during chronic, stable phase of disease. Allergen provocation studies in nose and lung support this hypothesis. These studies demonstrated persistent inflammation up to 2 weeks after allergen provocation long after symptoms had disappeared[30,40,41]. The presence of inflammatory cells *per se* in mucosal tissue may, therefore, only be an epiphenomenon of atopy.

In contrast to our histological findings in the bronchial mucosa, we found a significant correlation between blood eosinophils and bronchial obstruction ($FEV_1$/VC ratio) in atopic patients. In addition, skin-test reactivity (atopy score) was significantly related to $PC_{20}$ methacholine, indicating that allergy is a systemic disease with increased clinical expression in "severe" atopic subjects. Our findings are in line with those of Gergen *et al*.[42] who showed that the prevalence of asthma and rhinitis is positively correlated with the number of positive skin-prick test reactions to inhalant allergens. Moreover, Kerkhof *et al*. found that the presence of BHR or a high blood eosinophil count increased the risk of asthma in atopic subjects[43].

We propose that acute inflammation is related to nasal and/or pulmonary symptoms and influences airway caliber through the release of a variety of mediators. The relationship between chronic inflammation and clinical disease is, however, less straightforward. Our results suggest that, in addition to systemic factors, such as atopy and blood eosinophils, other factors, such as local repair mechanisms, tissue remodeling and the presence of signaling mediators, are relevant to the clinical expression in allergic airway disease.

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# 8

# Upper and lower airway remodeling aspects in allergic rhinitis and asthma

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*Submitted*

## 8.1 Summary

Airway remodeling is a hallmark of allergic asthma. Little is known about remodeling in atopic patients without asthma. Also, the role of remodeling in the upper airways is still unclear. Animal studies indicate that inflammatory mediators, such as interleukin (IL)-4 and IL-13, are likely to contribute to remodeling in allergic disease. We analyzed aspects of nasal and bronchial airway remodeling in atopic patients with persistent perennial nasal and/or bronchial symptomatology. Five experimental groups were formed: allergic asthma and rhinitis ($A^+R^+$; n = 19); allergic rhinitis, no asthma ($A^-R^+$; n = 18); allergic asthma, no rhinitis ($A^+R^-$; n = 3); atopic subjects, no asthma, no rhinitis ($A^-R^-$; n = 8) and nonallergic healthy control subjects (HC; n = 16). Nasal and bronchial biopsy specimens were collected in one session during stable disease. Immunohistochemistry was performed for the pro-inflammatory cytokines IL-4, IL-13 and vascular endothelium (CD31). The degree of epithelial loss, reticular basement membrane (RBM) thickness and subepithelial vascularity were assessed with a computer-assisted image analysis system. The relationships between clinical expression (atopy, rhinitis and/or asthma) and parameters of airway remodeling were assessed by multiple regression analysis.

In bronchial mucosa, thickness of the RBM was related to the presence of asthma ($p = 0.02$). Also, epithelial shedding and subepithelial vascularity were increased in allergic rhinitis patients with and without asthma compared to HC subjects. However, this was not related to asthma, rhinitis or atopy. No difference could be detected between asthma and rhinitis with regard to lower airways remodeling. In nasal mucosa, no difference was found in airway remodeling between patients and controls. The numbers of $IL\text{-}4^+$ and $IL\text{-}13^+$ cells were similar among the investigated groups in both nasal and bronchial mucosa. No significant correlations were found between airway remodeling and severity of symptoms or airway obstruction.

In conclusion, lower airways remodeling is related to the presence of the asthma phenotype. However, no significant difference could be found between asthmatic and rhinitis patients. Airway remodeling was not observed in the upper airways in our patients. Also, no evidence was found for the involvement of IL-4 and IL-13 in the ongoing process of tissue remodeling.

## 8.2 Introduction

Allergic rhinitis and asthma are two entities of the atopic syndrome that commonly occur together[1]. The discrimination between allergic rhinitis and asthma can be difficult, since symptom perception is widely variable; lung function can be normal in mild asthmatics and, although BHR is a constant feature of asthma, it is also frequently present in allergic rhinitis[2].

Chronic airway inflammation has been considered an important hallmark in both asthma and rhinitis. Lately, this association between cellular aspects of inflammation and clinical expression of allergic airways disease has been subject of discussion. Inflammatory cells and mediators have been found in broncho-alveolar lavage (BAL) fluid, mucosal biopsies and induced sputum of nonasthmatic allergic rhinitis patients, as well[3-5]. Also, eosinophil infiltration has been demonstrated in the upper airways of asthmatic patients, even in the absence of rhinitis[6]. Therefore, factors other than the local inflammatory cell infiltrate are likely to be important in the clinical expression of allergic airway disease.

Airway remodeling is mentioned as one of the factors accounting for airflow obstruction and bronchial hyperresponsiveness in allergic asthma. Structural changes in the bronchial wall, typical for asthma, are loss of epithelial lining[7,8], reticular basement membrane thickening[9,10] and increased vascularity in the subepithelial layer[11,12]. The T helper (Th) 2-derived cytokines IL-4, IL-5 and IL-13 are thought to play an important role in the initiation of the airway remodeling process.

Little is known about airway remodeling in atopic patients without asthma. Moreover, only few studies have investigated structural changes in the upper airways of atopic patients. Although there is no fundamental difference between the structure of the ciliated epithelium in upper and lower airways, epithelial fragility and increased basement membrane thickening are found in bronchial mucosa of asthmatic patients[8], but not in nasal mucosa of allergic rhinitis patients[13]. The nasal lamina propria has a subepithelial network of capillaries, arterio-venous shunts and venous sinusoids, which can change in dimension and modulate upper airway resistance[14,15]. In bronchial

lamina propria, the degree of vascularization is less compared to the nose, but subepithelial vascularity may increase in asthmatic patients[11,12].

The present study is aimed at analyzing both upper and lower airway remodeling features in atopic patients with persistent perennial nasal and/or bronchial symptomatology. Multiple regression analysis was used to study the relationship between clinical manifestations of allergic disease and features of airway remodeling in nasal and mucosal biopsy specimens.

## 8.3 Materials and methods

### *8.3.1 Subject groups and study design*

Inclusion of subjects started in June 1998 and was completed in February 2000. Patients were selected on the basis of persistent perennial nasal and/or bronchial symptomatology. Five experimental groups were formed: allergic rhinitis and asthma ($A^+R^+$; n = 19); allergic rhinitis, no asthma ($A^-R^+$; n = 18); allergic asthma, no rhinitis ($A^+R^-$; n = 3); atopic subjects, no asthma, no rhinitis ($A^-R^-$; n = 8) and nonallergic healthy control subjects (HC; n = 16). Diagnosis of asthma was made according to ATS criteria[16]. Diagnosis of allergic rhinitis was based on a positive skin-prick test for house dust mite and a history of perennial rhinitis symptoms for at least two years[17]. Allergy was assessed by skin-prick test. All subjects were tested for a panel of 14 common inhalant allergens (Vivodiagnost, ALK Benelux BV, Groningen, the Netherlands). A skin-prick test was considered positive when a wheal diameter > 3 mm was recorded after 15 minutes. Other allergens, such as dander or pollen, were allowed. Controls had a negative skin prick test. The atopy score[18] was determined as follows: 0 = no different from saline control, + = 1 - 2 mm larger than control, ++ = 3 - 5 mm larger than control, +++ = 6 - 8 mm larger than control, ++++ = > 8 mm larger than control. For each subject, a composite score was obtained.

All subjects were non-smokers. Treatment with short-acting bronchodilators was allowed up to 8 hours and long-acting bronchodilators and antihistamines up to 48 hours prior to investigation. Treatment with sodium cromoglycate, astemizole, inhaled or intranasal corticosteroids was stopped at least 6 weeks before entering the study.

None of the study subjects had used oral or systemic corticosteroids 6 months prior to the study.

All patients and control subjects were in stable clinical condition, according to the GINA guidelines[19]: no subject had experienced an infection of the respiratory tract, an acute asthmatic attack or hospital admission 6 weeks prior to investigation. All participants gave informed written consent to the study, which was approved by the medical ethics committee of the Erasmus university Medical Center Rotterdam, the Netherlands.

**TABEL 1 PATIENT CHARACTERISTICS**

| | HC | $A^-R^-$ | $A^-R^+$ | $A^+R^-$ | $A^+R^+$ |
|---|---|---|---|---|---|
| **Number** | 16 | 8 | 18 | 3 | 19 |
| **Sex male** | 8 | 3 | 9 | 2 | 6 |
| **female** | 8 | 5 | 9 | 1 | 13 |
| **Age (yr)** | 23.5 (18 - 46) | 23.5 (23 - 31) | 29 (18 - 59) | 24 (21 - 25) | 27 (19 - 57) |
| **Atopy score (N+)** | 0 | 3 (2 - 9) | 8 (3 - 20) | 5 (4 - 10) | 14 (7 - 27) |
| **Positive family history (%)** | 37.5 | 37.5 | 81 | 100 | 79 |
| **$PC_{20}$ (mg/ml)** | 40 (32.4 - 40) | 40 (16.9 - 40) | 40 (9.3 - 40) | 0.4 (0.1 - 4.7) | 1.9 (0.2 - 6.1) |
| **VC(%)** | 104 (73 - 123) | 100 (85 - 122) | 100 (86 - 120) | 104 (98 - 112) | 108 (88 - 126) |
| **$FEV_1$(%)** | 106 (75 - 118) | 106 (83 - 122) | 103 (78 - 129) | 93 (87 - 102) | 101 (62 - 128) |
| **$FEV_1$/VC (%)** | 101 (89 - 114) | 102 (94 - 110) | 102 (92 - 118) | 91 (75 - 100) | 94 (70 - 104) |
| **PNIF (L/min)** | 232 (100 - 320) | 187 (155 - 230) | 180 (120 - 290) | 205 (200 - 350) | 180 (80 - 250) |
| **VAS nose (mm)** | 9 (0 - 38) | 19 (0 - 46) | 53 (5 - 229) | 20 (3 - 59) | 74 (10 - 371) |
| **VAS lung (mm)** | 0 (0 - 31) | 9 (0 - 41) | 8 (0 - 46) | 17 (4 - 206) | 33 (0 - 259) |

*Abbreviations used:* HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. $PC_{20}$ = The provocative concentration of methacholine causing a 20 % decrease in $FEV_1$. VC = vital capacity. $FEV_1$ = forced expiratory volume in 1 s. PNIF = peak nasal inspiratory flow. VAS = visual analogue scale. Data are expressed as medians and range.

### *8.3.2 Clinical parameters*

Signs and symptoms were scored at the beginning of the visit on a 10-cm visual analogue scale (VAS). Symptoms were divided into ocular and nasal complaints (rhinorrhea, nasal itching, sneezing, and nasal blockage) and pulmonary complaints (wheezing, coughing, shortness of breath and exercise intolerance).

Upper and lower airways obstruction were determined with peak nasal inspiratory flow (PNIF) and forced expiratory volume in 1 s ($FEV_1$) measurements, respectively. PNIF was measured by a Youlten peak nasal inspiratory flow meter (Armstrong Industries, Inc., Northbrook, IL). $FEV_1$ was determined by standard spirometry. All study subjects had a $FEV_1$ > 60 % of predicted. The provocative concentration of methacholine causing a 20 % decrease in $FEV_1$ ($PC_{20}$ methacholine) was ≤ 8 mg/ml in asthmatic patients. All nonasthmatic patients had values > 8 mg/ml. Methacholine was administered according to a standardized tidal breathing method[20].

### *8.3.3 Bronchial biopsies*

Collection of bronchial and nasal biopsy specimens were performed on the same day. All bronchial biopsies were taken by the same pulmonary physician (S.E.O.). After intramuscular premedication with atropine dinitrate (0.5 mg), oropharyngeal anesthesia was accomplished with topical xylocaine spray 1%. Next, the vocal cords, trachea and bronchial tree were anaesthetized with oxybuprocaine. The fiberoptic bronchoscope (BF, type P20 D; Olympus Tokyo, Japan) was introduced into the airway via the oral route and mucosal biopsies were taken from the segmental carinae of the left and right lung. Bronchial biopsies were embedded in Tissue-Tek II Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA), frozen and stored at –150 $^0$C.

### *8.3.4 Nasal biopsies*

All biopsy specimens of the nasal mucosa were taken by the same investigator (G.J.B.). First, local anesthesia was induced by placing a cotton-wool carrier with 50 to 100 mg of cocaine and 3 drops of epinephrine (1:1,000) under the inferior turbinate, without touching the biopsy site. Secondly, a mucosal biopsy sample was obtained from the lower edge of the inferior turbinate about 2 cm posterior to the edge, using a Gerritsma forceps with a cup diameter of 2.5 mm[21]. The nasal biopsies were embedded in Tissue-Tek II OCT compound, frozen and stored at – 150 $^0$C.

### *8.3.5 Monoclonal Antibodies*

The monoclonal antibodies (mAbs) used in this study were anti-human endothelium (CD31) antibody (IgG1, 1 μg/ml, clone nr. EN4, Sanbio, NL), anti-human IL-4 antibody (IgG1, 20 μg/ml, clone nr. 1-41-1, Novartis, Vienna, Austria), anti-human IL-13 antibody (IgG polyclonal, 0.4 μg/ml, Pepro Tech Inc. Rocky Hill, NJ)

### *8.3.6 Immunohistochemical staining*

Each tissue specimen was cut into serial 6 $\mu$m thick sections. The mAb stainings were developed with the supersensitive immuno-alkaline phosphatase (ss-AP) method as previously described[22]. Isotype-specific control staining was done with Mouse $IgG_1$ antibody to *Aspergillus niger* glucose oxidase (Dako cat. nr. X 0931) at the same protein concentration as the specific antibody.

### *8.3.7 Microscopic assessment of immunohistochemical staining*

Biopsies were coded and two sections, at least 120 μm apart, were counted blind for each antibody as has been described previously[22]. Bronchial and nasal sections were divided into epithelium and subepithelium (area 100 μm deep into the lamina propria along the length of the epithelial basement membrane). Positively stained inflammatory cells localized in epithelium and subepithelium were counted along the basement membrane, which had to be undamaged for a length of at least 1 mm before being accepted for evaluation. Cell numbers in epithelium were expressed as positively stained cells per mm basement membrane (BM); subepithelial cell numbers were expressed as the number of cells per $mm^2$, using an Axioskop 20 microscope (Zeiss, Jena, Germany) with an eyepiece graticule at a magnification of x 200.

### *8.3.8 Quantification of remodeling factors*

Nasal and bronchial sections were recorded using a computer assisted image analysis system (Leica, Rijswijk, the Netherlands). This method is based on densitometric differences between positively stained tissue and background after setting thresholds

for color, saturation and intensity[23]. If the total area studied was less than 100,000 $\mu m^2$ for a given subject, this subject was excluded from further analysis.

*Reticular basement membrane thickness.* Thickness of the reticular basement membrane was interactively measured on 400 x magnified images at regular intervals following recommendations described recently[24].

*Epithelial shedding.* The reticular basement membrane occupancy with epithelium, plotted as ratio of occupied membrane length divided by the total membrane length, was determined for the investigated groups at 400 x magnification .

*Subepithelial vascularity.* Vessels with a distinct lumen staining positive for CD31 were counted on 200 x magnified images in an area 100 μm deep in the lamina propria along the length of the epithelial basement membrane.

### *8.3.9 Statistical analysis*

Data were analyzed using multiple linear regression analysis with stepwise selection of the independent variables. When outcome variables were positively skewed, they were ln-transformed prior to the analysis. The independent variables were presence of atopy (Y/N), asthma (Y/N) and rhinitis (Y/N). As the variables asthma and rhinitis were not mutually exclusive, also their interaction was taken into account. All three explanatory variables being equal to "N" defined the healthy control group. If the interaction between asthma and rhinitis appeared not to be significant at the 5% level, it was eliminated from the model. For each outcome variable a regression model was estimated. The outcome variables were the absolute numbers of each inflammatory cell in the blood, nasal or bronchial mucosa. As there were many outcome variables dealt with, models were considered significant if their overall p-value was less than 0.02. Correlations were evaluated with Spearman's rank correlation test. Data are presented as medians ± range or mean ± SEM.

## 8.4 Results

### *8.4.1 Subject characteristics*

The subject groups were comparable for sex, age, vital capacity (VC) and $FEV_1$ (Table 1). The subject groups were comparable for sex, age, vital capacity (VC) and $FEV_1$

(Table 1). By definition, asthmatic patients had a significantly decreased $PC_{20}$ (methacholine), lower $FEV_1$/VC ratio and more pulmonary symptoms compared to nonasthmatic subjects. Also, rhinitis patients had more nasal symptoms than non-rhinitic subjects. No difference in PNIF and total nasal symptom score was detectable between patients with and without asthma.

By definition, HC subjects had an atopy score of 0. Patients with asthma and rhinitis had a higher atopy score than patients with rhinitis only. A positive family history for allergy was found more frequently in rhinitis patients with and without asthma than in control subjects.

### *8.4.2 mucosal biopsies*

*general description* We collected one nasal and several bronchial mucosa biopsy specimens per patient. Hundred and twenty-one specimens reached criteria for evaluation. The mean length of evaluable basement membrane was 5.3 mm ± 0.20 (SEM) in bronchial mucosa and 6.6 mm ± 0.30 in nasal mucosa. Epithelium and/or subepithelium could not be evaluated in six bronchial and one nasal sample. These samples were excluded from the study.

Since the $A^-R^-$ (n = 8) and $A^+R^-$ (n = 3) group were to small in number to be statistically analyzed as separate groups, we studied the relationship between the clinical phenotypes (atopy, asthma and rhinitis) and inflammation parameters.

*Nasal biopsy specimens*. The results of multiple regression analysis are summarized in Table 2. Three patients had epithelial loss of more than 50 % in the nasal mucosa (1 control, 2 rhinitis subjects), whereas in most subjects the epithelium was completely intact. Median values were between 84 and 94 % in the investigated groups (Figure 1a).

The reticular basement membrane thickness (RBM) in the nasal mucosa was similar in patient and control groups with a median value between 7.9 and 8.6 μm (Figure 2a).

The number of $CD31^+$ vessels in the nasal subepithelium tended to be higher in atopic patients (Figure 3a and 4a).

Also numbers of IL-$4^+$ and IL-$13^+$ cells were not different in the investigated groups in both nasal epithelium and subepithelium.

*Bronchial biopsy specimens*. The results of the bronchial biopsies are summarized in Table 2. In all five groups, even in HC subjects, some degree of epithelial damage was present in the lower airways (Figure 1b). A significant proportion of the variability of epithelial loss was explained by the multiple regression model including the presence of atopy or asthma or rhinitis ($r^2 = 0.16$, $p = 0.02$). No difference could be detected between the two phenotypes of allergic airways disease.

**TABEL 2 Results of multiple regression analysis**

| | | partial correlation coefficients | | | | |
|---|---|---|---|---|---|---|
| | | ATOPY | ASTHMA | RHINITIS | $r^2$ | p-value† |
| **nose** | **vascularity** | 0.26 | 0.18 | -0.15 | 0.09 | ns |
| | **thickness RBM** | 0.07 | 0.08 | 0.004 | 0.02 | ns |
| | **epithelial loss** | 0.03 | 0.005 | 0.07 | 0.02 | ns |
| | **IL-4 epi** | -0.09 | -0.02 | 0.13 | 0.008 | ns |
| | **IL-4 subepi** | -0.17 | -0.08 | 0.19 | 0.02 | ns |
| | **IL-13 epi** | -0.15 | 0.14 | -0.01 | 0.02 | ns |
| | **IL-13 subepi** | -0.07 | 0.16 | -0.15 | 0.04 | ns |
| **lung** | **vascularity** | 0.06 | 0.07 | -0.04 | 0.007 | ns |
| | **thickness RBM** | 0.16 | 0.3 | 0.21 | 0.29 | <0.001 |
| | **epithelial loss** | -0.06 | -0.18 | -0.24 | 0.16 | 0.02 |
| | **IL-4 epi** | -0.23 | 0.06 | 0.24 | 0.04 | ns |
| | **IL-4 subepi** | 0.07 | 0.07 | -0.004 | 0.01 | ns |
| | **IL-13 epi** | -0.1 | -0.05 | 0.25 | 0.04 | ns |
| | **IL-13 subepi** | -0.21 | 0.19 | 0.04 | 0.04 | ns |

Independent variables: Atopy, asthma, rhinitis
Dependent variables: inflammatory cell numbers in epithelium and subepithelium
† = Analysis of variance (ANOVA)

Also, approximately one third of the variability of RBM thickness (Figure 2b) was explained by the presence of atopy or asthma or rhinitis ($r^2 = 0.29$, $p < 0.001$). The RBM thickness was directly related to the presence of asthma ($\beta = 0.3$, $p = 0.02$). Again no difference was found between allergic rhinitis and asthma patients in a multiple regression model.

The number of $CD31^+$ vessels in the bronchial subepithelium (Figure 3b and 4b) was generally higher in patients with asthma and rhinitis than in controls. However, after multiple regression analysis, this difference was not significant.

The numbers of $IL\text{-}4^+$ and $IL\text{-}13^+$ cells were similar among the investigated groups in both bronchial epithelium and subepithelium.

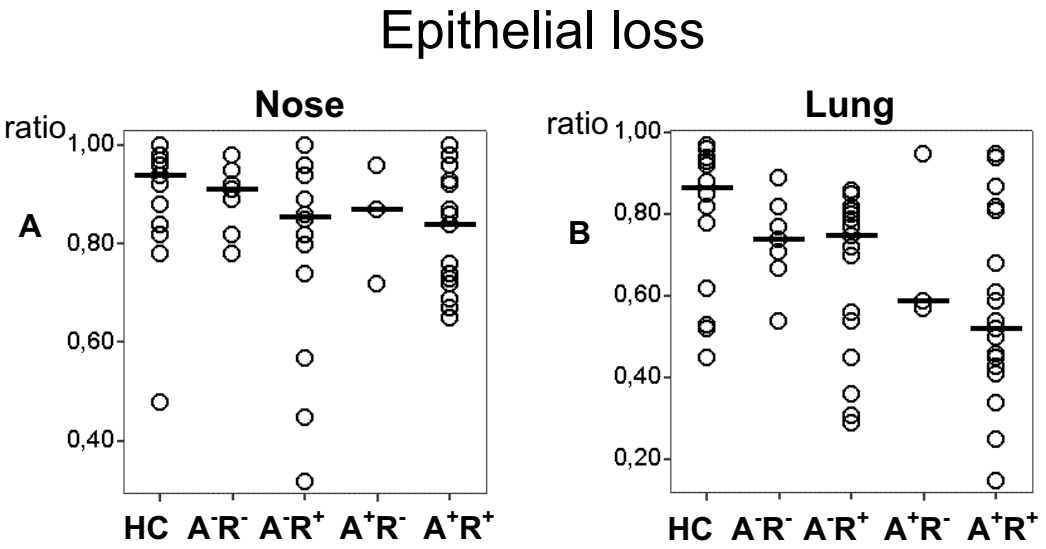


**Figure 1.** **A**: nasal mucosa, **B**: bronchial mucosa.

*Abbreviations used:* HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. Data are plotted as the ratio of occupied membrane length divided by the total membrane length. Horizontal bars represent medians and open circles represent individual values.

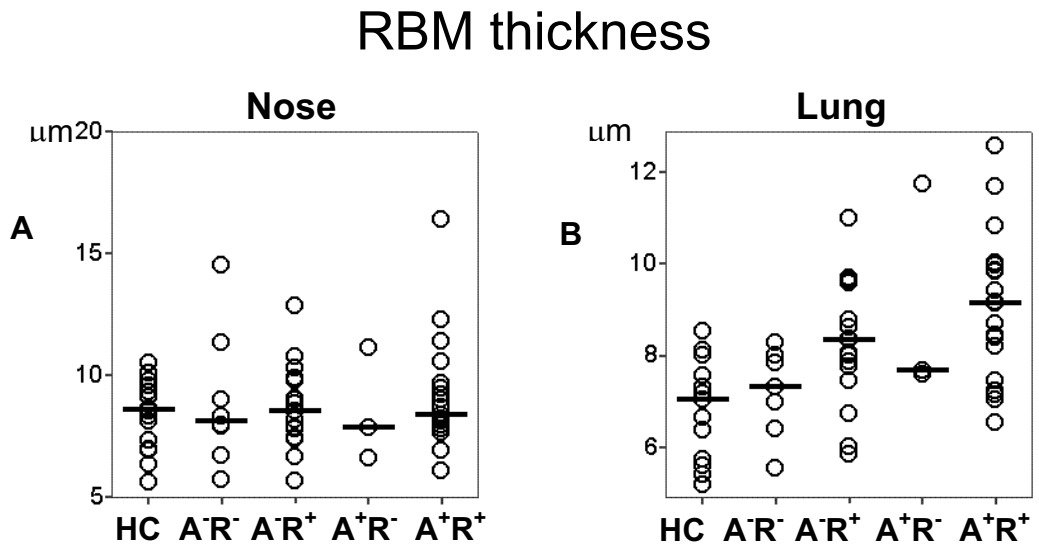


**Figure 2. A:** nasal mucosa, **B:** bronchial mucosa. Thickness of the reticular basement membrane was interactively measured on 400 x magnified images at regular intervals.

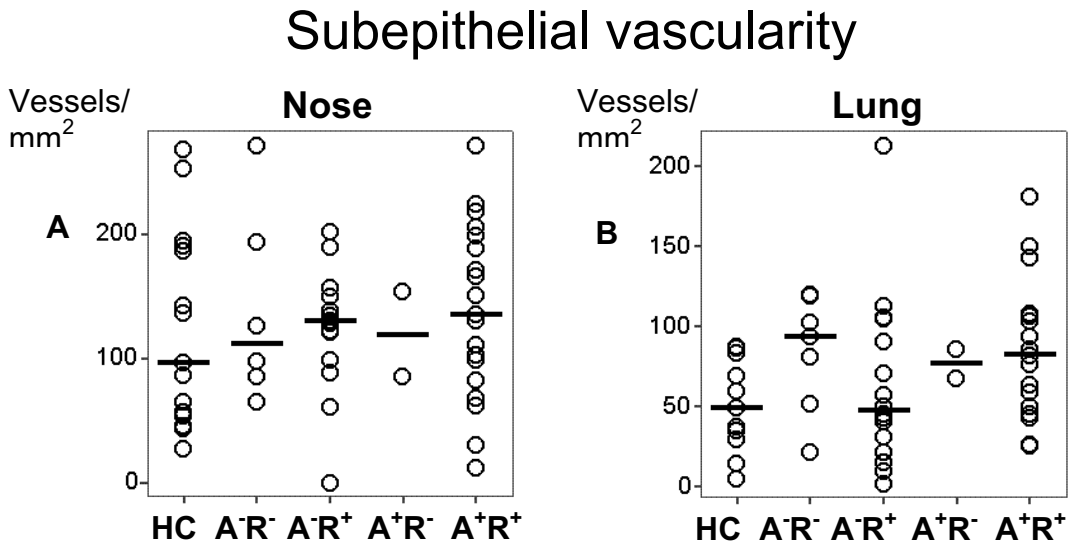


**Figure 3. A**: nasal mucosa, **B**: bronchial mucosa. Vessels with a distinct lumen staining positive for CD31 were counted on 20 x 10 magnified images in an area 100 µm deep in the lamina propria along the length of the reticular basement membrane.

*Abbreviations used:* RBM = reticular basement membrane; HC = healthy control subjects; $A^-R^-$ = atopic subjects, no asthma, no rhinitis; $A^-R^+$ = allergic rhinitis, no asthma; $A^+R^-$ = allergic asthma, no rhinitis; $A^+R^+$ = allergic asthma and rhinitis. Horizontal bars represent medians and open circles represent individual values.

## 8.5 Discussion

In this study, we demonstrated that airway remodeling, in terms of epithelial shedding and basement membrane thickening, is present in the lower airways of allergic patients. We were able to collect nasal and bronchial biopsy specimens from subjects representing the total range of the atopic spectrum. Asthmatic and nonasthmatic subjects were discriminated on the basis of frequent pulmonary symptoms and presence of BHR. This study is unique, in that remodeling features, cytokine profile and clinical findings in upper and lower airways are investigated simultaneously in these patient groups.

In a previous study, we were unable to demonstrate a relationship between mucosal inflammatory cells and manifestations of allergic airways disease[25]. No significant difference could be found with regard to the nasal and bronchial cellular infiltrate in allergic rhinitis patients with or without asthma, suggesting that factors other than the local inflammatory cell infiltrate are likely to be more important in the clinical expression of allergic rhinitis and asthma. Local repair mechanisms, tissue remodeling and the presence of signaling mediators could be relevant to the clinical expression in allergic airways disease.

Three aspects of airway remodeling were investigated in the current study: epithelial shedding, reticular basement membrane thickening and subepithelial vascularity. Our results demonstrate that airway remodeling is present in the bronchial mucosa of asthmatic patients, but also, to a lesser extent, in mucosa of nonasthmatic atopic patients. Thickening of the bronchial basement membrane was related to the presence of asthma. However, no difference could be found between asthmatics and allergic rhinitis patients in a multiple regression model. There was no evidence for remodeling in the upper airways.

Thickening of the bronchial reticular basement membrane due to subepithelial collagen deposition is considered as an important feature of allergic asthma. The degree of basement membrane thickening was positively correlated to disease severity in asthmatics[9,10]. However, this relationship is still subject of debate. Increased RBM thickness was present in recently diagnosed mild asthmatic disease[26], as well as nonasthmatic allergic subjects[27]. In fact in one study, no difference in basement

membrane thickness could be detected between several groups of patients with increasing severity of asthma and healthy controls[28]. In our study, we were able to identify asthma as the most outstanding factor responsible for increased RBM thickening. However, atopy and rhinitis appeared to have some contribution to RBM thickness, as well.

Increased loss of bronchial epithelium has previously been reported in asthmatic patients[8] and was found to be associated with BHR[8,29]. In our study, epithelial loss in the lower airways appeared to be a feature of allergic airways disease in general and was not specifically related to the presence of asthma. This increased epithelial fragility is thought to be the result of either cytotoxic damage by inflammatory cells[29,30] or disruption of intra-epithelial adhesive mechanisms[7,31]. The epithelial loss, which was seen in healthy controls, is thought to be an artifact due to the biopsy procedure[8].

The role of subepithelial vascularity in the pathophysiology of asthma is still controversial. There are very few reports in the literature that describe a relationship between increased subepithelial vascularity and asthma. Li *et al.* found an increased number of vessels per subepithelial area in asthmatic patients[11], whereas Carroll *et al.* detected similar numbers in asthmatics compared to controls and postulated that the number of blood vessels is only increased in proportion to increased airway wall area[12]. In our study, we did not observe significant differences in subepithelial vascularity between patients and controls. Different methodological approaches could account for the discrepancy. We used CD31, an endothelium marker, instead of Factor VIII. Factor VIII was found to be inferior to CD31 in lower airways mucosa in a previous study[23].

Data about structural changes in upper airway mucosa are rare. Although there is no fundamental difference between the structure of the ciliated epithelium in upper and lower airways, epithelial fragility and increased basement membrane thickening is found in bronchial mucosa of asthmatic patients[8], but usually not in nasal mucosa of allergic rhinitis patients[13,32]. Only one publication was found in the literature where Amin *et al.* described epithelial loss in patients with perennial rhinitis, suggesting that

some degree of remodeling may take place[33]. Our data do not support these findings. Only three subjects, distributed over various groups had more than 50% epithelial loss in their nasal mucosa. Also Chanez *et al.*, using the same biopsy technique in the upper as in the lower airways, found no significant difference between rhinitics and controls in nasal epithelial shedding[13]. Therefore, we suggest that epithelial shedding in the upper airways should be considered mostly as artifacts.

Eosinophilic inflammation has for a long time been regarded as the cause of the structural changes in the airway mucosa, since they are a source of degranulation products cytotoxic to the epithelial cells. The current study, as well as the previous study, challenge this hypothesis. No association could be found between numbers of inflammatory cells and aspects of airway remodeling. This lack of relationship is particularly clear in nasal mucosa, where we see no difference in epithelial integrity, basement membrane thickness and vascularity, despite increased numbers of inflammatory cells, such as eosinophils and dendritic cells. Although inhaled corticosteroids in asthma can totally abolish eosinophilic inflammation and restore

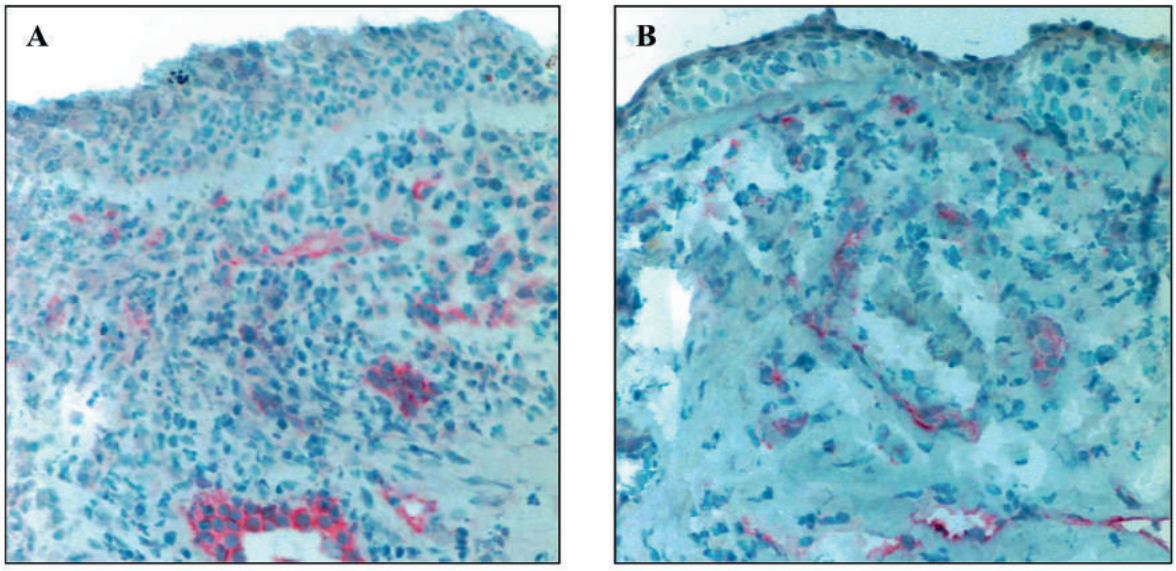


**Figure 4. A**: nasal mucosa, **B**: bronchial mucosa. Immunohistochemical staining for human endothelium (CD31). Counterstained with hematoxylin. Original magnification x 200.

epithelial lining to a normal level[13], thickness of basement membrane is generally not influenced[13,26].

IL-4 and IL-13, two cytokines that bind to the same receptor, are thought to regulate bronchial hyperresponsiveness and airway remodeling independent of eosinophilic inflammation[34-39]. In our experimental groups, we analyzed IL-4 and IL-13 expression in both nasal and bronchial mucosa. However, no significant difference could be detected between patients and controls. This may be explained by either the stable phase of disease in which most of our patients were at the time of sampling, or the short half-life of released cytokines in the tissue. Although we can not rule out signaling cytokines as the orchestrating mediators in airway remodeling, we should perhaps look more at growth factors and tissue repair mechanisms.

Myofibroblasts may play a key role in the remodeling process. Increased numbers of activated myofibroblasts were found in asthmatic airways after allergen challenge[40], and their number was correlated with the size of the reticular basement membrane[41]. In nasal mucosa, on the other hand, myofibroblasts are not readily detectable[42]. On the other hand, the contribution of growth factors as VEGF, FGF should not be underestimated. Therefore, these factors will be included in subsequent evaluations of the samples.

In conclusion, we have demonstrated structural changes in the lower airways of allergic asthmatic and nonasthmatic patients. Thickness of reticular basement membrane and epithelial fragility are altered in allergic airways disease, although no significant difference was found between patients with rhinitis and patients with asthma. In the upper airways, we have found no tissue remodeling in allergic rhinitis patients. Although animal models suggest that signaling factors, such as IL-4 and IL-13, could orchestrate airway remodeling, we could not detect differences in IL-4 and IL-13 expression between allergic patients and controls. Future studies need to address the question whether growth factors and myofibroblasts may be discriminative factors with regard to remodeling in upper and lower airways.

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# 9

# General discussion and conclusions

Nose and lungs are anatomically and functionally closely related organs. From this point of view, the relationship between allergic rhinitis and asthma seems rather obvious. Several epidemiological studies have indeed shown that allergic rhinitis often co-exists with, and even may precede allergic asthma. Therefore, allergic rhinitis is regarded as a risk factor for the development of asthma, especially in the presence of bronchial hyperresponsiveness (BHR).

Allergic rhinitis and asthma can be discriminated on the basis of clinical history, variable airway obstruction and BHR. This distinction is sometimes difficult to make since symptom perception is widely variable; lung function can be normal in mild asthmatics and, although BHR is a constant feature of asthma, it is also frequently present in allergic rhinitis.

Several studies have demonstrated that asthma and rhinitis are characterized by a similar inflammatory process. However, pathophysiologic interactions between upper and lower airways are still not entirely understood.

In this thesis, the following questions were addressed:

- Is local allergen exposition essential for the induction of airway mucosal inflammation?
- Does the systemic circulation contribute to the interaction between nose and lung?
- Is mucosal inflammation associated with symptomatology and changes in airway calibre?

*Generalized airway inflammation after local allergen provocation*

Few studies have investigated upper and lower airways simultaneously in atopic patients. One study compared nasal and bronchial inflammation in asthmatic patients with perennial rhinitis and found more eosinophilia and airway remodelling in bronchial than in nasal mucosa[1]. Nonasthmatic allergic subjects were not included in this study. Until now, no studies have been done comparing upper and lower inflammatory features in an allergen provocation model. We are the first to demonstrate that generalized airway inflammation is also present in nonasthmatic

allergic rhinitis patients after local allergen provocation, irrespective of the site of the challenge.

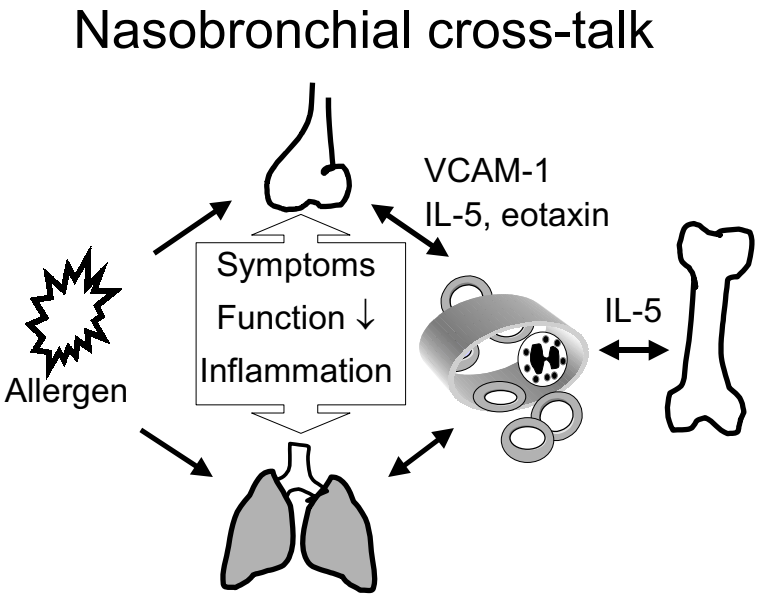


**Figure 1.** The systemic circulation as an important pathway in the interaction between upper and lower airways

Bronchoscopy and biopsy *per se* do not result in generalized bronchial inflammation[2]. To minimize the intraluminal spread of antigen after SBP, the bronchoscope was held in the wedged position. It is also very unlikely that allergen spilled into the nose after SBP, since bronchoscopy was performed via the oral route and did not lead to excessive coughing. This is supported by a small study which addressed the issue of cross-contamination of nose and lungs with a radioactive tracer. In a pilot-study, a radionuclide was placed bronchoscopically in the bronchial tree in four patients and was still clearly visible in the same position after 24 hours, suggesting that contamination of the nose through intraluminal spread is unlikely to take place[3].

Also, after NP, allergen spill from the nose into the lower airways is not likely to happen. Inhalation studies with radio-labeled allergen have shown no deposition of allergen in the lungs after nasal allergen application, following the technique we used[4]. Although postnasal drainage of inflammatory mediators into the lower airways

can not be excluded entirely, it is not likely to play a role in normally functioning human airways[3].

In our cross-sectional study, we found a good correlation between inflammatory cell numbers in nasal and those in bronchial mucosa, which suggests that these cells are recruited into the tissue via a common, or at least similar, mechanism. These results are in accordance with our previous work.

From our results, we conclude that local allergen exposition is not essential for the induction of airway mucosal inflammation. Upper and lower airways are closely connected and have a mutual influence on each other, resulting in a similar allergic response. However, a clear difference was observed in the time course and the degree of clinical and immuno-pathological findings between the nose and bronchi of allergic subjects after local allergen challenge.

### *9.2.2 Systemic circulation as a pathway in the interaction between nose and lungs*

Blood eosinophilia is a frequent finding in allergic rhinitis[5] and asthma[5-7]. Nasal provocation with methacholine in asthmatic patients with rhinitis resulted in an increase in lower airway resistance that could be blocked by premedication of nasal mucosa with phenylephrine, suggesting a role for systemic mediators in the induction of lower airway resistance[8]. Furthermore, studies in animals[9-11] and patients with allergic rhinitis, asthma and other atopic diseases[12-14] have consistently shown a rise in circulating inflammatory cells and progenitors after allergen inhalation followed by recruitment to sites of allergic inflammation. The results of our studies are in line with these findings. In peripheral blood, a significant increase was observed in IL-5 levels and the number of circulating eosinophils after SBP and NP (Figure 1). Also, in our cross-sectional study, we found that numbers of blood eosinophils were associated with clinical manifestations in both upper and lower airways disease. We speculate that the absorption of inflammatory mediators, such as IL-5, at the site of allergen challenge, results in the release of eosinophils from the bone marrow into the blood and, subsequently, in their migration into the nasal and bronchial mucosa under the influence of chemotactic agents and endothelial adhesion molecules.

Several other mechanisms have been suggested to play a role in lower airway dysfunction among patients with rhinitis: i.e. altered breathing pattern, pulmonary aspiration of nasal contents and the nasal-bronchial reflex[15].

Nasal breathing is definitely important for the filtering and conditioning of the inhaled air. However, in allergen provocation studies, the degree of nasal blockage was not found to be related to changes in bronchial hyperresponsiveness[4].

Since studies with radio-labelled allergen did not show deposition of allergen in the lungs after nasal allergen application[3,4], allergen spill or mucus transport from the nose to the lungs or vice versa are not very likely to have influenced the results of our study.

Although exposure of the nasal mucosa to cold dry air results in immediate bronchoconstriction in asthmatic patients[16], no direct effect on $FEV_1$ could be detected by most authors after nasal allergen challenge[4,17,18]. Corren et al. demonstrated increased BHR 30 minutes after NP suggesting a role for a nasobronchial reflex mechanism[4]. However, since increased BHR was still present after 4½ hours, other mechanisms, such as the systemic mediators, are more likely to contribute to the interaction.

### *9.2.3 Mucosal inflammation and clinical manifestations*

Eosinophilic and basophilic infiltration in the lower airways has been associated with the severity of asthma[7,19,20]. SBP was previously used to study local inflammatory processes in allergic rhinitis patients[21,22]. In our provocation studies, we found an allergic inflammatory response, similar to asthma, in the lower airways of allergic rhinitis patients without bronchial hyperresponsiveness. Significant changes in pulmonary symptom score and lung function were found in the rhinitis group until 24 hours after SBP and, to a lesser extent, after NP. Our data demonstrate that even in allergic rhinitis patients without asthma, bronchial mucosal inflammation correlated with the pulmonary symptom score. Increased numbers of eosinophils (EG2) were previously demonstrated in the lower airways of atopic, non-asthmatic subjects, along

with decreased pulmonary function. Moreover, it was shown that bronchial eosinophilia and clinical expression were dependent on the local dose of allergen[21].

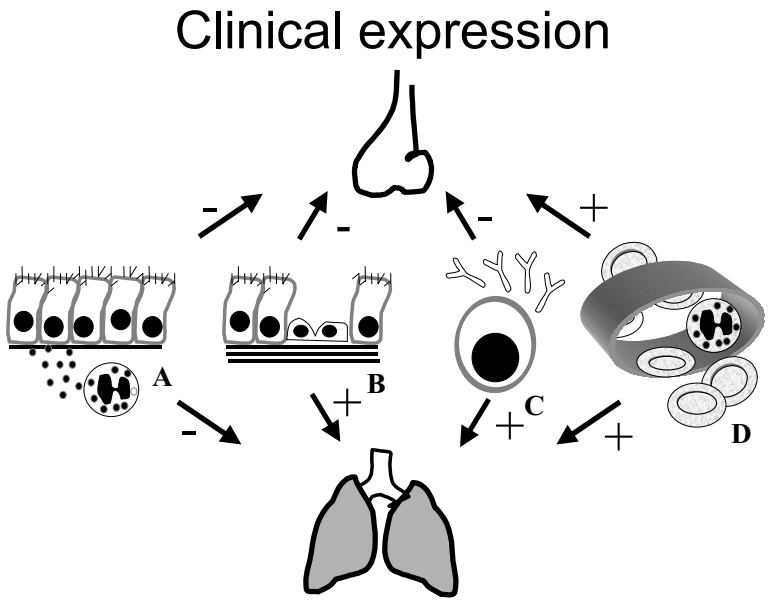


**Figure 2.** Aspects of inflammation (**A:** mucosal inflammation, **B:** airway remodeling, **C:** severity of atopy, and **D:** blood eosinophilia) in perennial allergic patients and their relationship to clinical manifestations in nose and lungs.

Do these findings indicate that the allergic rhinitis patients actually have asthma? We conclude from our studies that this is indeed the case. However, the threshold above which an allergic response is initiated in the bronchi in daily life is probably higher in patients with allergic rhinitis than in those with clinical asthma. It should be realised that size of the inhaled allergen, as well as duration and severity of the allergen exposure could also affect the clinical manifestation[23].

This issue was also addressed in a cross-sectional study that we performed in atopic patients with persistent perennial nasal and/or bronchial symptomatology. Allergic inflammation and airway remodeling were present in bronchial mucosa of nonasthmatic allergic rhinitis patients with house dust mite allergy. Although inflammatory cell numbers were higher and the reticular basement membrane was thicker in the asthmatic group, no relationship was found between aspects of the cellular infiltrate and tissue remodeling or clinical manifestations (Figure 2). We speculate that inflammatory cells are in an “active” mode during acute inflammation

(as, for example, in our provocation studies) and become "quiescent" during the chronic, stable phase of disease. Other allergen provocation studies in nose and lung support this hypothesis. These studies demonstrated persistent inflammation up to 2 weeks after allergen provocation, long after symptoms had disappeared[24-26].

We suppose that the presence of inflammatory cells *per se* in mucosal tissue may be a feature of atopy in general. Therefore, the quality of the inflammatory response may discriminate the chronic from the acute phase of disease, and may be more likely to show an association with clinical disease. To study this aspect of allergic inflammation, we looked at the expression of IL-4 and IL-13 in nasal and bronchial mucosa. IL-4 and IL-13 are thought to regulate bronchial hyperresponsiveness and airway remodeling, independent of eosinophilic inflammation[27-32]. However, no significant difference could be detected regarding these cytokines between patients and controls. This could be due to either the stable phase of disease in which most of our patients were at the time of biopsy, or the short half-life of the released cytokines in the tissue.

In the nose, the relation between allergic inflammation and symptoms is less clear: no association could be found between mucosal eosinophils and clinical parameters in our allergen provocation studies in seasonal allergic rhinitis patients, as well as in the cross-sectional study in perennial allergic rhinitis patients. The majority of the studies addressing the relationship between inflammation and clinical disease have been performed in seasonal allergic rhinitis patients. Higher numbers of eosinophils, basophils, mast cells and dendritic cells were present in the nasal subepithelium after NP[26,33] and natural exposure[34]. However, changes in eosinophils and mast cells were related to nasal symptoms in the acute stage of allergic inflammation only[35,36]. Few studies have investigated the allergic response in perennial allergic rhinitis patients. They show that mucosal eosinophilia is less marked in perennial than in seasonal disease and usually not related to symptom expression[37].

## 9.3 Concluding remarks

The relationship between allergic rhinitis and asthma has been well established in epidemiological studies and clinical trials. However, no attempt has been made thus far to study the kinetics and association of inflammatory parameters and clinical findings in upper and lower airways of patients simultaneously. Allergen provocation studies are a good model to study the nose-lung interaction in allergic airway disease. In this thesis, we have reviewed the results of a nasal and endobronchial provocation study in allergic rhinitis patients without pre-existent asthma. In addition, we have compared upper and lower airway inflammation in perennial allergic patients with nasal and/or pulmonary symptomatology. We have demonstrated that local allergen challenge induces allergic inflammation in the entire airway system. We suggest that the systemic pathway, involving bloodstream and bone marrow, is an important mechanism in the nose-lung cross-link. Therefore, upper and lower airways need to be regarded as one functional entity in allergic rhinitis, even in the absence of clinical asthma.

In the allergen provocation studies, representing “acute” inflammation, a good correlation was found between bronchial inflammation and lower airway symptoms. In the nose, we were not able to find a correlation between inflammatory parameters and clinical expression. We propose that acute inflammation, at least in the lower airways, is related to symptoms and influences airway caliber through the release of a variety of mediators.

The relationship between chronic inflammation and clinical disease is less straightforward. In perennial allergic patients, representing “chronic” inflammation, no association was seen between inflammatory cell numbers, aspects of remodeling and cytokines on one hand, and clinical manifestations on the other hand. Our results suggest that, in addition to systemic factors, such as atopy and blood eosinophils, other factors, such as local repair mechanisms and individual susceptibility could be relevant to the clinical expression in allergic airway disease. Future studies need to address these issues.

## 9.4 Directions for future research

It is clear that there is a close interaction between upper and lower airways. Nasal therapy can influence asthma symptoms in allergic rhinitis patients with concomitant asthma. However, the effect of nasal therapy on bronchial inflammation and aspects of airway remodeling is still unexplored. The observation that allergic rhinitis is ubiquitous in allergic asthma, and that nasal allergen exposure can indeed induce bronchial inflammation stress the importance of such clinical trials.

Our results have led us to hypothesize that allergic rhinitis patients do have asthma, but that the dose of allergen required to initiate an allergic response in the bronchi is probably higher in allergic rhinitics than in clinical asthma patients. Therefore, it would be interesting to do a threshold-study in atopic patients with various expression of allergic airways disease to find out about the relationship between allergen exposure and the development of clinical symptoms.

Also, the question remains to be solved what causes the discrepancy between upper and lower airways, with regard to airway remodeling. Since mucosal inflammation can be present in both nose and bronchi, it is not very likely to play an important role in the development of airway remodeling. We speculate that intrinsic factors in the airway epithelium or local repair mechanisms may contribute to the different response of upper and lower airways to airway stimuli. We are currently investigating the effect of growth factors on structural changes in the airways.

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# 10

Summary

The main subject of this thesis is the interaction between upper and lower airways in allergic airways disease. A two-way allergen provocation model was used to gain more insight into the mechanisms involved in nasobronchial cross-talk. Furthermore, cellular and remodeling aspects of upper and lower airways were analyzed in atopic patients with persistent perennial nasal and/or bronchial symptomatology.

In Chapter 1, the historical and epidemiological aspects of the relationship between allergic rhinitis and asthma are reviewed. The importance of the interaction between upper and lower airways for daily clinical practice is emphasized. It is postulated that immunopathological mechanisms may play a pivotal role in nasobronchial cross-talk.

In Chapter 2, structure and function of upper and lower airways are described. Several interaction mechanisms between nose and lungs are proposed, such as mouth breathing, aspiration of nasal contents, nasobronchial reflex and systemic circulation. Allergen provocation is introduced as a useful tool to investigate clinical and immunopathological aspects of nasobronchial interaction in allergic airways disease.

In Chapter 3, the aims of the studies are presented. This thesis comprises studies aimed at: Firstly, analyzing the events involved in nasobronchial interaction, using a two-way allergen provocation model in patients with allergic rhinitis without pre-existent asthma. Secondly, investigating the relation between allergic inflammation and clinical manifestations in atopic patients with persistent perennial nasal and/or bronchial symptomatology.

In Chapter 4, we studied the effect of segmental bronchial provocation (SBP) on allergic inflammation in blood, nasal and bronchial mucosa and related this to clinical findings in upper and lower airways. No reports in the literature have been found describing signs of allergic inflammation in a remote and "upstream" organ after local allergen challenge. SBP resulted in immediate and persistent lower airways symptoms in seasonal allergic rhinitis subjects without bronchial hyperresponsiveness. Also, pulmonary function, assessed with $FEV_1$, was significantly decreased in rhinitis

patients 1 hour and 24 hours after SBP. SBP induced mucosal inflammation - characterized by increased numbers of eosinophils (MBP) - in the lower airways. Interestingly, the allergic response was more widespread: eosinophilia was also detectable in peripheral blood. Moreover, we found increased numbers of eosinophils, IL-$5^+$ and eotaxin$^+$ cells in the nasal mucosa 24 hours after SBP, together with signs and symptoms of allergic rhinitis, indicating that the nose was involved in the allergic response, as well.

In conclusion, we have found an allergic inflammatory response, similar to asthma, in the lower airways of rhinitis patients without bronchial hyperresponsiveness after SBP. Moreover, endobronchial allergen provocation resulted in a generalized allergic airway response and induced increased numbers of eosinophils in the peripheral blood suggesting that the systemic circulation is involved in the interaction between nose and lungs.

In Chapter 5, the role of mast cells and basophils was investigated in the same SBP model as was previously described in Chapter 4. Mast cells and basophils are metachromatically staining cells, which are believed to play an important role in the initiation and control of upper and lower respiratory allergy. The number of basophils (BB1) significantly increased after SBP in nasal and bronchial mucosa of allergic rhinitis patients. In contrast, the numbers of mast cells (tryptase/chymase) significantly decreased in nasal mucosa of allergic rhinitis patients at 24 hours after SBP. In blood, the number of basophils decreased and the level of interleukin (IL)-5 increased in atopic patients.

This study shows that SBP in nonasthmatic allergic rhinitis patients results in reduced numbers of mast cells in the nose, possibly as a result of enhanced degranulation. At the same time, there is evidence for an influx of basophils from the blood into the nasal and bronchial mucosa. IL-5, an important mediator in the release of inflammatory cells from the bone marrow in the blood, was found to be present in the blood 24 hours after SBP. This finding further supports the idea that the systemic circulation may be an important pathway in nasobronchial cross-talk.

In Chapter 6, we examined immunopathological and clinical findings in upper and lower airways following nasal allergen provocation (NP). Nasal and pulmonary peak flow, as well as upper and lower airway symptom scores, were recorded at two hour intervals after NP. NP induced a profound clinical response in upper and lower airways of nonasthmatic allergic rhinitis subjects. Allergic rhinitis patients almost immediately developed nasal and pulmonary symptoms, followed by a more gradual reduction in upper and lower airway function.
Eosinophil migration is dependent on the expression of cytokines, chemokines and adhesion molecules. Increased expression of endothelial adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and eosinophilic (MBP) allergic inflammation was detected in the nasal and bronchial mucosa of rhinitis patients at 24 hours after NP. In the blood, a significant increase was found in serum IL-5 and in the number of circulating eosinophils after NP. In the bronchial mucosa, tissue eosinophilia was associated with the increased expression of endothelial VCAM-1, suggesting that recruitment of inflammatory cells to the mucosa is not a local phenomenon in allergic airway disease.
In conclusion, we were able to induce an allergic inflammatory response, similar to asthma, in the lower airways of nonasthmatic AR patients after nasal allergen provocation. We speculate that the absorption of inflammatory mediators at the site of allergen challenge results in release of eosinophils from the bone marrow into the blood and, subsequently, in their migration into the nasal and bronchial mucosa under the influence of endothelial adhesion molecules.

In Chapter 7, we report on a cross-sectional study where we looked at cellular aspects of upper and lower airways inflammation in atopic patients with various clinical manifestations. Local airway inflammation is considered important in the clinical expression of both asthma and rhinitis. Five experimental groups were formed: allergic asthma and rhinitis, allergic rhinitis without asthma, allergic asthma without rhinitis, atopic subjects without asthma or rhinitis and nonallergic healthy control subjects. Nasal and bronchial biopsy specimens were collected in one session during stable disease.

In nasal and bronchial mucosa, numbers of eosinophils (MBP) and dendritic cells (CD1a) were higher in allergic patients with symptoms than in nonsymptomatic atopics and control subjects. No difference could be detected, however, in the number of eosinophils, basophils (BB1), mast cells (CD117), dendritic cells and macrophages (CD68) between rhinitis patients with or without asthma in both nasal and bronchial mucosa. In atopic patients, no correlation was found between inflammatory cell numbers and airway function parameters or clinical symptom scores.
In conclusion, nasal and bronchial inflammation is a feature of allergic airways disease, irrespective of the clinical manifestations. Our results suggest that factors other than local inflammatory cell infiltrate contribute to the clinical expression of atopic disease.

Chapter 8 describes airway remodeling in nasal and bronchial mucosal sections in the investigated groups mentioned in the previous chapter. Little is known about tissue remodeling in nonasthmatic atopic patients, or about structural changes in the upper airways. Therefore, we assessed epithelial shedding, reticular basement membrane (RBM) thickening and increased subepithelial vascularity with the use of a computer assisted image analysis system. We hypothesized that there would be a relationship with signaling factors, such as interleukin (IL)-4 and IL-13, which are likely to contribute to remodeling in allergic disease according to some animal models.
In bronchial mucosa, epithelial loss was increased in allergic patients with symptoms compared to control subjects. The RBM was thicker in allergic patients with rhinitis and/or asthma than in asymptomatic atopics and controls. The number of vessels (CD31) in the bronchial subepithelium was higher in asthmatic patients compared to controls. No difference was found regarding airway remodeling between rhinitis patients with or without asthma. In nasal mucosa, no difference could be detected in airway remodeling between allergic patients and controls. The numbers of IL-$4^+$ and IL-$13^+$ cells were similar among the investigated groups in both nasal and bronchial mucosa.
In conclusion, lower airway remodeling is present in atopic patients, irrespective of their clinical manifestations. In contrast, no structural changes were detected in the

upper airways. In stable allergic disease, we could find no evidence for the involvement of IL-4 and IL-13 in clinical expression or tissue remodeling. We speculate that tissue remodeling and airway inflammation are present in bronchial mucosa of atopic patients before the expression of clinical disease.

# 11

# Samenvatting

## 11.1 Samenvatting

Neus en longen zijn anatomisch en fysiologisch nauw verwante organen. Daarom lijkt de relatie tussen allergische rhinitis en astma nogal voor de hand te liggen. Diverse epidemiologische onderzoeken hebben inderdaad aangetoond dat astma vaak samengaat met, en voorafgegaan wordt door allergische rhinitis. Allergische rhinitis wordt dan ook als een belangrijke risico factor gezien voor de ontwikkeling van asthma, met name wanneer ook al bronchiale hyperreactiviteit (BHR) aanwezig is.
Allergische rhinitis en astma worden onderscheiden op grond van het klinische verhaal, variabele luchtwegvernauwing en BHR. Dit onderscheid valt soms moeilijk te maken. De perceptie van klachten kan nogal verschillen van patiënt tot patiënt. De longfunctie kan volledig normaal zijn in mensen met een milde vorm van astma. Hoewel BHR per definitie aanwezig is in astma, kan het ook voorkomen bij allergische rhinitis patiënten.
Verschillende studies laten zien dat astma en rhinitis worden gekenmerkt door een gelijksoortig ontstekingsproces. Desondanks is over de pathofysiologische interacties tussen bovenste en onderste luchtwegen nog maar weinig bekend.
Het doel van dit proefschrift was om de interactie tussen bovenste en onderste luchtwegen te onderzoeken in allergische rhinitis patiënten met behulp van een allergeen provocatie model. Daarnaast wilden we de slijmvliesontsteking in onderste en bovenste luchtwegen analyseren bij atopische patiënten met persisterende, perenniale klachten van neus en/of longen.

In hoofdstuk 1 geven we een overzicht van de historische en epidemiologische aspecten van de relatie tussen allergische rhinitis en astma. We benadrukken het belang van de interactie tussen bovenste en onderste luchtwegen voor de kliniek en stellen dat immunopathologische mechanismen zeer waarschijnlijk een belangrijke rol spelen in de interactie tussen neus en longen.

In hoofdstuk 2 beschrijven we de anatomie en fysiologie van de bovenste en onderste luchtwegen. Een aantal interactie-mechanismen wordt besproken, zoals mond ademhaling, aspiratie van neussecreet, de nasobronchiale reflex en de systemische

circulatie (de bloedbaan). We behandelen de principes van het allergeen provocatie model. Dit model is geschikt om klinische en immunopathologische aspecten van de interactie tussen neus en longen in luchtwegallergieën te onderzoeken.

In hoofdstuk 3 worden de doelstellingen besproken. Dit proefschrift omvat drie hoofdstudies. Om meer inzicht te verkrijgen in de interactie tussen neus en longen, hebben we in de eerste twee studies de allergische ontstekingsrespons bestudeerd in onderste en bovenste luchtwegen na segmentale bronchoprovocatie of neusprovocatie in allergische rhinitis patïenten zonder astma.
In de derde studie onderzochten we de relatie tussen allergische ontsteking en klinische manifestaties in atopische patiënten met persisterende, perenniale neus- en/of longklachten.

In hoofdstuk 4 komt het effect van segmentale bronchoprovocatie (SBP) op allergische ontsteking in bloed, neus- en bronchusslijmvlies aan de orde, en deze gegevens worden gerelateerd aan klinische bevindingen in bovenste en onderste luchtwegen. Wij hebben geen eerdere beschrijvingen in de literatuur kunnen vinden van allergische ontsteking in een ander “stroomopwaarts” gelegen orgaan na plaatselijke allergeen provocatie. SBP leidde tot directe en persisterende lagere luchtwegklachten in niet-astmatische patiënten met allergische rhinitis ten gevolge van graspollen. Ook de longfunctie, vastgesteld met $FEV_1$, was significant minder in rhinitispatiënten 1 uur en 24 uur na SBP. SBP veroorzaakte slijmvliesontsteking, gekenmerkt door een toename in het aantal eosinofielen (MBP), in de lagere luchtwegen. Echter deze slijmvliesontsteking was zeer uitgebreid: eosinofilie kon ook worden aangetoond in het bloed. Daarnaast vonden we een toegenomen aantal eosinofielen, IL-$5^+$ en eotaxine$^+$ cellen in het neusslijmvlies 24 uur na SBP, samengaand met rhinitisklachten, wat aangeeft dat de neus ook betrokken was bij de allergische reactie.
Concluderend hebben we een allergische ontstekingsrespons, vergelijkbaar met astma, aangetoond in de lagere luchtwegen van rhinitispatiënten zonder BHR na SBP. Daarnaast resulteerde SBP in een gegeneraliseerde allergische luchtwegreactie en

leidde het tot een toename in eosinofielen in het bloed, hetgeen suggereert dat de bloedbaan betrokken is bij de interactie tussen neus en longen.

De rol van mestcellen en basofielen werd onderzocht in hoofdstuk 5 gebruikmakend van dezelfde studieopzet als besproken is in hoofdstuk 4. Mestcellen en basofielen zijn metachromatisch aankleurende cellen, die geacht worden betrokken te zijn bij de initiatie en controle van luchtwegallergieën. Het aantal basofielen (BB1) nam significant toe na SBP in neus- en bronchusslijmvlies van allregische rhinitispatiënten. Het aantal mestcellen (tryptase/chymase) daarentegen verminderde significant in het neusslijmvlies van allergische rhinitispatiënten 24 uur na SBP. In het bloed nam het aantal basofielen af, terwijl de concentratie IL-5 steeg in atopische patiënten.
Deze studie laat zien dat SBP in allergische rhinitispatiënten zonder astma resulteerde in een afname van de hoeveelheid mestcellen in de neus, waarschijnlijk als gevolg van toegenomen degranulatie. Gelijktijdig vond er migratie plaats van basofielen vanuit het bloed naar het neus- en longslijmvlies. IL-5, een belangrijke mediator bij het vrijkomen van ontstekingscellen vanuit het beenmerg in de bloedbaan, was verhoogd aanwezig in het bloed 24 uur na SBP. Deze bevinding onderstreept nogmaals het idee dat de bloedbaan een belangrijke rol speelt in de interactie tussen neus en longen.

In hoofdstuk 6 onderzochten we de immunopathologische en klinische bevindingen in bovenste en onderste luchtwegen na neusprovocatie (NP). Neus- en long *peak flow*, evenals symptoomscores van luchtwegklachten werden bijgehouden om de twee uur na NP. NP veroorzaakte een duidelijke klinische respons in bovenste en onderste luchtwegen van allergische rhinitispatiënten zonder astma: zij kregen bijna onmiddellijk neus- en longklachten, gevolgd door een meer geleidelijke afname in neus- en longfunctie.
De migratie van eosinofielen is afhankelijk van de expressie van cytokines, chemokines, en adhesiemoleculen. Wij namen een toegenomen expressie waar van endotheliale adhesiemoleculen (ICAM-1, VCAM-1 en E-selectine) en eosinofiele (MBP) ontsteking in neus- en longslijmvlies van rhinitispatiënten 24 uur na NP. In het bloed werd een significante toename gezien van het serum IL-5 en het aantal

circulerende eosinofielen na NP. In het bronchusslijmvlies correleerde eosinofilie met de toegenomen expressie van VCAM-1, hetgeen impliceert dat ophoping van eosinofielen in het slijmvlies niet een plaatselijk fenomeen is in allergische luchtwegziekten.
De conclusie is dat een allergische ontstekingsrespons, lijkend op astma, kan worden uitgelokt in de lagere luchtwegen van niet-astmatische allergische rhinitispatiënten na neusprovocatie. Wij denken dat de absorptie van ontstekingsmediatoren vanuit het geprovoceerde orgaan leidt tot het vrijkomen van eosinofielen vanuit het beenmerg in de bloedbaan en vervolgens resulteert in hun migratie naar neus- en bronchusslijmvlies onder invloed van endotheliale adhesiemoleculen.

In hoofdstuk 7 tonen we de resultaten van een cross-sectionele studie, waarin we hebben gekeken naar de cellulaire aspecten van bovenste en onderste luchtwegontsteking in atopische patiënten met verschillende klinische manifestaties. Lokale luchtwegontsteking wordt verantwoordelijk gehouden voor de klinische expressie van zowel astma als rhinitis. Vijf experimentele groepen werden gevormd: allergische rhinitis en astma, allergische rhinitis zonder astma, astma zonder rhinitis, atopisch zonder astma of rhinitis en niet-allergische gezonde controles. Neus- en bronchusbiopten werden verzameld gedurende één sessie wanneer de ziekte stabiel was.
In zowel neus- als bronchusslijmvlies was het aantal eosinofielen (MBP) en dendritische cellen (CD1a) hoger in allergische patiënten met luchtwegklachten dan in niet-symptomatische proefpersonen. We konden geen verschil aantonen in het aantal eosinofielen, basofielen (BB1), mestcellen (CD117), dendritische cellen en macrofagen (CD68) tussen rhinitispatiënten met of zonder astma, in zowel neus als longen. Het aantal ontstekingscellen in allergische patiënten was niet gecorreleerd met luchtwegsymptomen en functieparameters.
In conclusie kunnen we stellen dat neus- en bronchusontsteking voorkomen bij luchtwegallergieën ongeacht de klinische presentatie. Onze resultaten doen vermoeden dat factoren, anders dan het ontstekingscelinfiltraat, verantwoordelijk zijn voor de klinische presentatie van atopie.

Hoofdstuk 8 beschrijft de structurele veranderingen (airway remodeling) in het neus- en bronchusslijmvlies van de onderzoeksgroepen genoemd in het vorige hoofdstuk. Er is weinig bekend over *airway remodeling* in niet-astmatische atopische patiënten, evenals het voorkomen in de bovenste luchtwegen. Daarom, hebben we epitheelverlies, basale membraan (BM) verdikking en subepitheliale doorbloeding gemeten met behulp van een computergestuurd beeldanalyse systeem. Het was onze hypothese dat er een relatie zou zijn met signaalactoren, zoals IL-4 en IL-13, die zouden bijdragen aan het *airway remodeling* proces volgens sommige diermodellen.
In bronchusslijmvlies was meer epitheelverlies aanwezig in allergische patiënten met luchtwegsymptomen in vergelijking tot gezonde controles. De BM was dikker in allergische patiënten met rhinitis en/of astma dan in asymptomatische atopische proefpersonen en controles. Het aantal bloedvaten (CD31) in het bronchusslijmvlies was hoger in astmapatiënten dan in controles. We konden geen verschil aantonen, wat betreft *airway remodeling*, tussen rhinitispatiënten met of zonder astma. In de neus werd geen verschil gezien in *airway remodeling* tussen allergische patiënten en controles. Het aantal IL-$4^+$ en IL-$13^+$ cellen was gelijk in alle onderzoeksgroepen in zowel neus- als bronchusslijmvlies.
Concluderend is er sprake van *airway remodeling* in atopische patiënten, ongeacht de klinische presentatie. Daarentegen werden er geen structurele veranderingen aangetroffen in de bovenste luchtwegen. In stabiele, allergische ziekte konden we geen bewijs vinden voor de betrokkenheid van IL-4 en IL-13 bij de klinische expressie en *airway remodeling*. Wij veronderstellen dat *airway remodeling* en luchtwegonsteking aanwezig zijn in het bronchusslijmvlies van atopische patiënten reeds voor de eerste presentatie van klachten.

## 11.2 Conclusies

In dit proefschrift hebben we de resultaten beschreven van een neus- en een bronchusprovocatiestudie in allergische rhinitispatiënten zonder preëxistent astma. Daarnaast hebben we de ontsteking van de bovenste en onderste luchtwegen vergeleken in perenniale allergische patiënten met neus- en/of longklachten.

De belangrijkste conclusies uit deze studies zijn:

1. Lokale allergeenprovocatie in neus of longen van allergische rhinitis patiënten veroorzaakt een allergische ontstekingsreactie in de gehele luchtwegen, gekenmerkt door een toename in eosinofiele en basofiele granulocyten, IL-5 en adhesiemoleculen.
2. De systemische circulatie is een belangrijk mechanisme in de interactie tussen neus en longen. Allergeen provocatie in allergische rhinitis patiënten leidt tot een verhoogd aantal eosinofiele granulocyten en een toegenomen IL-5 concentratie in het bloed.
3. Bij allergische patiënten met perenniale symptomen van rhinitis en/of asthma is geen relatie aantoonbaar tussen lokale ontstekingsaspecten en klinische manifestaties.

**List of abbreviations**

AR : Allergic rhinitis
BAL : Bronchoalveolar lavage
BHR : Bronchial hyperresponsiveness
EAR : Early phase of the allergic response
FcεRI : high affinity receptor for IgE
$FEV_1$ : Forced expiratory volume in 1 s
FGF : Fibroblast growth factor
ICAM-1 : Intercellular adhesion molecule-1
IL : Interleukin
LAR : Late phase of the allergic response
LTs : Leukotriens
mAb : Monoclonal antibody
MBP : Major basic protein
MC : Mast cell
NP : Nasal allergen provocation
$PC_{20}$ : Provocative concentration of methacholine causing a 20% fall in $FEV_1$
PAF : platelet activating factor
PEF : Peak expiratory flow
PGs : prostaglandins
PNIF : Peak nasal inspiratory flow
RBM : Reticular basement membrane
SBP : Segmental bronchial provocation
VAS : Visual analogue scale
VCAM-1 : Vascular cell adhesion molecule-1
VEGF : Vascular endothelium growth factor

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**Curriculum vitae**

Gerrit Johannes Braunstahl werd geboren op 20 november 1968 te ’s Gravenhage. Het diploma Gymnasium β behaalde hij in 1987 aan het Christelijk Lyceum in Apeldoorn. Van 1987 tot 1993 studeerde hij geneeskunde aan de Rijksuniversiteit Groningen. Het arts-examen behaalde hij in 1996. Tijdens zijn studie en co-schappen liep hij onderzoeksstages in Valladolid (1993 Spanje) en Hamilton (1995 Canada). Van juni 1996 tot oktober 1997 was hij werkzaam als arts-assistent Interne Geneeskunde in ziekenhuis “De Weezenlanden” te Zwolle. Het astmaonderzoek aan de McMaster University (begeleiders prof. Koëter en prof. O’Byrne) in Hamilton en het prettige werken op de afdeling Longziekten in Zwolle (Dr. Van der Star en Dr. Westermann) waren aanleiding om in juli 1997 te solliciteren voor een baan als arts-onderzoeker bij de afdeling Longziekten van het Dijkzigt Ziekenhuis in Rotterdam. Na een korte periode als AGNIO op de afdeling begon hij in januari 1998 met het promotie-onderzoek (begeleiders prof. Hoogsteden en Dr. Fokkens). Vanaf januari 2001 doet hij als AGIO longziekten zijn vooropleiding Interne Geneeskunde in het St-Franciscus Gasthuis te Rotterdam (opleiders Dr. Tjen en prof. Hoogsteden).