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Immunopathological and Structural Alterations in Difficult to Control Asthma

Bergqvist, Anders

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ANDERS BERGQVIST | DEPARTMENT OF RESPIRATORY MEDICINE AND ALLERGOLOGY
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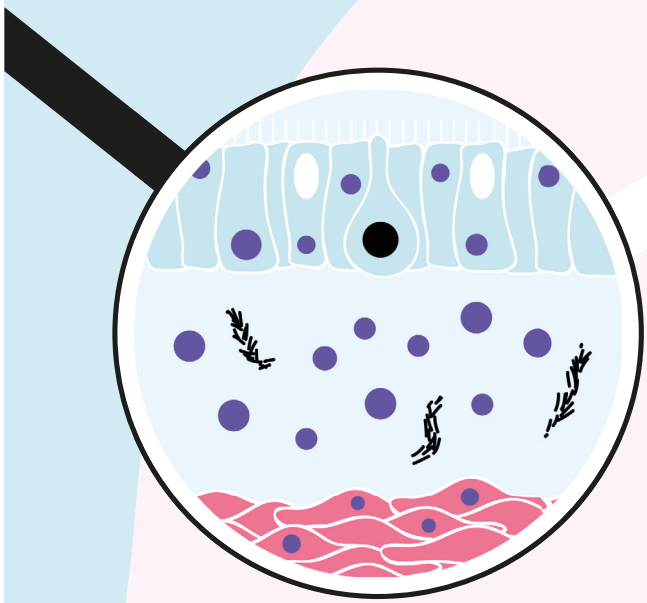

Immunopathological and Structural Alterations in Difficult to Control Asthma

By

Anders Bergqvist

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Abstract

Asthma is a chronic respiratory disorder affecting an estimated 300 million people worldwide. Most patients with asthma can be controlled with bronchodilators and low to moderate dose inhaled corticosteroids. However, an estimated 5-10% of the population presents a more troublesome disease, often referred to as severe asthma. The aim of present thesis was to study the relationship between histopathological alterations and clinical control in patients with different severities of asthma. To study this, a detailed immunohistochemical analysis was performed on lung biopsies obtained from moderate asthmatics (n = 24 in Paper I), severe asthmatics (n = 25 in Paper II), and uncontrolled severe asthmatics undergoing bronchial thermoplasty treatment (n = 15 and n = 20 in Paper III and IV, respectively). In Paper I, we show that allergic airway inflammation extends to the peripheral airways specifically in patients that are poorly controlled. This suggests that targeting peripheral airway inflammation, for example with extrafine-particle formulations of inhaled corticosteroids, may benefit patients that remain symptomatic despite standard inhaled corticosteroid treatment. In Paper II, we found that symptomatic severe asthma is associated with lower number of eosinophils and no apparent signs of chronic inflammation as compared with stable severe asthma. However, we detected stretches of bronchoepithelial metaplasia in the former patient category suggesting that external assaults, possible episodic pathogen infections, may play an important role in this form of asthma. In Paper III, we show that bronchial thermoplasty markedly improves several elements of clinical control in patients with uncontrolled severe asthma. The clinical improvements were associated with a down-regulation of structures involved in airway narrowing and hyperreactivity, including airway smooth muscle, neuroendocrine epithelial cells, and nerve fibres. In Paper IV, we show that bronchial thermoplasty treatment likewise has long-lasting immunological effects as evident by a reduction of key bronchial immune cells including mast cell populations and T helper cells. These changes may to some extent explain the clinical benefits associated with bronchial thermoplasty, although this remains to be investigated. In summary, the results in this thesis provide new histopathological data that are associated with clinical control in asthma.

Key words: Severe asthma, bronchial thermoplasty, airway smooth muscle, neuroendocrine cells, inflammation, immune cells, remodelling

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By

Anders Bergqvist

LUND
UNIVERSITY

Main supervisor: Professor Jonas Erjefält

Unit of Airway Inflammation
Department of Experimental Medical Sciences
Faculty of Medicine, Lund University
2015

Main supervisor: Professor Jonas Erjefält

Unit of Airway Inflammation
Department of Experimental Medical Sciences
Lund University

Co-supervisor: Professor Leif Bjermer

Department of Respiratory Medicine and Allergology,
Lund University Hospital

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Contents

List of Papers

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

I. Bergqvist A, Andersson CK, Mori M, Walls AF, Bjermer L, and Erjefält JS.

Alveolar T-helper type-2 immunity in atopic asthma is associated with poor clinical control. *Clin Sci (Lond)* 2015; 128: 47 – 56.

II. Bergqvist A, Andersson CK, Hoffmann HJ, Mori M, Shikhagaie M, Krohn IK, Dahl R, Bjermer L, and Erjefält JS.

Marked epithelial cell pathology and leukocyte paucity in persistently symptomatic severe asthma. *Am J Respir Crit Care Med* 2013; 188: 1475 – 7.

III. Pretolani M,⋆ Bergqvist A,⋆ Dombret MC, Thabut G, Knapp D, Hamidi F, Alavoine L, Taillé C, Chanez P, Erjefält JS, and Aubier M.

Effectiveness of bronchial thermoplasty in patients with severe refractory asthma: clinical and histopathological correlations. (submitted)

IV. Bergqvist A, Pretolani M, Dombret MC, Thabut G, Knapp D, Alavoine L, Taillé C, Chanez P, Bjermer L, Aubier M, and Erjefält JS.

Immunological effects induced by bronchial thermoplasty in patients with severe refractory asthma. (working manuscript)

⋆) Equal contribution.

Additional papers contributing to this thesis

Sverrild A, Bergqvist A, Baines KJ, Porsbjerg C, Andersson CK, Thomsen SF, Hoffmann HJ, Gibson P, Erjefält JS, and Backer V. Airway responsiveness to mannitol in asthma is associated with chymase-positive mast cells and eosinophilic airway inflammation. *Clin Exp Allergy* 2015 Aug 7. doi: 10.1111/cea.12609 [Epub ahead of print].

(178)⋆

Andersson CK, Bergqvist A, Mori M, Mauad T, Bjermer L, and Erjefält JS. Mast cell associated alveolar inflammation in atopic uncontrolled asthma. *J Allergy Clin Immunol* 2011; 127: 905 – 12.

(179)⋆

⋆) Thesis reference No.

Selected Abbreviations

ACT	Asthma Control Test
AQLQ	Asthma Quality of Life Questionnaire
ASM	Airway smooth muscle
ATS	American Thoracic Society
BT	Bronchial thermoplasty
FcεRI	High-affinity receptor for IgE
FEV_1	Forced expiratory volume in 1 second
GINA	Global Initiative for Asthma
ICS	Inhaled corticosteroids
ICU	Intensive care unit
IgE	Immunoglobulin E
NEC	Neuroendocrine epithelial cell
OCS	Oral corticosteroids
PGP 9.5	Protein gene product 9.5
SA	Severe asthma
SBM	Subepithelial basement membrane
Th1	T helper type 1
Th2	T helper type 2

Background

Normal anatomy and histology of the airways

The airways of the lungs can be divided into two functional compartments; the conducting airways and the respiratory airways. The conducting airways begin outside the lungs with the nose/mouth and end at the terminal bronchioles. Their main function is to transport air to the respiratory bronchioles and alveoli, where blood is oxygenated and cleared from carbon dioxide. In order to allow effective gaseous exchange, the airways of the lungs branch out to reach approximately 700 million alveoli.

Bronchial airways

The bronchial airways represent the large conducting airways. Originating from the trachea, the left and right main bronchi enter the lungs where they later branch into secondary and tertiary bronchi. The bronchial wall consists of several layers, which can be organised into epithelium, lamina propria, muscularis mucosa, and submucosa (Figure 1).

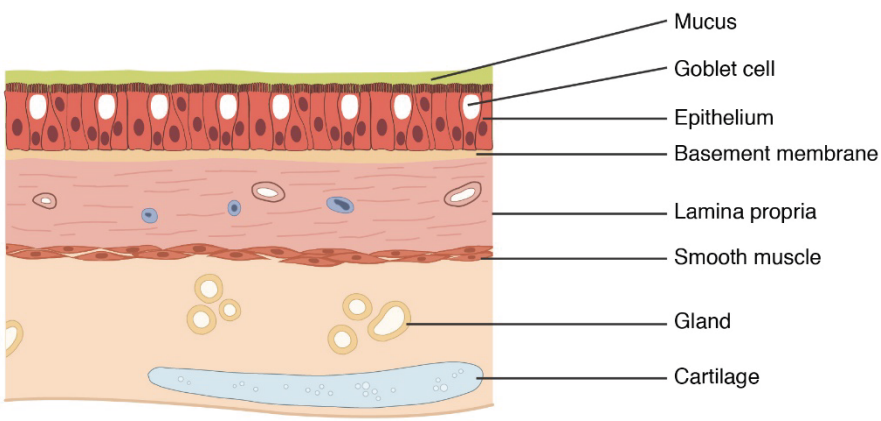

Figure 1.
Main structures of the bronchial wall.

Bronchial epithelium

The luminal surface of bronchial airways is covered by a pseudostratified ciliated columnar epithelium consisting of four different cell types: ciliated columnar cells, mucus-producing goblet cells, basal cells, and neuroendocrine cells. Cilia are hair-like projections on the apical surface of ciliated cells. Their main function is to distribute mucus across the epithelial surface and to filter incoming air from unwanted material. The latter process is carried out by mucociliary clearance, where particles such as dust or bacteria adhere to mucus and get transported to the pharynx by ciliary movement.[1] From the pharynx, the mucus can be swallowed to the stomach for digestion or spitted out. Neuroendocrine cells in the epithelium respond to neuronal input and are capable of releasing a variety of bioactive amines and peptides that can affect autonomic nerve terminals or vasculature.[2] Adjacent epithelial cells are connected tightly to each other by various tight junctions and the epithelium itself is connected to the basal lamina by hemidesmosome formations found on the surface of basal cells.[3] Besides anchoring the epithelium, basal cells are believed to be stem cells that can differentiate to other epithelial cells when needed.[4]

Lamina propria

The lamina propria is a layer of connective tissue that is found beneath the epithelium. Closest to the epithelium lies the basal lamina and the lamina reticularis, which together form the subepithelial basement membrane (SBM). This thin membrane consists of several collagens, laminin, and perlecan that are highly cross-linked to form a dense extracellular matrix of connective tissue. The composition of connective tissue beneath the basement membrane is on the other hand loose and elastic, allowing many cells to infiltrate and populate this region. Blood vessels, lymph vessels, glands, and autonomic nervous system components are also found in the lamina propria.

Muscularis mucosa

Beneath the lamina propria comes a layer of smooth muscle, which separates the lamina propria form the submucosa. The physiological role of airway smooth muscle is currently unclear, as no consensus exists regarding what useful function contraction might play. Some experts argue that this is a vestigial organ similar to the appendix with no obvious physiological consequences if eliminated.[5] In any regards, abnormal smooth muscle function can cause airflow obstruction for example in patients with asthma.[6]

Submucosa

The bronchial submucosa contains cartilage that supports the bronchi and limits the degree of airway narrowing during smooth muscle contraction. Serous and mucous glands can also be found in this region.

Bronchioles

The tertiary bronchi branch into bronchioles (or 'small airways') which in turn branch into terminal and respiratory bronchioles. Bronchioles and terminal bronchioles belong to the conducting airways whereas respiratory bronchioles belong to the respiratory airways. The airway wall can be organised into epithelium, lamina propria and muscularis mucosa.

Bronchiolar epithelium

The bronchiolar epithelium starts as ciliated/columnar but changes to ciliated/cuboidal as the luminal diameter decreases. Goblet cells are present in the large bronchioles, but not in the terminal or respiratory bronchioles that follow. Instead, the epithelium here contains clara cells, which are non-ciliated cells that secrete surfactant in order to reduce surface tension during respiration. Clara cells also have detoxifying functions and are capable of dividing and differentiating into other epithelial cells.[7] Similar to bronchi, adjacent epithelial cells are connected tightly to each other and to the basal lamina.

Lamina propria and muscularis mucosa

The lamina propria and muscularis mucosa are analogous to that of the bronchi, with the exception that glands cannot be found.

Alveoli

Alveoli are found scattered in the walls of respiratory bronchioles and in small clusters called alveolar sacs at the end of bronchioles. The alveoli are gas exchange units where carbon dioxide is removed from blood and replaced with oxygen (Figure 2). It is estimated that the combined surface area of gas diffusion in the alveoli reach above 100 square metres.[8] The alveolar wall, which is distinctively different from that of the bronchi or bronchioles, can be organised into epithelium, basal lamina, interstitium, and capillary endothelium.

Alveolar epithelium

The alveolar epithelium consists of type I and II pneumocytes. Although type I pneumocytes are fewer than type II pneumocytes (40% vs. 60%), they cover 96% of the luminal surface.[9] Their main function is to form a structural barrier that allows carbon dioxide and oxygen to diffuse between the lumen and capillaries. The remaining 4% of the luminal surface is covered by type II pneumocytes, which secrete surfactant to reduce the surface tension during respiration. Type II pneumocytes are also capable of dividing and differentiating into type I pneumocytes. Similar to bronchi and bronchioles, the epithelial cells are connected to each other and to a basal lamina.

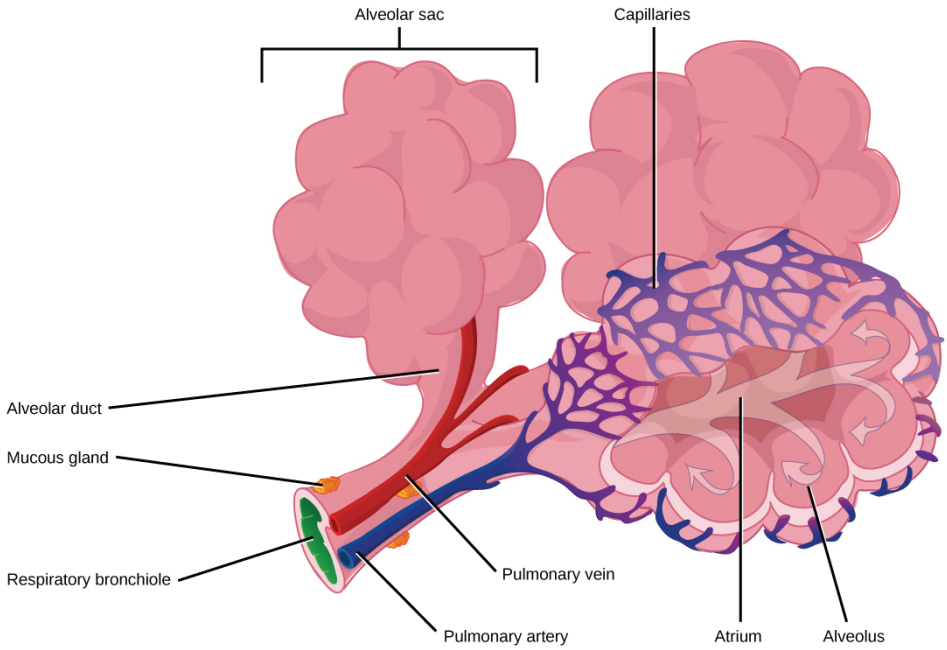

Figure 2.
Bronchioles lead air to the respiratory zone, where gas exchange occurs.

Basal lamina, interstitium and capillary endothelium

The basal lamina of the epithelium is most often fused with the basal lamina of the capillary endothelium. This results in a very thin layer where most of the gaseous exchange is believed to occur. In the remaining regions, the basal lamina of alveoli and endothelium is separated by an interstitium (or 'alveolar septum') where elastic fibres, collagen, and various cell types can be found. Adjacent alveoli may share the same interstitium with each other.

Introduction to asthma

Asthma is a chronic respiratory disease affecting an estimated 300 million people worldwide. It is more common in developed countries than developing ones, with prevalence rates ranging between 1 to 18%. Asthma affects people of all ages: it is one of the most common chronic diseases of childhood, adolescence and adulthood. The disease can have a significant impact on school or work performance and daily life activities. Unfortunately asthma can also lead to death, especially if untreated. To raise awareness of asthma, the Global Initiative for Asthma (GINA) was initiated in 1993 in collaboration with several collaborators, including the World Health Organization (WHO).

Definition

As of 2015, GINA defines asthma as follows: "Asthma is a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation".[10] Symptoms of asthma are usually triggered by non-specific factors such as exercise, exposure to allergens, change in weather, or respiratory infections. They may resolve spontaneously or in response to medication. Patients with asthma may also experience episodic flare-ups (exacerbations) that may be life-threatening. The diagnosis of asthma is based primarily on the pattern of symptoms. Measuring variability in expiratory airflow obstruction is recommended to aid diagnosis. Briefly, this can be assessed during spirometry by measuring the volume of inhaled and exhaled air over time. An increase in forced expiratory volume in one second (FEV_1) of $\geq$ 12% and $\geq$ 200 mL after the inhalation of a bronchodilator indicates variable airflow obstruction due to asthma.[10] Airway hyperresponsiveness (excessive airway narrowing in response to a provoking stimulus) is a characteristic feature of asthma, although not exclusive to the disease. This can also be assessed during spirometry by measuring the decline in the predicted value of FEV_1 following the administration of a provoking stimulus such as methacholine or histamine.

Risk factors and etiology

Asthma usually starts at young age. About half of all patients with asthma have an onset of their disease before the age of ten. A family history of asthma, indicative symptoms during preschool years, and allergic sensitisation (atopy) are strong predictors of asthma in childhood and adolescence. In adult-onset asthma, a family history of the disease is often non-existent and the occurrence of atopy is not higher than in the general population. Risk factors for adult-onset asthma include occupational exposure to sensitising agents or irritants, smoking, obesity, female sex hormones, and respiratory infections.[11] The etiology of asthma is not clear. However, the disease is likely caused by a complex interplay between genetic factors and environmental exposures happening at critical time points during the course of life.[12]

Pathophysiology

The most prominent symptoms of asthma such as wheeze, breathlessness, chest tightness, and cough are caused by airflow obstruction. Factors that contribute to airflow obstruction in asthma include bronchoconstriction (bronchial airway narrowing due to smooth muscle contraction), mucus overproduction leading to

congestion or mucus plugs, and swelling of the bronchial wall resulting in airway closure, as well as plasma extravasation.[6,13] (Figure 3). Bronchoconstriction is the primary physiological event leading to airflow obstruction in asthma.

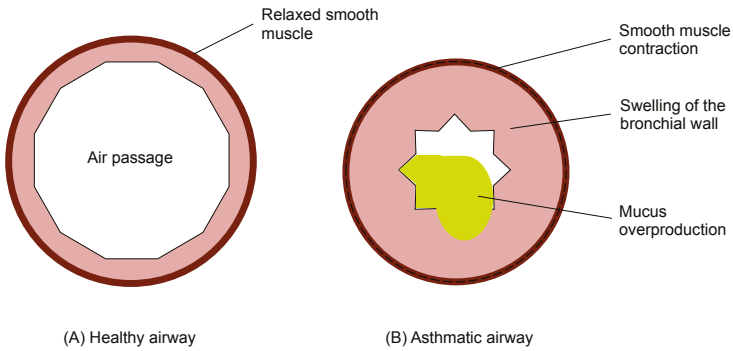

Figure 3.
Healthy airway (A) and events leading to airflow obstruction in asthma (B).

Histopathological changes in asthma

The asthmatic airways are structurally altered and infiltrated with inflammatory cells. These histopathological changes are mainly thought to be restricted to the bronchial airways, but may spread to include the small airways and alveoli. The most prominent histopathological changes and their role in the pathophysiology of asthma are summarised in the coming sections.

Structural changes

Airway wall thickening

Post-mortem studies of fatal and non-fatal asthma[14,15] and high-resolution computed tomography studies involving non-deceased asthmatics[16-18] have shown that the airway wall is significantly thicker in asthmatics as compared with healthy control subjects. Factors that contribute to wall thickening in asthma include edema, subepithelial fibrosis, airway smooth muscle enlargement, and epithelial alterations. The pathophysiological significance of wall thickening in asthma is not completely understood. *In vitro* studies have indicated that the thickening response can increase the extent of luminal narrowing caused by a given degree of smooth muscle contraction.[19,20] However, a negative correlation between wall thickening and airway hyperresponsiveness has later been demonstrated, indicating that it may have a protective role instead.[21] In addition to this, it has

been shown that wall thickening is less pronounced in asthmatics with highly variable airflow obstruction as compared with asthmatics that present partial or fixed airflow obstruction.[22]

Subepithelial fibrosis

Subepithelial fibrosis in asthma is characterised by thickening of the subepithelial basement membrane (SBM). The thickening is caused by an increased deposition of extracellular matrix proteins, particularly fibronectin and different collagens.[23] SBM thickening is in an early feature of asthma. It has been shown to occur in children with asthma, and to a similar degree to that seen in adult patients.[24] The role of SBM thickening in the pathophysiology of asthma is unclear. It has inconsistently been associated with airway hyperresponsiveness and fixed airflow obstruction.[25-28]

Airway smooth muscle enlargement

The airway smooth muscle layer is increased by 50-200% in fatal asthma and by 25-55% in non-fatal asthma as compared with normal control cases[29] (Figure 4). The enlargement is caused by hyperplasia (increased cell numbers) and possibly by hypertrophy (increased cell size) of smooth muscle cells. Deposition of extracellular matrix proteins between the muscle cells has also been suggested to play a role. Although abnormal smooth muscle function is fundamental to the pathophysiology of asthma, the functional significance of its enlargement is not entirely clear. *In vitro* studies have shown that increased smooth muscle thickness allow greater contraction upon stimulation.[30] In this study it was concluded that smooth muscle enlargement is likely the primary cause of airway hyperresponsiveness in asthma. However, intrinsic changes of smooth muscle cells (such as increased contractility) and/or altered mechanical properties of the airway wall during muscle contraction may also contribute to airway hyperresponsiveness.[31] Two bronchial biopsy studies have shown that airway smooth muscle enlargement is associated with fixed airflow obstruction in asthma.[28,32]

Mucus metaplasia

Goblet cell hyperplasia and an increased amount of submucosal glands are characteristic features of asthma. These abnormalities facilitate mucus overproduction, which can lead to airflow obstruction due to mucus plugging.[33]

Increased vascularisation

Bronchial biopsy studies have reported increased vascularisation in the lamina propria layer in patients with asthma as compared with normal control subjects.[34-36] This change is considered to worsen asthma by predisposing the airways to an influx of inflammatory cells from the blood stream. Moreover, an increase

microvasulature in asthmatic airways may also contribute to the aggravated plasma extravasation response seen in asthmatic patients.[13]

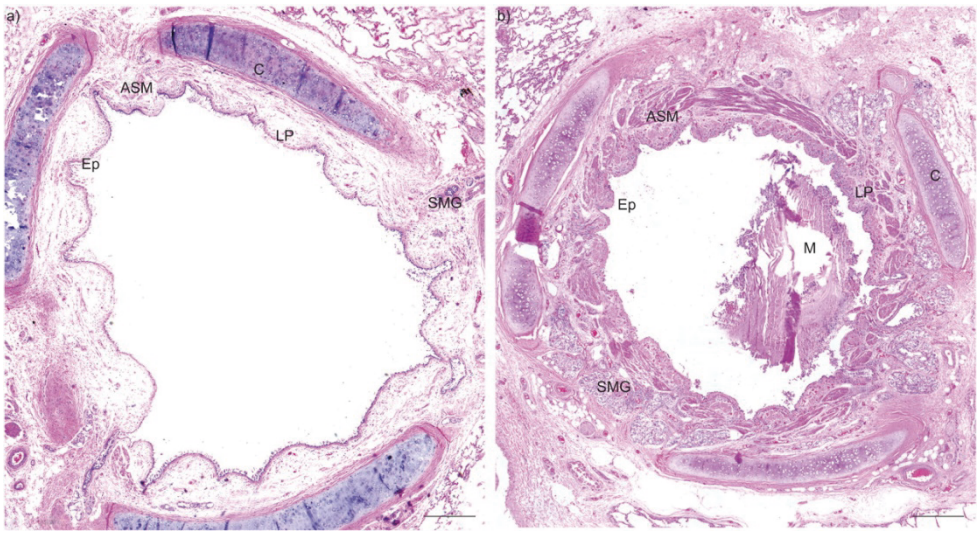

Figure 4.
Bronchial airway from a normal control subject (a) and a fatal asthma patient (b). Fatal asthma in particular is associated with a significant thickening of airway smooth muscle (ASM). Cartilage (C), epithelium (Ep), lamina propria (LP), mucus plug (M), and submucosal glands (SMG) are also shown in the pictures. Haematoxylin and eosin staining. Scale bars = 200 μm. Reproduced with permission of the European Respiratory Society ©. Eur Respir J 2008 32:1, 61-69; published ahead of print 2008, doi: 10.1183/ 09031936.00147807.

Epithelial alterations

Other epithelial alterations associated with asthma include epithelial shedding, loss of cilia, and disruption of tight junctions.[37] These changes make it easier for foreign material to reach the basal cells or the underlying airway tissue. As such, an impaired epithelial barrier function may increase the risk of infection and facilitate immunological reactions to environmental factors such as allergens or irritants.

Th2 inflammation

Airway inflammation in asthma is a multi-cellular process that mainly involves T helper type 2 (Th2) cells, eosinophils, and mast cells. Infiltration of eosinophils into the airway tissue is the most striking feature of the disease. This type of inflammation is linked to type 1 hypersensitivity reactions and chronic allergic diseases.[38] Intriguingly, studies have shown that also non-atopic asthma is associated with this type of inflammation.[39-42] Allergen-induced asthma is orchestrated by Th2 cells through their release of IL-4, IL-5 and IL-13 (Figure 5).

In brief, allergen exposure trigger mast cell degranulation and the release of several potent mediators that are capable of inducing bronchoconstriction, mucus overproduction, and edema formation i.e. all characteristic features of asthma. This early asthmatic response, which is thought to represent a type 1 hypersensitivity reaction, is followed by a late-phase response. During the late-phase response, Th2 cells are reactivated and the disease adopts an immunologically complex form characterised by eosinophilic infiltration and chronic mast cell activation. The functional role of each individual cell population involved in allergen-induced asthma is summarised below.

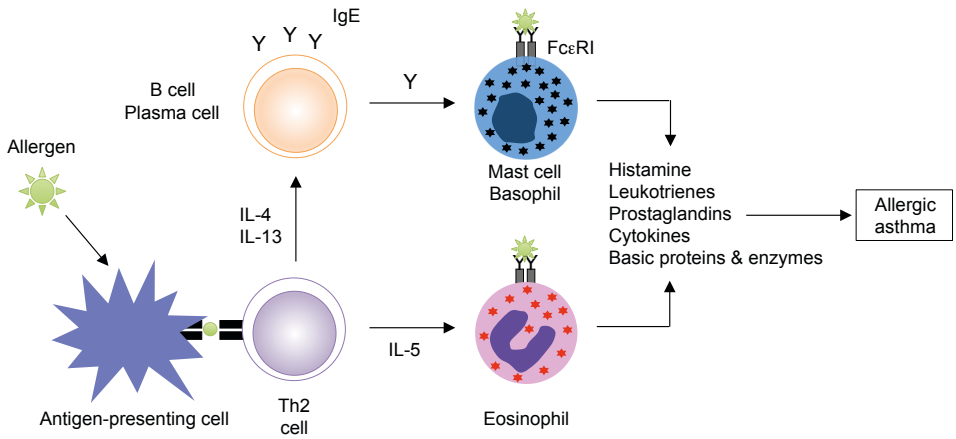

Figure 5.
Schematic illustration of the Th2 inflammatory response in allergic asthma. Drawing by Bergqvist A, adapted from Holgate ST.[43]

Antigen-presenting cells

Antigen-presenting cells can internalize allergens and present allergen fragments (antigens) on their surface in order to trigger an immunological reaction. Dendritic cells are the principal antigen-presenting cells in the human airways. These specialised cells are situated in the airway epithelium and subepithelium throughout the respiratory tract.[44] During the sensitisation phase, dendritic cells that have taken up and processed allergens migrate to the nearest draining lymph node where initial antigen presentation take place.[38] The antigens are loaded onto MHC class II molecules and presented to undifferentiated T helper cells. The ability of dendritic cells to secrete IL-12 during antigen presentation determines the balance between T helper type 1 (Th1) and Th2 responses. Thus, a high degree of IL-12 secretion favours Th1 differentiation whereas a low degree of IL-12 secretion favours Th2 differentiation.[45] After sensitisation, dendritic cells can migrate back to the site of allergen exposure and reactivate Th2 cells by sustained antigen presentation.[38]

Epithelial cells

Epithelial cells (not shown in figure 5) can enhance Th2 inflammation by secreting TSLP, IL-25 and IL-33. Studies have identified TSLP as an important cytokine that can activate dendritic cells during antigen presentation.[46,47] Recently, IL-25 and IL-33 have been shown to activate type 2 innate lymphoid cells.[48] These cells can orchestrate a Th2 type of inflammation by releasing high amounts of IL-5 and IL-13.[49-51] The role of type 2 innate lymphoid cells in asthma and other immunological settings is currently an area under intense research.

Th2 cells

Following antigen presentation, Th2 cells interact with B cells to stimulate the production of allergen-specific IgE antibodies. This process is depended on the ability of Th2 cells to secrete IL-4 and IL-13. Th2 cells also recruit eosinophils to the site of allergen exposure by releasing IL-5. Among the aforementioned cytokines, IL-13 has also been shown to induce mucus overproduction,[52,53] increase epithelial permeability,[54] and stimulate the proliferation of airway smooth muscle cells.[55] Th2 cells are difficult to detect in tissue samples due to the complexity of T-cell biology. However, bronchial biopsy studies have reported increased mRNA expression of Th2 cytokines and GATA-3 (a Th2 promoting transcription factor) in patients with asthma as compared with normal control subjects.[56-59]

B cells

B cells that have been primed to secrete allergen-specific IgE clonally expand and differentiate into plasma B cells. These specialised B cells are capable of secreting high amounts of soluble IgE, which can bind to FcεRI expressed on the surface of mast cells and basophils.[38]

Mast cells

FcεRI cross-linking upon allergen binding trigger mast cell degranulation and the release of histamine, leukotrienes, and prostaglandins. These preformed mediators are potent inducers of bronchoconstriction, mucus overproduction, and edema formation. The significance of these mediators has been demonstrated during experimental allergen challenge studies where inhibition of these mediators attenuates the early fall in FEV_1.[60-63] Inhibition of IgE has been shown to attenuate both the early and late fall in FEV_1, demonstrating the critical role of IgE in lung function decline following allergen exposure.[64,65] Mast cells are also considered to promote late-phase inflammation by releasing a plethora of cytokines that can recruit inflammatory cells to the site of allergen exposure.[66] Importantly, studies have shown that mast cells appear to be chronically activated in asthma, as manifested by a constitutive release of different mediators.[67-69] With this in mind, it should be mentioned that mast cells can be activated by non-IgE dependent factors including Toll-like receptor ligands,[70-73] complement factors,[74] and

neuropeptides.[75,76] Although the total number of infiltrating mast cells does not appear to differ between asthmatics and non-asthmatics,[77] studies have shown that mast cells localize to different regions in asthma, Most notably is the infiltration of mast cells in airway smooth muscle bundles.[78] Since this is not as evident in normal controls or patients with eosinophilic bronchitis (which shares several immunopathological features with asthma), it is considered to be an important determinant of asthma.[79] Indeed, mast cells in close proximity to airway smooth muscle cells can be expected to induce bronchoconstriction as described above and stimulate smooth muscle cell proliferation through cytokine release.[66] Mast cells in asthma have also been shown to infiltrate the epithelium and submucosal glands, where they can stimulate mucus overproduction.[80-82]

Basophils

Basophils express FcεRI and contain histamine that can be released upon allergen binding. They normally circulate in the blood stream as opposed to mast cells that are tissue resident. The precise role of basophils in asthma is not yet clear. Studies have reported a small infiltration of basophils in the airways of asthmatics as compared to normal control subjects.[83,84]

Eosinophils

The inflammation in asthma is characterised by a prominent infiltration of eosinophils into the airways.[77,85-87] Eosinophils are recruited to the airways by IL-5, which also promotes the survival and activation of these cells. The granules of eosinophils contain cytotoxic proteins that can damage the airway epithelium.[88,89] Eosinophils are also a rich source of mediators that are involved in fibrogenesis and angiogenesis.[90] Eosinophil activation does not seem to be dependent on IgE, indicating that IL-5 and other factors are responsible for activation.[91] A critical role of eosinophils in the pathophysiology of asthma has been questioned following the results of IL-5 inhibition showing that a significant reduction of eosinophils was not accompanied by symptom improvements.[92-94] However, later studies reported symptom improvements following IL-5 inhibition, which may indicate that a meaningful depletion of eosinophils was not achieved in the earlier studies.[95,96] Despite being a hallmarked feature of asthma, it has been estimated that 25% of patients with asthma have normal levels of eosinophils.[97] This has lead to the concept that asthma can be divided into eosinophilic (Th2-associated) and non-eosinophilic (non-Th2 associated) subtypes.[98,99]

Neutrophilic inflammation

Neutrophils are phagocytic cells that can engulf and destroy foreign agents, particularly pathogens. Being highly motile and abundant in the blood, neutrophils are the first cells to arrive at the site of an infection. Their granules contain high

levels of reactive oxygen species and antimicrobial proteins, which are used to destroy ingested material during phagocytosis.[100] Increased infiltration of neutrophils into the airways has been observed in severe asthmatics with normal levels of eosinophils as compared to healthy control subjects.[101] Other studies have reported increased number of neutrophils in sputum (coughed up mucus) particularly in patients with severe asthma.[102,103] These findings are unfortunately difficult to interpret as the pharmacological treatment of severe asthma may act to promote the survival of neutrophils.[98,104] Nevertheless, neutrophilic inflammation can worsen asthma by damaging airway epithelium and inducing mucus overproduction as has previously been suggested.[105]

Paucigranulocytic asthma

Histopathological changes in paucigranulocytic asthma (defined as normal levels of eosinophils and neutrophils) are unknown. This form of asthma could be driven by abnormal smooth function that is not necessarily dependent on immunological components.[43,98]

Treatment of asthma

The goals of asthma management are to achieve good symptom control, maintain normal activity levels, and to minimize future risks of exacerbations, fixed airflow obstruction and side-effects of treatment.[10] Asthma can be managed by avoidance of provoking factors and by treatment. The main pharmacological treatment options, which can be classified as relievers and controllers, are reviewed below.

Relievers

Reliever medications are usually bronchodilators that quickly alleviate symptoms of bronchospasm. These medications are also helpful in preventing exercise-induced asthma symptoms. There are two types of bronchodilators: short-acting β_2 agonists (SABA) and anticholinergics. SABA bind to β_2 receptors expressed on the surface of smooth muscle cells. By activating these receptors, SABA induce muscle relaxation through downstream signalling. Anticholinergics enable muscle relaxation by blocking the action of acetylcholine, which is a neurotransmitter that can be released from parasympathetic nerve endings. SABA are often more effective than anticholinergics, and should be used on an as-needed basis only. Daily use indicates deterioration of asthma control and may lead to side effects such as tremor and tachycardia.[106] During exacerbations, systemic administration

of corticosteroids may also alleviate acute symptoms of asthma. However, these drugs are normally used only as controllers.

Controllers

Inhaled corticosteroids

Inhaled corticosteroids (ICS) are the most effective anti-inflammatory medications in the treatment of asthma. These medicines are to be taken on a daily basis for long-term asthma management. ICS have consistently been shown to reduce asthma symptoms, improve quality of life, improve lung function, decrease airway hyperresponsiveness, reduce asthma exacerbations, decrease the risk of death, and reduce the need for relievers.[107] If discontinued, deterioration of clinical control follows within weeks to months.[108,109] At a molecular level, corticosteroids enter the cell cytoplasm where they bind to glucocorticoid receptors. The activated receptors then translocate to the cell nucleus where they bind to glucocorticoid responsive elements. This consequently inhibits the synthesis of inflammatory cytokines and promotes synthesis of anti-inflammatory cytokines.[110] In biopsy studies, clinical benefits of corticosteroids are accompanied with reduced numbers of T cells, eosinophils, mast cells and dendritic cells in the bronchial mucosa.[85,111-113] Corticosteroids follow a log-dose linear effect, meaning that most of the clinical benefits are seen at low-moderate doses.[114] Therefore, add-on therapies are generally preferable to increasing the dose of ICS. Side-effects associated with high dose of ICS include slow wound healing, adrenal suppression, decreased bone mineral density, cataracts, and glaucoma.[115]

Long-acting β_2 agonists

Long-acting β_2 agonists (LABA) have a longer duration than SABAs (approximately 12 hours versus 4-6 hours) and are most effective when combined with ICS.[106] Indeed, adding a LABA to a daily regimen of ICS improves symptom control, improves lung function, reduces asthma exacerbations, decreases nocturnal (nighttime) asthma, and reduces the need for SABA.[116-118] High doses of LABA may lead to side-effects such as tremor and tachycardia.

Leukotriene modifiers

Leukotriene modifiers include receptor antagonists and 5-liopoxygenase inhibitors. The receptor antagonists are designed to prevent the action of leukotrienes whereas 5-liopoxygenase inhibitors are designed to prevent the synthesis of leukotrienes. Treatment with leukotriene modifiers is associated with reduced symptoms, improved lung function, reduced airway inflammation, and fewer exacerbations.[119-122] However, their role in the treatment of asthma is not clear as several studies have shown that leukotriene modifiers in combination with ICS are less effective than LABA in combination with ICS.[123-126]

Oral corticosteroids

Some patients with asthma may require systemic administrations of corticosteroids such as oral corticosteroids (OCS) to achieve clinical control.[127] However, long-term treatment with OCS is limited due to the risk significant side-effects such as osteoporosis, hypertension, diabetes, obesity, hypothalamic-pituitary-adrenal axis suppression, slow wound healing, muscle weakness, cataracts, and glaucoma.[128]

Anti-IgE

Patients that are not controlled with high doses of ICS and have elevated serum levels of IgE may benefit from anti-IgE treatment.[129-131] This treatment is made of humanized monoclonal antibodies that prevent mast cell activation by inhibiting soluble IgE and membrane-bound IgE on B cells.[132] Specifically, the anti-IgE antibodies bind to the epitope on IgE molecules that overlaps with the FcεRI binding site. In addition to this, depletion of soluble IgE is linked with a down-regulation of FcεRI expression on the surface of cells.[133,134] Treatment with anti-IgE is generally well-tolerated but its widespread use is limited due to high economical costs.

Bronchial thermoplasty

Bronchial thermoplasty (BT) is a novel non-drug device-based therapy that delivers temperature-controlled radiofrequency energy to the airway wall in a series of bronchoscopy procedures. The treatment aims to reduce airway smooth muscle mass in order to diminish bronchoconstriction. At 3 months and 1 year after treatment, studies have shown that BT results in improved asthma control, improved quality of life, fewer exacerbations, as well as reduced need for SABA and corticosteroids.[135-137] The short-term side effects of BT consist primarily of symptoms typical of asthma lasting up to 7 days after treatment.[138] Long-term safety data up to 5 years show no adverse side effects.[139,140] The mechanisms by which BT leads to clinical improvements are unclear. A reduction of smooth muscle mass following BT has recently been confirmed in patients with asthma,[141,142] yet the relationship to clinical improvements has not been evaluated. Heat energy produced during BT can potentially alter other structural components, meaning that additional mechanisms may underlie clinical improvements.

Difficult to control asthma

Classifications

Most patients with asthma can be controlled with β_2 agonists with or without low doses of ICS. However, an estimated 5-10% of the asthma population presents a

more troublesome disease often referred to as 'severe asthma'. Severe asthma has recently been defined as "asthma that requires treatment with high dose inhaled corticosteroids plus a second controller and/or systemic corticosteroids to prevent it from becoming "uncontrolled" or that remains "uncontrolled" despite this therapy".[127] By the same task force, uncontrolled asthma was defined as at least one of the following: "(1) Poor symptom control: ACQ >1.5, or ACT <20 (or "not well controlled" by NAEPP/GINA guidelines). (2) Frequent severe exacerbations: two or more bursts of systemic corticosteroids (>3 days each) in the previous year. (3) Serious exacerbations: at least one hospitalisation, ICU stay, or mechanical ventilation in the previous year. (4) Airflow limitation: after appropriate bronchodilator withhold FEV_1 <80% predicted (in the face of reduced FEV_1/FVC defined as less than the lower limit of normal".[127] ACT in the first criteria stands for the Asthma Control Test. This is a patient questionnaire form that can be used to classify asthma based on clinical control. It has been shown that an ACT score of <20 can be used as a threshold value to identify patients with poorly controlled asthma.[143] A score between 20 and the maximal value of 25 identifies patients with well-controlled asthma. In present thesis, ACT score was used as one tool to identify patients with poorly controlled asthma. In this context it should be mentioned that patients with poorly controlled asthma according to ACT may not necessarily meet the criteria for severe asthma as described above. Therefore, in present thesis the term 'difficult to control asthma' refers to patients that are poorly controlled despite treatment with ICS with our without OCS.

Mechanisms

Poor adherence to treatment is a significant problem in chronic disease that require long-term therapy, including asthma.[144] However, asthma is a complex disorder and experts agree that it is important to gain more insights into the underlying mechanisms in order to develop new therapeutic strategies.[98,145] Factors associated with difficult to control asthma include steroid resistance, peripheral airway inflammation, respiratory infections, and irreversible structural changes.[146]

Corticosteroid resistance

Corticosteroid resistance at a molecular level has been suggested to play an important role in patients with severe, difficult to control asthma.[147] This is supported by *in vitro* studies showing that circulating cells form such patients are less responsive to steroids than cells from well-controlled asthmatics.[148-151] The notion that some patients with severe asthma continue to present Th2 inflammation despite heavy treatment with ICS and OCS provides additional support.[101] Factors that may contribute to steroid resistance include (1) down-regulation in binding affinity to the glucocorticoid receptor, (2) up-regulation of the inactive isoform receptor for glucocorticoids, (3) interferences during the

activation of glucocorticoid responsive elements, or (4) lack of co-receptor activity. These and other potential mechanisms have been extensively described.[147] A more speculative underlying cause of steroid resistance involves type 2 innate lymphoid cells, which have been suggested to be more steroid-resistant as compared with Th2 cells.

Peripheral airway inflammation

Autopsy studies have shown that inflammation and structural changes in asthma extend to the peripheral airways.[152,153] In non-deceased asthmatics, transbronchial biopsy sampling has been identified as a tool to evaluate peripheral inflammation.[154] Using this technique, it has been shown that T helper cells and eosinophils infiltrate the alveolar tissue at night specifically in patients with nocturnal asthma.[155,156] The contribution of peripheral airway inflammation in the pathophysiology of asthma is unclear. However, small airway inflammation has been linked to air trapping and to the severe asthma phenotype.[157,158] Because conventional ICS have poor access to the peripheral airways,[159,160] inflammation in this region might explain the increased efficacy of extrafine-particle formulations of ICS and systemic administration of corticosteroids.[161]

Respiratory infections and fungal sensitisation

Respiratory infections are strongly associated with asthma exacerbations, with the common cold virus identified as being especially important.[162,163] Virus-induced exacerbations in asthma are accompanied by neutrophilic airway inflammation[164] and increased infiltration of T cells in the bronchial mucosa that is poorly responsive to ICS treatment.[165] Accumulating data suggest that viral infection and allergy act synergistically to increase the risk of an exacerbation that neither alone can produce.[166,167] This could be due to allergic Th2 polarization, as it has been shown that Th1 polarization (as measured by interferon-γ/IL-5 mRNA ratio) in sputum is associated with milder colds and a faster clearance of the virus.[168] Certain bacteria are also associated with asthma exacerbations, particularly *Mycoplasma pneumonia* and *Chlamydia pneumonia*.[169] Fungal sensitisation is strongly associated with an increased risk of developing fatal or near-fatal asthmatic attacks.[170-172] In contrast to viral and bacterial infections, fungal allergens evoke a Th2 inflammatory response. Of importance is that many fungal allergens contain proteases that can aggravate inflammation and damage the airway epithelium.[173,174] These effects are likely to explain the link between fungal sensitisation and fatal exacerbations.

Irreversible structural changes

Irreversible structural changes of the airways including airway smooth muscle enlargement, fibrosis, mucus metaplasia, and increased vascularity are believed to contribute to fixed airflow obstruction in asthma. While corticosteroids have been shown to reduce accelerated lung function decline in asthma,[175] they appear to

have little effect on structural changes.[176] Although the *in vivo* effect of corticosteroids on smooth muscle enlargement has not been evaluated, they fail to inhibit proliferation of bronchial smooth muscle cells obtained from those with asthma.[177] Bronchoconstriction plays a fundamental role in the pathophysiology of asthma and studies have shown that airway smooth muscle enlargement is associated with lower lung function.[28,32] To date, BT is the only FDA-approved treatment that directly targets structural changes in asthma. However, the mechanism of action of BT is not fully understood.

Aims

The general aim of this thesis was to study the relationship between histopathological alterations and clinical control in patients with asthma. Our specific aims were as follows:

Paper I

- To investigate if poorly controlled asthma is associated with inflammation in the alveolar parenchyma, a region where conventional ICS have poor access.

Paper II

- To compare bronchial airway inflammation between stable severe asthmatics and symptomatic severe asthmatics.
- To study structural changes that may be primary or secondary to the underlying severity of the disease.

Paper III

- To study the long-term effects of bronchial thermoplasty on bronchial structures and their association with clinical outcome in patients with uncontrolled severe asthma.

Paper IV

- To study the long-term effects of bronchial thermoplasty on markers of airway inflammation in patients with uncontrolled severe asthma.

Methods

This section provides an overview of the methods that were used. Further details can be found in the individual papers appended at the end of this thesis.

Study groups

Lung biopsies were obtained from healthy volunteers and patients with different severities of asthma. The clinical characteristics are presented in Table 1 on the next page.

Paper I

This study included 8 healthy volunteers, 12 patients with well-controlled atopic asthma, and 12 patients with poorly controlled atopic asthma. Asthma was diagnosed according to GINA guidelines. A standardised skin-prick test was used to screen for allergic sensitisation to ten different aeroallergens. Atopy was defined as a positive response (weal reaction larger or equal to histamine) to at least one them. An ACT score of 19 or less was used to identify patients with poorly controlled asthma.[143] From each subject, 5 bronchial and 5 transbronchial biopsies were taken at the Department of Respiratory Medicine, Lund University Hospital. The study was approved by the ethics committee in Lund, Sweden (LU412-03) and all subjects gave written and informed consent.

Paper II

This study included 25 patients with severe asthma according to ATS workshop criteria.[146] The asthmatics could be divided into two groups based on symptom profile according to patient journals, which included patient self-assessment data: 15 were classified as stable and 10 were classified as symptomatic. All patients were on daily doses of OCS. From each subject, 2-4 bronchial biopsies were taken at the Ear- Nose and Throat Department, Aarhus University Hospital. The ethics committee in Aarhus, Denmark approved the study and all subjects gave informed consent.

Table 1. Clinical characteristics and study design.

	Paper I			Paper II		Paper III	Paper IV
	Healthy controls	Well-controlled asthma	Poorly controlled asthma	Stable severe asthma	Symptomatic severe asthma	Severe refractory asthma†	Severe refractory asthma†
Sample size (n)	8	12	12	15	10	15	20
Females (%)	63	33	50	67	50	53	45
Age (years)	25.4 (±6.0)	28.5 (±6.1)	47.3 (±8.5)	44.9 (±15.8)	46.3 (±12.0)	46.9 (±11.9)	47.6 (±12.9)
BMI (kg/m^2)	22.9 (±2.5)	22.3 (±1.7)	24.4 (±2.4)	28.0 (±5.1)	30.2 (±4.6)	28.2 (±4.6)	28.5 (±5.0)
FEV_1 pred. (%)	99.1 (±14.5)	89.4 (±11.4)	82.7 (±14.9)	65.2 (±17.4)	64.7 (±18.8)	67.1 (±19.5)	66.4 (±18.3)
ACT score	-	21.6 (1.7)	13.6 (±4.3)	-	-	8.5 (±2.8)	8.5 (±2.7)
Atopy (% yes)	0	100	100	66	44	67	55
ICS (μg/day)	-	766 (±358)	809 (±268)	3094 (±1156)	3280 (±1494)	2133 (±516)	2100 (±447)
OCS (mg/day)	-	-	-	19.7 (±14.6)	20.0 (±13.7)	31.5 (±11.1)	33.0 (±12.2)
Biopsies taken per subject (n)	BX: 5, TX: 5			BX: 2 – 4		BX: 20‡	
Bronchoscope	Flexible			Rigid		Flexible	
Anatomical region	BX: First or second bifurcation in the right lung TX: Right lower lung			First bifurcation in the right lung		Right lower lobe (n = 3), upper lobes (n = 2), middle lobe (n = 2), lower left lobe (n = 3)	
Average No. biopsies analysed per subject	BX: 2, TX: 2			1		20	
Histological parameters analysed	T helper cell populations, T cytotoxic cells, mast cell populations, eosinophils, neutrophils, B-cells, macrophages, natural killer cells, basophils			T helper cell populations, T cytotoxic cells, mast cell populations, eosinophils, neutrophils, B-cells, macrophages, natural killer cells, basophils, epithelial morphology		Paper III: Airway smooth muscle mass, nerve fibres, neuroendocrine cells, lymph and blood vessels, epithelial morphology, SBM, glands, eosinophils, neutrophils Paper IV: T helper cells, T cytotoxic cells, mast cell populations, eosinophils, neutrophils, B-cells, macrophages, plasma B cells, basophils	

†Clinical data at inclusion, ‡10 biopsies taken before BT and 10 biopsies taken at 3 months after BT. Abbreviations: ACT=asthma control test, BMI=body mass index, BT=bronchial thermoplasty, BX=bronchial biopsy, FEV_1=forced expiratory volume in 1 s, ICS=inhaled corticosteroids (beclomethasone dipropionate equivalent), OCS=oral corticosteroids (prednisolone), SBM=subepithelial basement membrane, TX=transbronchial biopsy. Values are presented as mean (±SD) unless otherwise stated.

Paper III and IV

Paper III and IV included 15 and 20 patients, respectively, with severe asthma undergoing BT treatment at the Department of Pneumology A, Bichat University Hospital. Key inclusion criteria were: uncontrolled severe asthma, assessed by ACT ≤ 15, despite optimal management and maximal medical treatment for at least 12 months before entry; pre-bronchodilatory FEV_1 > 30% and < 80% of predicted, and at least 3 exacerbations, defined as worsening of asthma symptoms requiring OCS during the year before entry. Patients underwent 3 session of BT treatment, separated by one-month intervals. Heat-activation was delivered in the right lower lobe, the left lower lobe and the two upper lobes. No heat-activation was delivered in the middle lobe. A total of 20 biopsies were taken per patient (10 before BT and 10 after BT). The study was approved by the CPP Ile-de-France 1 Ethics committee (No. 2012-sept-13003) and all subjects gave written and informed consent. This trial is registered with ClinicalTrials.gov, identification number NCT01777360.

Tissue processing

Harvested biopsies were fixated in 4 or 10% buffered formaldehyde, dehydrated and embedded in paraffin at the departments where they had been obtained. The purpose of fixation is to minimize necrotic degradation that occurs once tissue is detached from its source of nutrients. Biopsies with well-preserved morphology were then selected for serial sectioning and immunohistochemistry. In Paper I, the obtained biopsies were sectioned and stained with haematoxylin (a basic dye that stains cell nuclei blue) in order to identify bronchial biopsies with well-preserved lamina propria and transbronchial biopsies with well-preserved alveolar parenchyma. A maximum of 2 bronchial and 2 transbronchial biopsies per individual were selected. In Paper II, a rigid bronchoscope was used to obtain large bronchial biopsies. A total of 25 biopsies (representing one biopsy per patient) were selected and sent to our laboratory. In Paper III and IV, all biopsies were serially sectioned and sent to our laboratory. To prepare samples for immunohistochemistry, tissue sections (stored at 4 °C) were subjected to heat-induced antigen retrieval. The purpose of this is to make antigens accessible for antibody binding as tissue fixation might introduce conformational changes of proteins.

Immunohistochemistry

Immunohistochemistry is a staining method used to detect antigens (such as proteins) in tissue sections. The method is based on the ability of a primary antibody to recognise a specific antigen (such as the cell markers used in this thesis). Once the primary antibody has bound its antigen, an enzyme-labelled secondary antibody is usually required to detect the formation. In present thesis, the secondary antibody was either directly or indirectly labelled with horseradish peroxidase (HRP) or alkaline phosphatase (AP). By adding enzyme-specific substrate chromogens, a coloured precipitate is produced around location of the antigen. The HRP-specific chromogens used in present thesis were DAB (3'3 diaminobenzide; Dako, Denmark) to produce a brown precipitate, Vina Green (Biocare Medical, USA) to produce a green precipitate and Deep Space Black (Biocare Medical) to produce a black precipitate. The AP-specific chromogen Permanent Red (Dako) was used to produce a red precipitate. The benefit of having several chromogens is that more than one antigen can be detected on the same tissue section. The primary antibodies that were used in this thesis have been routinely used for staining on human paraffin-embedded tissue sections in research and clinical diagnosis, or validated thoroughly in our laboratory. Antigen retrieval and antibody concentrations were optimised for each primary antibody to achieve a staining pattern in accordance with the literature. Blocking steps (such as inhibition of endogenous enzyme activities) were used to avoid false positive staining results.

Tissue analysis

Digitalisation of stained tissue sections

Stained tissue sections were digitalised in a slide-scanning robot operating with a 20x or 40x magnification lens (Aperio Technologies, USA). The generated images were analysed with different image analysis software's as outlined below.

Computerised image analysis

Stained sections were quantified by measuring the degree of immunoreactivity, i.e. the number of stained pixels divided by the total number of pixels. Briefly, a positive staining recognising algorithm is used to set a fixed threshold value for coloured pixels (Figure 6). Once set, the algorithm is applied on all sections stained for the specific marker(s). Single staining sections were analysed in

ImageScope (Aperio Technologies) whereas multiple-staining sections were analysed in Visiomorph DP (Visiopharm, Denmark). Computerised image analysis is more rapid and reproducible compared to manual assessment under a microscope.

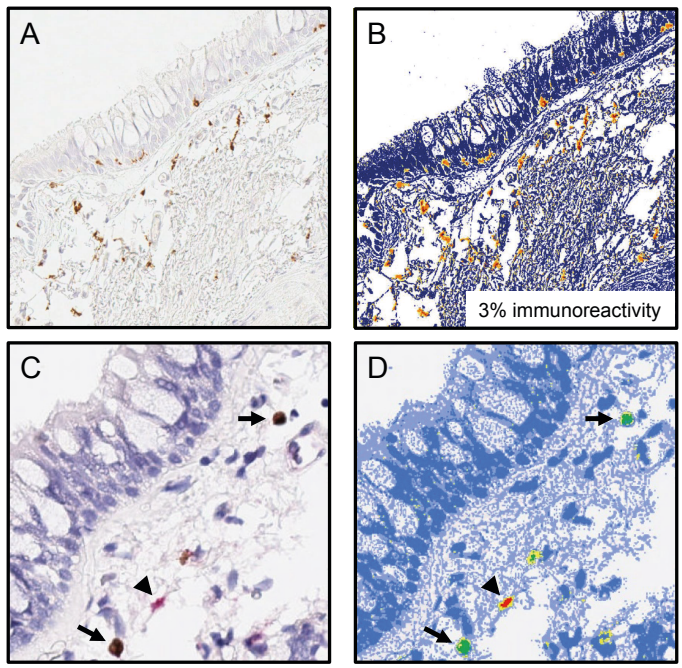

Figure 6.
(A) represents DAB stained cells that are detected in (B) using a positive staining algorithm in ImageScope. The accumulated number of orange (medium stained) and red (strong stained) pixels corresponded to 3% of the total number of coloured pixels, as automatically measured by the program. (C) represents DAB (arrows) and Permanent Red (arrowhead) stained cells that are separately detected in (D) using Visiomorph DP. Figure C and D are adapted from Sverrild A *et al*.[178]

Statistics

In Paper I and II, a non-parametric t-test was used to detect significant differences between two patient groups. The spearman rank (r_s) correlation test was used to detect significant correlations. All tests were two-tailed and $p < 0.05$ was considered significant. In Paper IV, a paired t-test was used to detect significant difference before and after treatment. The spearman rank (r_s) correlation test was used to detect significant correlations. All tests were two-tailed and $p < 0.05$ was considered significant. Details concerning statistics in Paper III can be found in the original manuscript appended at the end of present thesis.

Summary of Results

Paper I: Alveolar T-helper type-2 immunity in atopic asthma is associated with poor clinical control

Clinical findings

The clinical characteristics are presented in Table 1 in Methods. A statistical difference in age was found between the group of poorly controlled asthmatics (mean, 47 years) and the group of well-controlled asthmatics (mean, 29 years; $p < 0.0001$). In addition, BMI was slightly higher in the group of poorly controlled asthmatics (mean, 24.4 kg/m^2) compared with the group of well-controlled asthmatics (mean, 22.3 kg/m^2; $p < 0.05$). The dose of ICS did not differ between the two groups (mean dose budesonide, 630 µg/day; $p = 0.7$) nor did the predicted value of FEV_1 (mean 86.1 %; $p = 0.2$).

Leukocyte infiltration in the bronchial airways

No statistical difference in expression of T helper cells, T cytotoxic cells, B-cells, natural killer cells, macrophages, neutrophils, eosinophils, or basophils was found between the group of well-controlled asthmatics and the group of poorly controlled asthmatics.

Leukocyte infiltration in the alveolar parenchyma

The expression of T helper cells was statistically higher in the group of poorly controlled asthmatics compared with the group of well-controlled asthmatics ($p < 0.01$). Basophils were few in numbers, but statistically higher in the group of well-controlled asthmatics ($p < 0.05$). No statistical difference in the expression of T cytotoxic cells, B-cells, natural killer cells, macrophages, neutrophils, or eosinophils was found between the two groups.

Th2-scores and correlations to clinical control

To determine the degree of Th2 polarization, the log-value of the ratio between the number of Th2 and Th1 cells/mm^2 was used as a "Th2-score". In the bronchial airways, no statistical difference in Th2-score was found between the two groups ($p = 0.3$, Figure 7A). However, the alveolar Th2-score was statistically higher in the group of poorly controlled asthmatics ($p < 0.05$, Figure 7B). In contrast with bronchial Th2-score, alveolar Th2-score correlated statistically with ACT score in the pooled asthma group ($r_s = 0.62$, $p < 0.01$, Figure 7C and D respectively).

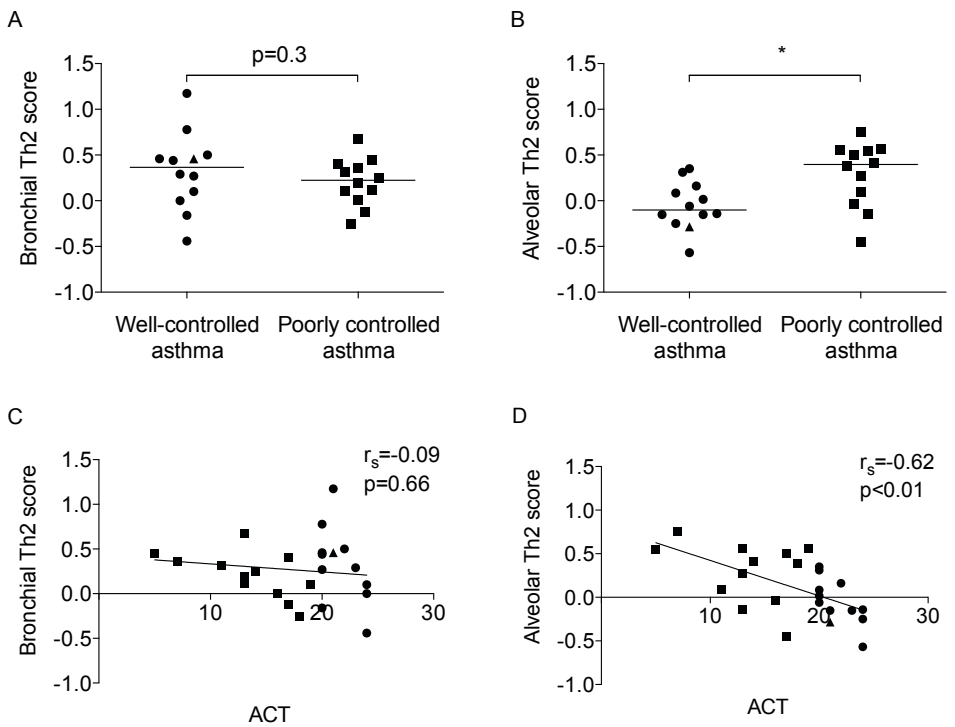

Figure 7.
Scattergrams showing Th2-scores (A and B) and correlations between Th2-scores and ACT score (C and D) in well-controlled and poorly controlled asthma in bronchial airways and alveolar parenchyma respectively. Each dot represents individual mean values and horizontal bars represent the median value for each patient group. The triangle represents the patient with well-controlled asthma who was treated with extrafine-particle formulation of ICS. *P < 0.05.

Infiltration of mast cells

We have previously shown that the poorly controlled asthmatics had an increased number of alveolar mast cells, highly positive for FcεRI and surface-bound IgE, as

compared with the healthy controls.[179] These results were in contrast with those obtained from the bronchial biopsies (Figure 8).

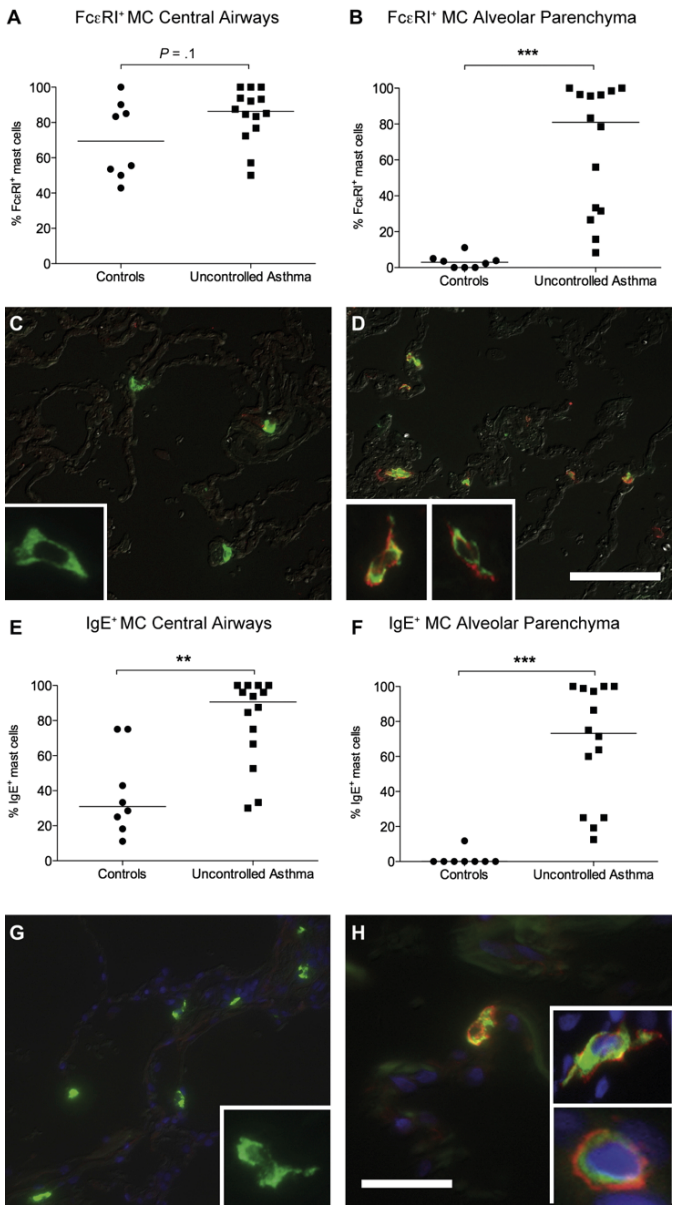

Figure 8

Scattergrams and micrographs showing mast cells positive for FcεRI (A-D) and IgE (E-H) in healthy controls and poorly controlled asthmatics in bronchial airways and alveolar parenchyma. Insets show mast cells double-positive for tryptase (green staining) and FcεRI or IgE (red staining). Each dot represents individual mean values and horizontal bars represent the median value for each group. Scale bars C, D, G = 50 μm; H = 25 μm. **P < 0.01 and ***P < 0.001. From Andersson CK *et al.*[179]

Paper II: Marked epithelial cell pathology and leukocyte paucity in persistently symptomatic severe asthma

Clinical findings

The clinical characteristics are presented in Table 1 in Methods. The two groups, stable SA (n = 15) and persistently symptomatic SA (n = 10), were matched in terms of gender, age (mean, 45 years; p = 1.0), BMI (mean, 28.8 kg/m^2; p = 0.3), dose of OCS (mean, 19.8 mg/day; p = 0.8), and the predicted value of FEV_1 (mean, 65.0%; p = 0.9).

Leukocyte infiltration in the bronchial airways

The group of symptomatic SA patients had statistically lower numbers of eosinophils (median 4,8 cells/mm^2) compared with the group of stable SA patients (median, 24.9 cells/mm^2; $p < 0.05$) (Table 2). In addition, the expression of Th2 cells and macrophages was lower in the group of symptomatic SA patients ($p < 0.05$ and $p < 0.01$, respectively). No statistical difference in the expression of T cytotoxic cells, mast cells, B-cells, natural killer cells, neutrophils, or basophils was found between the two groups.

Table 2. Leukocyte infiltration in the bronchial airways.

Leukocyte (Identification Marker[s])	Stable SA (*N = 15*)	Symptomatic SA (*N = 10*)	*P* Value*
T-helper cells ($CD4^+$)	1.3 (0.1–8.9)	0.8 (0.1–2.2)	0.37
Th2 cells ($CD4^+$ and $GATA3^+$), cells/mm^2	2.1 (0.0–7.3)	0.8 (0.0–2.2)	**<0.05**
T cytotoxic cells ($CD8^+$)	21.1 (6.0–56.5)	13.9 (3.5–71.3)	0.26
B-cells ($CD20^+$)	1.9 (0.4–14.9)	1.4 (0.3–11.8)	0.64
Natural killer cells ($CD57^+$)	4.2 (0.9–10.4)	1.9 (0.2–7.8)	0.13
Macrophages ($CD68^+$)	18.4 (7.5–82.0)	11.6 (4.9–34.3)	**<0.01**
Neutrophils (MPO^+)	4.3 (1.2–20.2)	2.6 (1.1–9.3)	0.93
Eosinophils ($EG2^+$), cells/mm^2	24.9 (0.6–101.4)	4.75 (0.0–61.6)	**<0.05**
Basophils ($BB1^+$), cells/mm^2	2.5 (0.0–25.5)	0.8 (0.0–4.8)	0.10
Total MC (MC_T + MC_{TC}), cells/mm^2	12.2 (2.3–57.2)	11.0 (1.1–65.9)	0.80
MC_T ($tryptase^+$ and $chymase^-$), cells/mm^2	6.1 (0.7–43.1)	6.0 (0.8–15.5)	0.89
MC_{TC} ($tryptase^+$ and $chymase^+$), cells/mm^2	3.9 (0.5–21.6)	3.8 (0.3–51.0)	0.45
% MC_{TC}	38.5 (17.5–83.6)	48.3 (25.0–77.8)	0.27

Definition of abbreviations: MC = mast cells; SA = severe asthma.

*Nonparametric Mann-Whitney test.

Values are presented as ‰ immunoreactivity and median (range) unless otherwise stated.

Epithelial morphology

The bronchial epithelium in both groups of severe asthmatics was abnormal. Metaplastic or squamous metaplastic epithelium was present in 77% of patients with stable SA and in 78% of patients with symptomatic SA. Furthermore, regenerating epithelium was present in 8% of patients with stable SA and 33% in patients with symptomatic SA. The epithelium in both groups was also associated with patchy areas with a high degree of cell proliferation, as revealed by the proliferation antigen Ki-67. The results are presented in Figure 9.

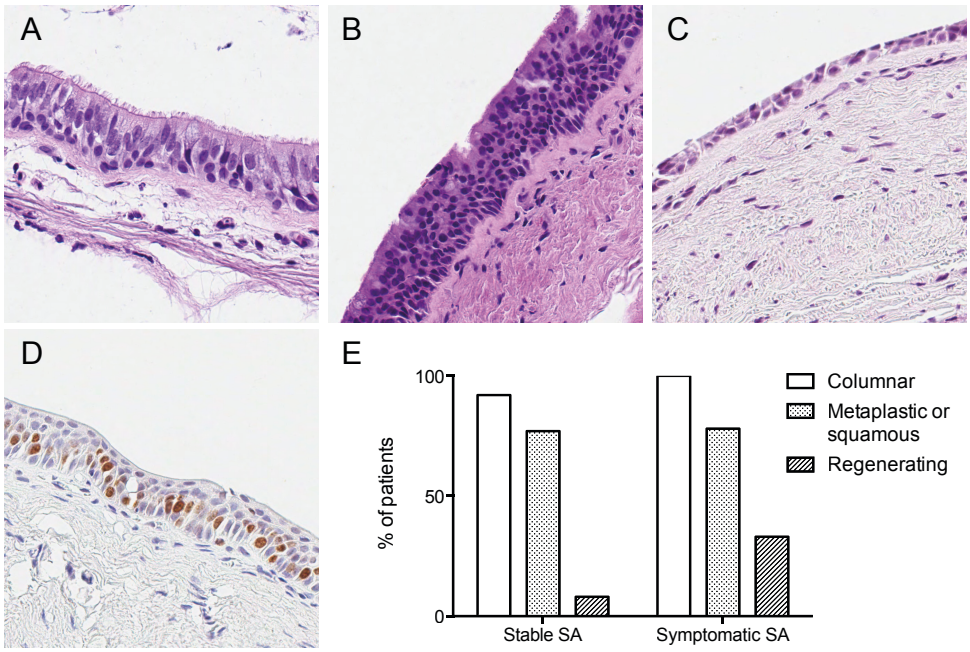

Figure 9.
Bright-field microscopic images exemplifying (A) normal columnar epithelium, (B) metaplastic epithelium, (C) regenerating epithelium, and (D) squamous metaplastic epithelium with a high degree of cell proliferation (Ki-67 positive cells in brown staining). (E) The number of patients presenting the different types of epitheliums. No statistical differences were found between the two groups.

Airway smooth muscle surface area

The surface area of airway smooth muscle was similar between stable SA (median [25-75 interquartile range] = 11.4% [6.4-23.2]) and symptomatic SA (median [25-75 interquartile range] = 15.6% [11.8-27.8]) (p=0.17) (data not shown).

Paper III: Effectiveness of bronchial thermoplasty in patients with severe refractory asthma: clinical and histopathological correlations

Clinical findings

Clinical effects of BT were examined at 3 and 12 months after treatment. ACT score improved from 8.5±2.8 (baseline) to 15.7±4.8 (at 3 months) to 16.4±6.9 (at 12 months) (overall $p < 0.001$). This was accompanied by a similar improvement in AQLQ score. The number of severe exacerbations (adjusted for 3 months) improved from 9.7±5.2 (baseline) to 0.7±1.1 (3 months) to 0.7±1.6 (12 months) (overall $p < 0.001$). This was accompanied with fewer hospitalisations for asthma, fewer visits to the emergency department, and fewer hospitalisations in the intensive care unit (ICU). Treatment with BT also resulted in reduced need for OCS, with a reduction from 31.5±11.1 mg/day (baseline) to 20.6±12.4 mg/day (3 months) to 13.8±5.2 mg/day (12 months) (overall $p = 0.002$). Furthermore, the number of patients requiring anti-IgE treatment went down from ten (at baseline) to none (at 3 and 12 months) (overall $p < 0.001$). A reduced need of anti-histamine and of nebulized anti-cholinergics and β_2 agonists was also observed ($p < 0.05$ and < 0.001 respectively).

Effect of BT on bronchial structures

At baseline, the surface area of airway smooth muscle (ASM) ranged from 9.1 to 30.3% (median [25-75 interquartile range] = 19.7% [16.2-21.8]). BT resulted in a significant reduction of the ASM area at 3 months, with a median [25-75 interquartile range] value of 5.2% [3.7-9.8] ($p < 0.001$) (Figure 10). This was accompanied by a significant increase of collagen deposition ($p < 0.003$). BT also resulted in a significant decrease of nerve fibres in the lamina propria region ($p < 0.001$), of ASM-associated nerve fibres ($p < 0.05$), and of epithelial neuroendocrine cells ($p < 0.05$) (Figure 10). BT marginally decreased SBM thickness (from 4.4 μm to 3.9 μm, $p < 0.05$) without affecting blood vessels, lymph vessels, submucosal glands, or epithelial morphology.

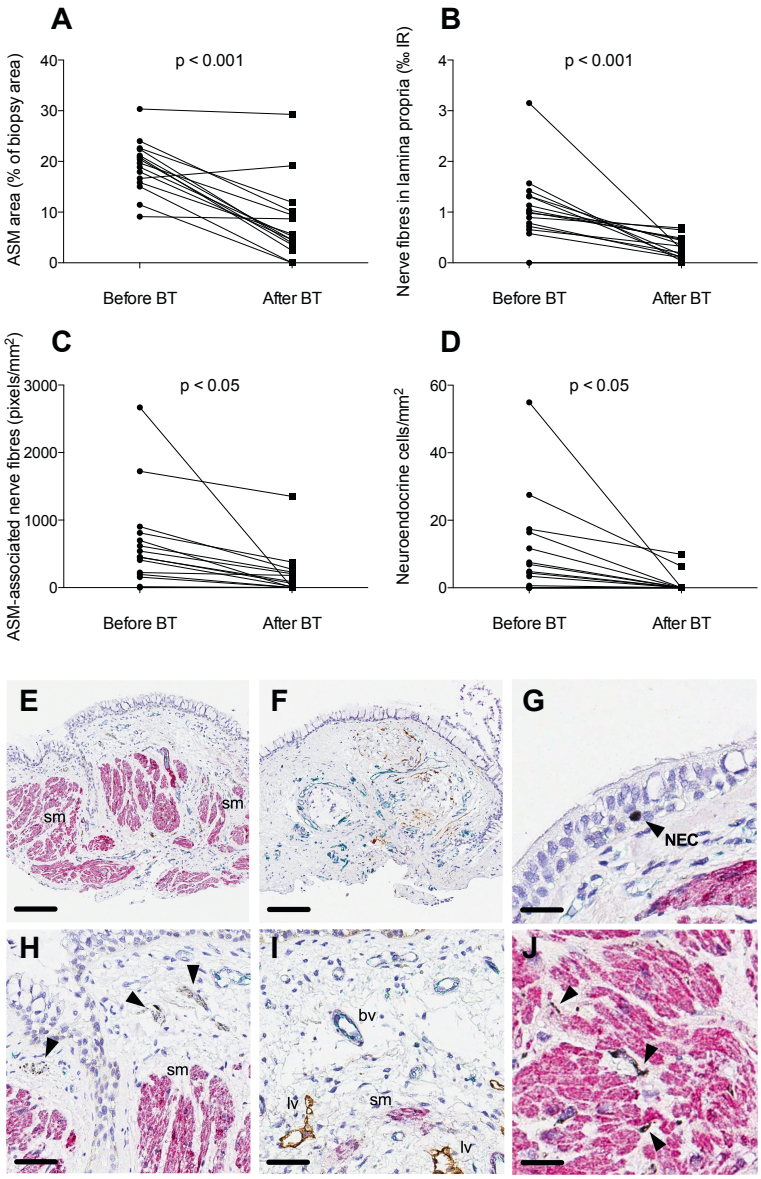

Figure 10.
Effect of BT on (A) airway smooth muscle surface area, (B) nerve fibres in the lamina propria, (C) ASM-associated nerve fibres, and (D) neuroendocrine epithelial cells. Each dot represent the median value derived from the set of biopsies analysed per patient. Micrographs (E) and (F) illustrate biopsies taken before and 3 months after BT, respectively. Micrograph (G) illustrates a neuroendocrine epithelial cell (arrowhead). Micrograph (H) and (I) illustrate nerve fibres (arrowheads) in the lamina propria before and after BT, respectively. Micrograph (J) illustrates ASM-associated nerve fibres (arrowheads). Scale bars = 250 µm in (E) and (F), 40 µm in (G), (H), (I) and (J). Smooth muscle (sm), blood vessles (bv), and lymph vessels (lv) are shown in the micrographs. IR = immunoreactivity.

Because 4 out of the 15 patients showed only partial clinical improvements after treatment, we performed a separate analysis where we compared their values to the BT-responsive patients (Figure 11). The group of partially responsive patients had a mean value of ASM area of 14.6%, as compared with 5.7% for the group of responsive patients ($p < 0.05$). There were no differences in terms of nerve fibres between the two groups. However, the number of neuroendocrine cells was statistically higher in group of partially responsive patients ($p < 0.05$).

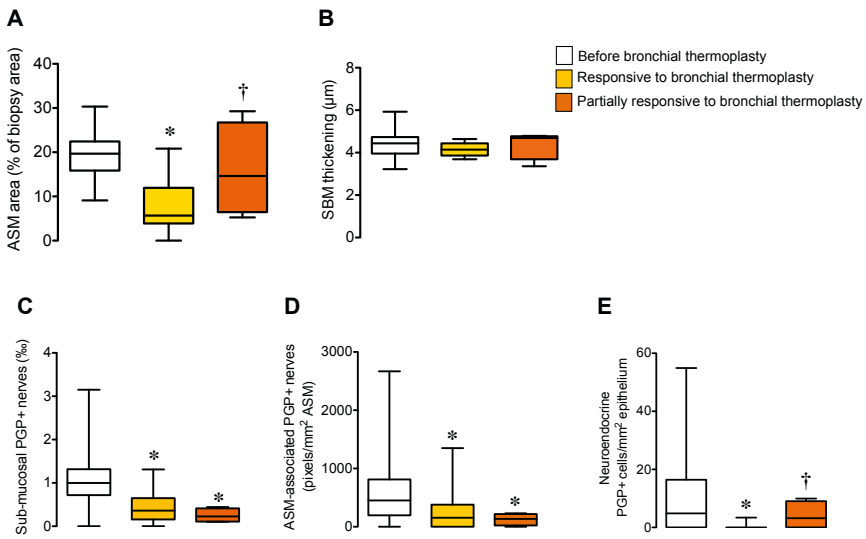

Figure 11.
Assessment of (A) airway smooth muscle (ASM) surface area, (B) subepithelial basement membrane (SBM) thickening, (C) nerve fibres in lamina propria, (D) ASM-associated nerve fibres, and (E) epithelial neuroendocrine epithelial cells before and at 3 months after BT treatment. Patients were separated into two groups based on clinical responsiveness: 11 patients were responsive (yellow bars) and 4 patients were partially responsive (red bars). Overall p values were < 0.01 (A), 0.31 (B), < 0.01 (C), 0.06 (D), and < 0.01 (E). * $p < 0.05$, as compared to values obtained before BT, † $p < 0.05$, as compared to patients responsive to BT.

Correlations to clinical outcomes

The structural changes correlated significantly with several measurements of clinical control at 3 and 12 months after treatment (Table 3). At 3 months, the surface area of ASM correlated significantly with ACT score ($p < 0.001$), with the number of severe exacerbations ($p < 0.001$), with the number of visits to the emergency department ($p < 0.001$) and with the number of hospitalisations for asthma ($p = 0.01$). Similar findings were found at 12 months. The reduction of neuroendocrine cells was the unique parameters that correlated significantly with all elements of control, including the number of hospitalisations in the ICU.

Table 3. Correlation analyses between histopathological parameters and asthma control after bronchial thermoplasty.

Parameter	Score on ACT		Score on AQLQ		No. of severe exacerbations		No. of visits to emergency department		No. of hospitalization for asthma		No. of hospitalization in ICU	
	r	P value [a]	r	P value	r	P value	r	P value	r	P value	r	P value
Results 3 months after bronchial thermoplasty												
ASM area	- 0.600	**< 0.001** [b]	- 0.321	0.08	0.69	**< 0.001**	0.616	**< 0.001**	0.457	**0.01**	0.309	0.10
SBM thickening	- 0.503	**0.005**	- 0.332	0.07	0.372	**0.04**	0.281	0.13	0.408	**0.03**	0.345	0.06
Submucosal PGP+ nerves	- 0.367	**0.04**	- 0.185	0.33	0.689	**< 0.001**	0.400	**0.03**	0.168	0.37	- 0.090	0.64
ASM-associated PGP+ nerves	- 0.187	0.32	- 0.041	0.83	0.433	**0.02**	0.219	0.25	0.154	0.42	- 0.052	0.78
PGP+ neuroendocrine cells	- 0.508	**0.004**	- 0.376	**0.04**	0.526	**0.003**	0.510	**0.004**	0.592	**< 0.001**	0.423	**0.02**
Results 12 months after bronchial thermoplasty												
ASM area	- 0.516	**0.003**	- 0.432	**0.02**	0.580	**< 0.001**	0.572	**< 0.001**	0.310	0.10	0.189	0.32
SBM thickening	- 0.388	**0.03**	- 0.364	**0.05**	0.341	0.07	0.296	0.11	0.386	**0.04**	0.255	0.17
Submucosal PGP+ nerves	- 0.236	0.21	- 0.232	0.22	- 0.667	**< 0.001**	0.518	**0.003**	0.202	0.29	0.001	0.98
ASM-associated PGP+ nerves	- 0.144	0.45	- 0.112	0.56	0.351	0.06	0.231	0.22	0.092	0.63	- 0.034	0.86
PGP+ neuroendocrine cells	- 0.502	**0.005**	- 0.452	**0.01**	0.506	**0.004**	0.538	**0.002**	0.501	**0.005**	0.387	**0.04**

Abbreviations: ACT, asthma control test; AQLQ, asthma quality of life questionnaire; ASM, airway smooth muscle; SBM, subepithelial basement membrane; ICU, intensive care unit; PGP, Protein Gene Product (nerve and epithelium neuroendocrine cell marker).

[a] Spearman's rank-order method and Benjamini and Hochberg correction

[b] Bold denotes significant correlation.

Paper IV: Immunological effects induced by bronchial thermoplasty in patients with severe refractory asthma

Effect of BT on mast cell populations

At 3 months after treatment, the total mast cell content in the lamina propria region decreased by 49% ($p < 0.001$) (Figure 12). This was accompanied by a significant decreased of both MC_T and MC_{TC} ($p < 0.01$ and $p < 0.05$ respectively). In the smooth muscle mass the expression of MC_T decreased by 58% ($p < 0.05$) whereas no statistical changes were observed for total mast cells or MC_{TC}. The expression of mast cells in the epithelium was not significantly modulated by BT. The relative proportion of MC_{TC} remained similar before and after BT treatment in all anatomical regions (data not shown).

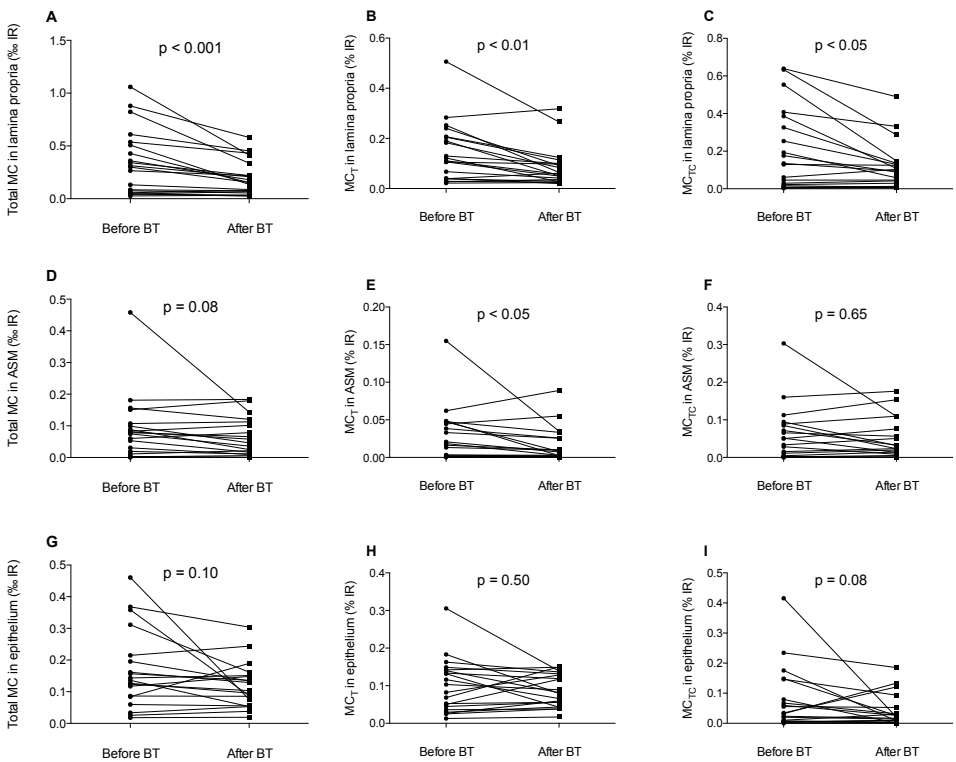

Figure 12.
Effect of BT on total MC, MC_T, and MC_{TC} in lamina propria (A, B and C respectively) in airway smooth muscle (D, E and F respectively) and in epithelium (G, H and I respectively). Each dot represent the median value derived from the set of biopsies analysed per patient. IR = immunoreactivity.

Effect of BT on other leukocyte populations

The expression of T cell subtypes in the lamina propria was significantly lower after BT treatment. T cytotoxic cells decreased by 30% ($p < 0.01$) whereas T helper cells decreased by 59% ($p < 0.001$) (see Figure 13). No change in the expression of eosinophils, B-cells, plasma cells, neutrophils, macrophages, or basophils was found after BT in the lamina propria.

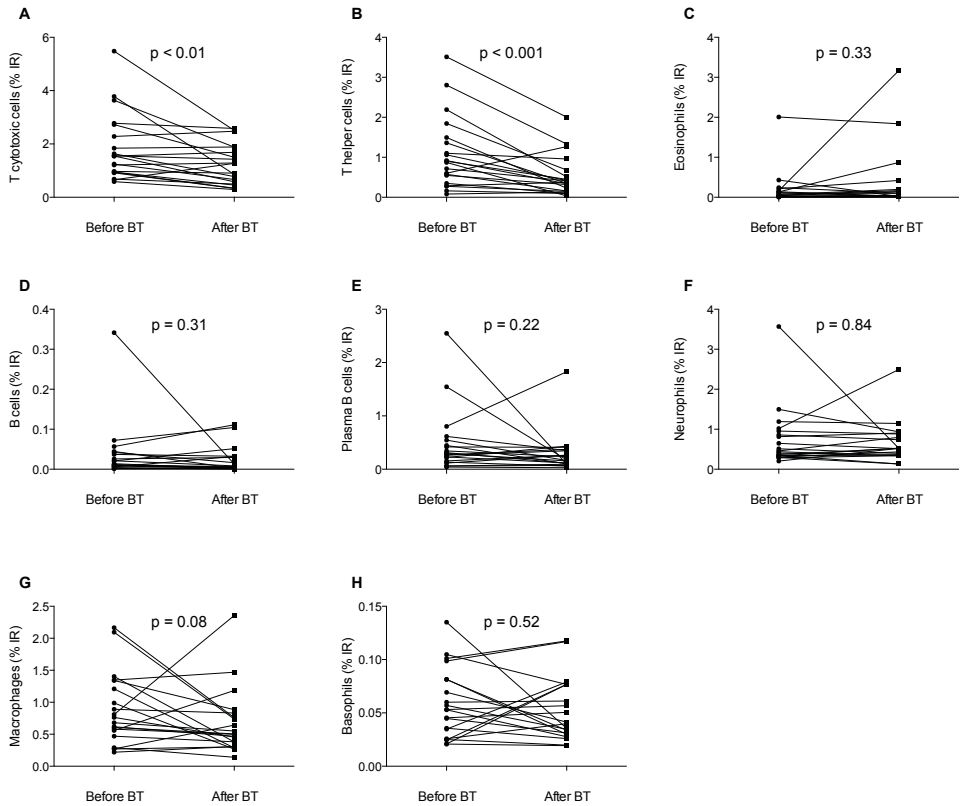

Figure 13.
Effect of BT on (A) T cytotoxic cells, (B) T helper cells, (C) eosinophils, (D) B cells, (E) plasma B cells, (F) neutrophils, (G) macrophages, and (H) on basophils in the lamina propria at 3 months after treatment. Each dot represent median value derived from the set of biopsies analysed per patient. IR = immunoreactivity.

Discussion

Alveolar Th2 inflammation in poorly controlled asthma (Paper I)

In this study, we found no difference in bronchial airway inflammation between poorly controlled and well-controlled asthma. These data suggest that ICS control bronchial inflammation in both groups. However, poorly controlled asthma was associated with a Th2 polarization of the alveolar parenchyma. This leads to the question whether allergic reactions may take place in this part of the lungs. Common aeroallergens such as house dust mite and pollen grains are likely too large to reach beyond the large airways. However, it has been shown that allergens of smaller particle size such as cat allergens can reach the small airways.[180] Moreover, air pollutants (such as diesel exhaust) may act as carriers to transport allergen fragments into the alveolar region.[181,182] We have previously shown that the poorly controlled asthmatics had an increased number of alveolar mast cells, highly positive for FcεRI and surface-bound IgE, as compared with the healthy controls.[179] Therefore, alveolar deposition of allergens could be expected to trigger mast cell degranulation in these patients. The consequence of such response remains to be investigated. Considering the combined surface area of alveoli and its close proximity to blood vessels, it could be speculated that activated leukocytes in this region could "leak" inflammatory cytokines into the blood stream. Cytokines released into the blood circulation can effect allergic airway responses according to mouse studies.[183,184] With regards to this, it would be interesting to evaluate if circulating cytokines from Th2 cells or mast cells can influence the clinical control of asthma. In summary, our data show that all immunological components are present to trigger an alveolar allergic reaction in patients with poorly controlled asthma. Although more research is needed in this field, our two studies provide a rationale to target peripheral airway inflammation in patients who remain symptomatic despite standard ICS treatment.

External assaults in eosinophilic-low severe asthma (Paper II)

In this study, we found that symptomatic severe asthma was associated with lower number of eosinophils as compared with stable severe asthma. Eosinophilic asthma is associated with a better responsiveness to ICS,[99,185] but it is likely that the latter group of patients had a severe underlying inflammation that required OCS to control, as has previously reported.[186] Surprisingly, all leukocyte

populations were numerically lower in the group of patients with symptomatic severe asthma. This suggest that no further clinical improvement is likely to be achieved by increasing the dose of OCS. In both patient groups, we found stretches of metaplastic epithelium, squamous metaplastic epithelium, and regenerating epithelium. Epithelial metaplasia is considered to be a precursor of squamous epithelial metaplasia, which in the airways can be induced by a variety of external assaults.[187] It can be induced by tobacco smoke and other agents, including viruses and bacteria.[188-190] Although our group of patients had a history of smoking, we detected squamous epithelium in one never-smoking individual and one ex-smoker since over ten years. Of note is that in Paper III, which included 13 never-smokers, 1 ex-smoker and 1 active smoker, we detected squamous epithelial metaplasia in 8 out of the 15 patients before BT treatment. These data indicate that squamous bronchoepithelial metaplasia in severe asthma can be induced by external assaults other than tobacco smoke. In the group of patients with eosinophilic-low symptomatic severe asthma, such assaults may include episodic pathogen infections where resulting epithelial alterations generally out-last acute inflammatory responses. In light of this, it has been shown that eosinophil-low severe asthma is more responsive to add-on treatment with antibiotics as compared with eosinophilic-high severe asthma.[191] In summary, our data suggest that eosinophilic-low severe asthma is: (1) more symptomatic and (2) not associated with on-going bronchial inflammation, as compared with eosinophilic-high severe asthma. Factors that trigger metaplasia and squamous metaplasia of the bronchoepithelium in symptomatic/uncontrolled severe asthmatics merit further investigation.

BT in uncontrolled severe asthma: clinical and histopathological correlations (Paper III)

In this study, we found that BT treatment drastically improved clinical control in a group of uncontrolled severe asthmatics. The clinical improvements persisted to 12 months after treatment, with a 92% decrease in the number of severe exacerbations, 90% fewer visits to the emergency department, 88% fewer visits to the ICU, and 93% and 62% improvements in ACT and AQLQ, respectively. It should be mentioned that these effects were of greater magnitude compared to previous studies where less severe asthmatics had been treated.[136,137] Consistent with these studies, we found that BT failed to alter pre or post-bronchodilator FEV_1. BT resulted in a significant reduction of airway smooth muscle surface area, as previously revealed.[141,142] Of novelty, we found this reduction correlated significantly with several measurements of clinical control. Unexpectedly, we found a significant decrease of nerve fibres in the lamina propria that also correlated with several elements of clinical control. Because we used a pan marker to detect neural structures (PGP 9.5), further work is needed understand this mechanism. It could be speculated that BT resulted in a depletion of cholinergic

nerve endings, which upon stimulation can induce bronchoconstriction and mucus secretion.[192] Also unexpectedly, we found that the number of neuroendocrine epithelial cells decreased by approximately 95% following BT treatment. The role of neuroendocrine cells in the pathophysiology of asthma is currently unclear. However, these specialised cells express chemoreceptors that can respond to stimulus such as O_2 concentration by modulating airway tone and control of breathing.[2] The reduction of neuroendocrine cells was the unique parameter that correlated with all elements of control, including visits to the ICU. In the 4 out of 15 patients that remained uncontrolled as assessed by ACT and AQLQ scores, BT failed to decrease airway smooth muscle surface area and the number of neuroendocrine cells. This indicates a causal relationship between the ablation of these structures and clinical benefit. Taken together, this study shows that BT is an effective treatment option in patients with uncontrolled severe asthma. The clinical improves were associated with a down-regulation of structures involved in airway narrowing and hyperreactivity including airway smooth muscle, neuroendocrine epithelial cells, and mucosal nerve fibres.

Long-term immunological effects of BT in uncontrolled severe asthma (Paper IV)

In this study, we found that BT resulted in a significant reduction of mast cell and T cell subtypes. Other leukocyte populations including eosinophils, B cell subtypes, neutrophils, macrophages, and basophils were not altered at 3 months after treatment. Previous work have reported the immunological effects of BT on inflammatory indices in BAL fluid at 3 and 6 weeks post treatment.[193] Our study was designed to look at more long-term immunomodulatory effects in the bronchial mucosa. Heat shock induced by BT can potentially damage the airway epithelium, which should trigger an inflammatory response. However, we found in Paper III that the structure of the epithelium at 3 months after treatment was similar to baseline conditions. Furthermore, in present study we found that immune cells associated with damage and repair, such as neutrophils and macrophages, were not elevated after treatment. Collectively, these data indicate that any heat-induced inflammation caused by BT resolves within 3 months. The down-regulation of mast cells in the lamina propria and to some extent in smooth muscle bundles could explain some of the clinical benefits associated with BT, although this remains to be investigated. BT treatment also resulted in a reduction of T helper cells in the lamina propria. This was not accompanied by a reduction of eosinophils, suggesting that BT has no long-term modulatory effect on Th2 associated inflammation in asthma. Due to the lack of neutrophilia, which is strongly associated with Th17 polarization, it could be speculated that Th1 cells were decreased by BT in present study. Th1 cells have been implicated in the pathogenesis of asthma although their role is not well understood.[194] Intriguingly, a recent mouse study has shown that the main effector cytokine of Th1 cells (IFN-

γ) can enhance mast cell activity and promote airway hyperresponsiveness.[195] Furthermore, a human study has shown that IFN-γ can induce the expression of a chemokine that attracts mast cells to smooth muscle bundles.[196] In present study, we also found a significant reduction of T cytotoxic cells following BT treatment. Their role in asthma is poorly understood, however T cytotoxic cells are also known to secrete IFN-γ. It is of note that down-regulation of leukocyte populations observed in present study took place despite a decreased need for daily OCS treatment. Taken together, this study shows that BT treatment has clear and long-lasting effect of mast cell and T cell populations. The relationship to clinical improvements remains to be investigated.

Conclusions

- Alveolar Th2 polarization is associated with poor clinical control (as assessed by ACT < 20) in patients with atopic asthma who are on standard ICS treatment.

- Eosinophilic-low severe asthma is: (1) more symptomatic and (2) not associated with on-going bronchial inflammation, as compared with eosinophilic-high severe asthma. Epithelial metaplasia in the former patient category suggests that external assaults, possibly episodic pathogen infections, may play an important role in this type of asthma.

- Bronchial thermoplasty is an effective treatment option in patients with uncontrolled severe asthma. Clinical improvements were associated with a down-regulation of structures involved in airway narrowing and hyper-responsiveness, including airway smooth muscle, neuroendocrine cells and nerve fibres.

- Bronchial thermoplasty treatment has clear and long-lasting immunological effects on the airway tissue in patients with uncontrolled severe asthma. Specifically, we show that the treatment is associated with a down-regulation of mast cell and T cell populations in the lamina propria region and in airway smooth muscle bundles.

Future Perspectives

The results in this thesis provide new histopathological data that are associated with clinical control in patients with asthma. During the course of our work, a number of questions have emerged that merit further investigation. Firstly, more research is needed to understand the role of peripheral airway inflammation in asthma. This is of particular interest since asthma is still mainly regarded and pharmacologically treated as an inflammatory disorder of the large conducting airways. Based on our results and others, inflammation in asthma may spread to include not only small airways but also alveoli. This gives a rationale to target peripheral airway inflammation in more severe forms of the disease, especially before adding oral corticosteroids to a daily treatment regimen. With regards to severe asthma, more research is needed to identify factors that may be involved in the eosinophilic-low subtype. We found that this symptomatic form of asthma is associated with bronchoepithelial metaplasia, possibly induced by episodic pathogen infections. The concept that infections may promote "asthma" is controversial. However, the study by Brusselle and co-workers showing that eosinophilic-low severe asthma is specifically responsive to antibiotic treatment indicate that pathogen infections may indeed play an important role in this form of asthma. With regards to novel therapeutic strategies for severe asthma, we show that bronchial thermoplasty is an effective treatment option for highly symptomatic patients. Apart from airway smooth muscle, we identify neuroendocrine epithelial cells and, to a lesser extent, nerve fibres as important targets whose down-regulation after treatment correlated with clinical efficacy. Because we used a pan marker to detect nerve fibres, follow-up studies are needed to identify what type of nerves that were affected. More studies are also needed to understand the role of neuroendocrine cells in asthma. Bronchial thermoplasty is also associated with a long-term down regulation of mast cell and T cell populations in the bronchial mucosa. Follow-up studies are needed to establish how these immunomodulatory effects are associated with clinical improvements. Bronchial thermoplasty was introduced in 2010 as a non-drug intervention for uncontrolled severe asthma. The first patient to receive this treatment in Sweden was in 2015, at the Department of Respiratory Medicine and Allergology, Lund University Hospital, Sweden.

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