Airway Inflammation during Clinical Remission of Atopic Asthma

Effect of anti-inflammatory therapy

Luchtwegontsteking tijdens klinische remissie van atopisch astma

Effect van ontstekingsremmende therapie

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Voor Angelique

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Chapter

0

Preface

"Panic.

Started too enthusiastic for a long run into the cold. I stop, but it is too late. A wheezing sound swells from my chest and accompanies the cough symphony. All my efforts to take a deep breath are in vain. I feel like choking. More than ever, I'm conscious of one of the vital functions of the human body known as respiration".

The unique character of respiration is that it is not only regulated by automatic centers located in the brainstem, but also by voluntary signals originating from the cortex. As a consequence of this, we are also aware of the occurrence of a discrepancy between supply and demand with respect to ventilatory function, a discrepancy very familiar to subjects with asthma under the term shortness of breath. I have the disputable honor to be among these subjects, that is to say when it comes to asthmatic symptoms experienced in early childhood. Fortunately, symptoms completely disappeared in early adulthood and, to the present day, never returned.

Have I grown out of asthma? Or are there merely no symptoms at the moment? And, if the latter is true, do I carry the risk of a relapse later in life and, moreover, should I take anti-inflammatory medication?

That is what this thesis is about….

Chapter

1

General introduction

1.1 Definition of asthma

Asthma has been well recognized since ancient times. Although there may be reference to asthma in Egyptian papyrus records from the second millennium BC, it was differentiated as a discrete disorder of breathing by the Greeks.

Figure 1: Hippocrates

The description of asthma as a disease with airflow limitation as the main component resulted in a definition derived from the first CIBA symposium in 1959:

"Asthma is a condition with widespread narrowing of the bronchial airways, which changes in severity over short periods of time, either spontaneously or under treatment, and is not due to cardiovascular disease"

This definition of asthma has been updated as aspects of its pathophysiology became recognized. In 1987, for example, the American Thoracic Society emphasized bronchial hyperresponsiveness in the definition of asthma (1). However, the growing evidence for the central role of inflammation in the pathogenesis of asthma led the 1991 National Asthma Education Program (Expert) Panel (NAEPP) Report from the National Institutes of Health (NIH) to define asthma as a lung disease with the following characteristics:

1 Variable airflow obstruction.

2 Airway inflammation.

3 Increased airway responsiveness to a variety of stimuli.

The concept of airway inflammation as central element in asthma was incorporated in a 1995 workshop report, which was revised in 2002, from the Global Initiative for Asthma (GINA), sponsored by the World Health Organization (WHO) and the NIH, where asthma was finally defined as:

"...a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow limitation that is often reversible, either spontaneously or with treatment." In 1997, the NAEPP expert panel report II guidelines subsequently included the variable presence of airway remodelling aspects in the working definition of asthma.

Presumably, because of the heterogeneity of the syndrome there will never be an all embracing definition of asthma, creating difficulty both in obtaining accurate numbers to determine the prevalence of asthma and in comparing the results of different studies.

1.2 Classification of asthma

"Physicians think they do a lot for a patient when they give his disease a name"

Immanuel Kant

Clinically usable classifications of disease are dependent on good descriptions of tissue morphology characteristics. So far, because of concerns with reference to the safety and ethics of employing invasive methods such as flexible bronchoscopy to obtain bronchial biopsies, the use of histopathology to define and classify asthma has lagged behind.

Atopic and non-atopic forms of asthma

Asthma may be subdivided according to the presence or absence of atopy. Atopy is defined as the enhanced sensitivity of individuals to common and otherwise innocuous aeroallergens such as house dust mites, pollen and allergenic substances of animal origin.

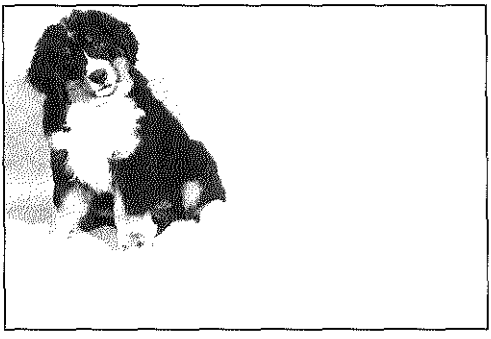


Figure 2: A source of allergens

Classification of asthma according to severity

The GINA workshop has proposed the first classification of asthma based on clinical features as well as treatment requirements. This classification makes use of asthmatic symptoms, spirometry values, peakflow variability and the necessity of using short acting β_2-agonists. Asthma is categorized as either intermittent or persistent. Persistent asthma is further subdivided into mild, moderate and severe. Although there is much overlap among these groups – and some patients do not fit easily into one category – this classification provides a useful guide for physicians in assessing their patients' asthma severity and prescribing the correct treatment.

Classification of asthma

	Clinical features	Medication required
I	**Intermittend** Intermittent symptoms < once a week. Brief exacerbations. Nocturnal symptoms < twice/month. Normal lung function between episodes.	None necessary
II	**Mild persistend** Symptoms > once a week, but < once a day. Nocturnal symptoms > twice a month, but < once a week. Normal lung function between episodes.	Inhaled glucocorticosteroid (≤ 500 µg BDP or equivalent) Consider theophylline, cromone, or leukotriene modifier.
III	**Moderate persistend** Symptoms daily. Exacerbations affect activity and sleep. Nocturnal symptoms at least once a week. FEV_1 60 - 80 % predicted or PEF 60 - 80 % of personal best	Inhaled glucocorticosteroid (200 – 1000 µg BDP or equivalent) plus long-acting.inhaled β_2-agonist. Consider theophylline, leukotriene modifier, long-acting oral β_2-agonist or higher dose inhaled glucocorticosteroid.
IV	**Severe persistent** Symptoms daily. Frequent exacerbations. Frequent nocturnal asthma symptoms. FEV_1 < 60 % predicted or PEF < 60 % of personal best	Inhaled glucocorticosteroid (> 1000 µg BDP or equivalent) plus long-acting.inhaled β_2-agonist plus theophylline, leukotriene modifier, long-acting oral β_2-agonist or oral glucocorticosteroid.

Based on US DHSS, NIH, NHLBI, Asthma Management and Prevention. Global Initiative for Asthma. A practical guide for public health officials and health care professionals. NIH publication no. 96-3659A, December 1995, revised in 2002.

1.3 Epidemiological aspects

Prevalence

The literature is replete with studies reporting the prevalence of asthma from different countries around the world. However, comparison of these prevalence estimates, ranging from 1% to 30%, has been difficult given the different methods used between studies. One multicenter study, the International Study of Asthma and Allergy in Children (ISAAC) has addressed this issue, and confirmed the wide variations in prevalence of asthma symptoms, even though assessed with the same instruments, in children worldwide (figure). There is substantial evidence that asthma prevalence is increasing throughout the world (2).

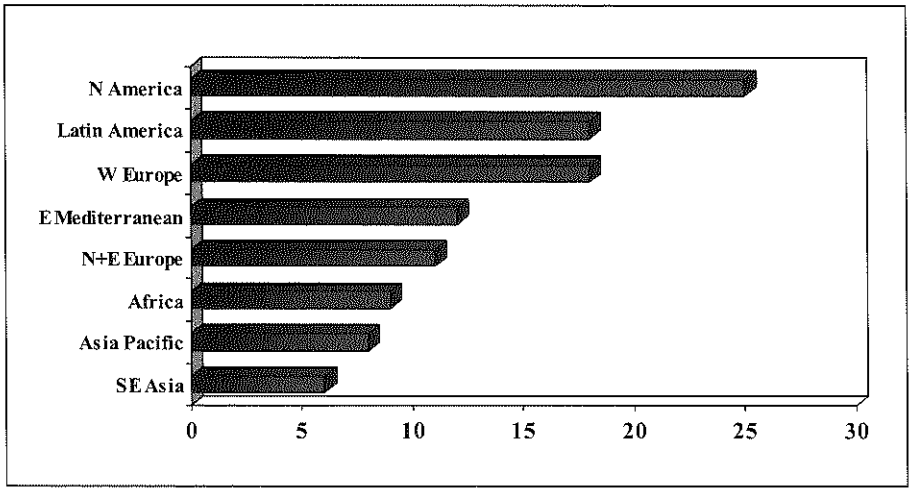


Figure 3: Prevalence (%) of asthma worldwide.50

The rate of increase in prevalence is such that it can only be rooted in environmental changes and not in genetic factors, although it is assumed that the latter are important for individual susceptibility to develop asthma (3). Next to the increase in asthma prevalence, there has been a similar rise in asthma severity, as suggested from hospital admission records (4).

Despite the improvement in asthma management, of which the emphasis on antiinflammatory treatment may have played a major role in the gradual decline in mortality (5), the reduction in deaths has been small and disappointing (6). There are probably various reasons for the failure to further decrease mortality due to asthma, including socioeconomic factors, lack of appreciation of disease severity by both patients and doctors resulting in non-compliance, and an overall increase in patients with asthma.

Natural history of asthma

Our understanding of the natural history of asthma has improved through the more specified definitions of asthma phenotypes that resulted from large longitudinal cohort studies. Risk factors for the development of childhood asthma including atopic status (7), genetic and familial factors (8), respiratory infections (9), and outdoor and indoor pollution are now more clearly appreciated. New information on the relation of viral wheezing episodes in infancy to later asthma-like syndromes is evolving (10, 11). Data from these studies suggest that recurrent obstructive symptoms remit in a large number of children who develop these symptoms during the first 3 years of life, and low lung function seems to be the main risk factor for these transient episodes. On the other hand,

children who will go on to develop persistent wheezing beyond infancy and early childhood usually have a family history of asthma and allergies and present with allergic symptoms very early in life (12, 13). Furthermore, epidemiological studies have shown that between 30 and 70 percent of those children with (early onset) atopic asthma markedly improve or become asymptomatic by early adulthood (13, 14). However, a considerable proportion of asthmatics in "clinical remission" will have a relapse later in life (14, 15), making the eventual "remission" rate in the middle aged and elderly small and presumably dependent on initial degree of bronchial hyperresponsiveness (BHR), Forced Expiratory Volume in one second (FEV_1), and smoking behaviour (16-18). Besides, if asthma persists into adulthood, the likelihood of remission seems to be even lower (18). It is intriguing whether early use of steroids or other newer modalities of therapy can alter the natural history of asthma.

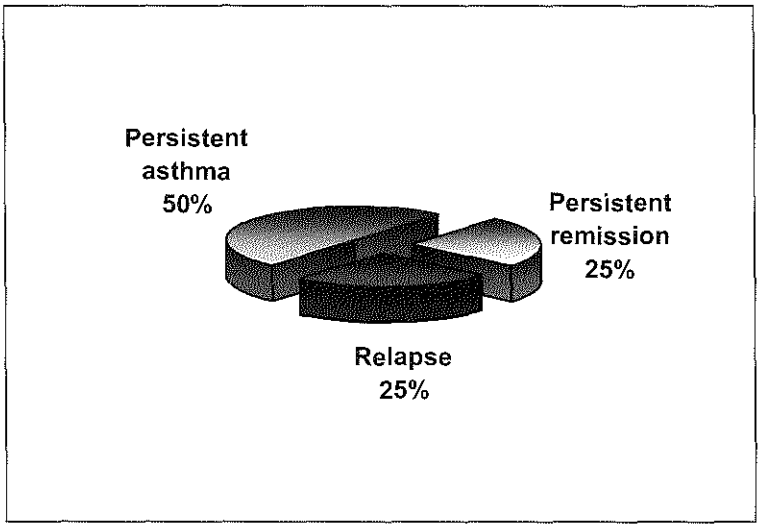


Figure 4: Natural history of atopic asthma

Effect of asthma on lung function

Little is known about the effects of asthma on lung function, particularly early in life. In studies concerning pulmonary function of asthmatic children in later adulthood, persistently lower lung function values were found in subjects with more severe asthma in childhood (19-21). Even so, the duration of asthma seems to correlate with the degree of impairment in lung function. Baseline data from 1041 children with mild to moderate asthma in the Childhood Asthma Management Program (CAMP) study found a significant correlation between asthma duration and lower lung function, greater bronchial hyperresponsiveness, more asthma symptoms, and greater use of medication. A rapid decline in lung function of asthmatics as compared with healthy controls was

confirmed in another study, where the decline in FEV_1 among subjects with asthma was 38 ml per year, compared with 22 ml per year in those without asthma (22).

1.4 Pathogenesis of asthma

Histology

The airways are lined with mucosa consisting of epithelium, basement membrane/reticular basement membrane (RBM), and lamina propria. The subepithelium is a cell-rich area 100 μm deep in the lamina propria. A spirally oriented smooth muscle layer surrounds the bronchial mucosa.

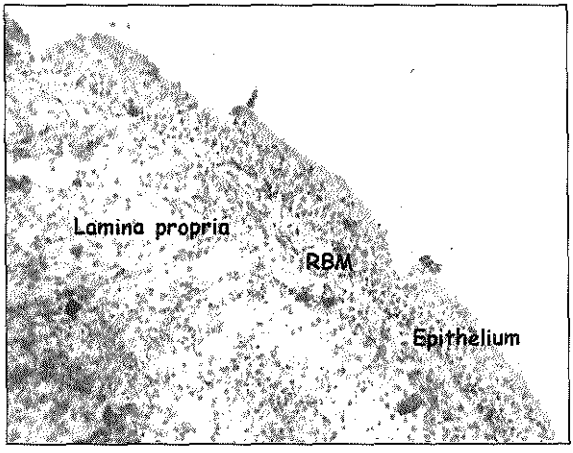


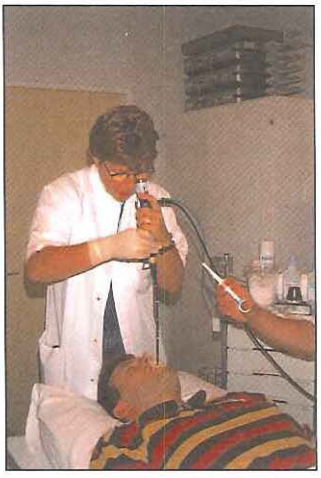
Figure 6: Bronchial mucosa

Mucosal inflammation

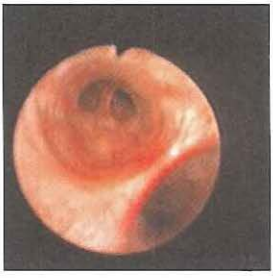
"Inflammation is not itself considered to be a disease but a salutary operation...but when it cannot accomplish that salutary purpose, it does mischief"

John Hunter

Although the inflammatory basis of asthma is well established, the mechanisms involved remain incompletely understood (23). Airway inflammation involves a complex network of interactions between inflammatory and structural cells and their mediators. Fibreoptic bronchoscopy has greatly improved our understanding of the pathogenesis of asthma (24). It is, however, important to appreciate in this regard that asthma is a syndrome of signs, symptoms, and laboratory abnormalities that is probably composed of many diseases, each with its own genetic and biochemical characteristics. The common denominator and primary physiologic abnormality in asthma is airway obstruction, which is most likely due to cellular inflammation and subsequent cytokine production, oedema, and smooth muscle infiltration with mast cells.



a



b

Figure 7: Flexible bronchoscopy (a) and example of an inside view (b).
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Inflammation is an early event in asthma, and infiltrating cells are uniformly present in airway biopsies obtained from newly diagnosed patients (25). Abundant evidence suggests that allergen-reactive type 2 T helper (Th2) cells, with eosinophils and mast cells as most important effector cells, orchestrate asthmatic airway inflammation (26, 27). The level of eosinophilia in the airway wall is, hereby, apparently the most distinguishing feature of bronchial asthma that seems to be correlated with asthma severity (28). Along with interleukin (IL)-5, a Th2 cytokine, and eotaxin, a chemoattractant for eosinophils, eosinophil-derived products such as major basic protein (MBP) have been put forward as markers of eosinophil participation in the pathogenesis of asthma (29). Also, elevated numbers of mast cells have been found in the bronchial mucosa of atopic and non-atopic asthmatic subjects (30, 31). Release of mediators from mast cells, such as granule-associated tryptase and chymase, leads to immediate bronchoconstriction and enhances airway inflammation (31).

How numbers and activity levels of the different cell types and mediators in the bronchial mucosa relate to less invasive markers of airway inflammation, is, up till now, still matter of debate (32-34).

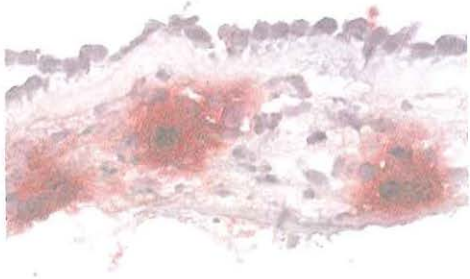


Figure 8: Bronchial mucosa showing extensive deposition of eosinophil derived major basic protein (red) beneath the reticular basement membrane.

Airway remodeling

In conjunction with, or because of, the inflammatory process structural changes occur that are known as airway remodeling (23). These structural changes of the airway walls occur early in the course of the disease (35, 36). As a result of airway remodeling, alterations in the airway epithelium, reticular basement membrane, subepithelium and submucosa develop, leading to thickening of the smooth muscle layer and airway wall. Moreover, these morphological changes may not be completely reversible (37). Attempts to delineate the physiologic consequences of such specific structural aberrations are at an early stage. Probably, (irreversible) airway narrowing and bronchial hyperresponsiveness may result from these changes in the mucosa and submucosa (38-42). The exact physiological consequences of airway wall thickening are, however, still incompletely understood and require more detailed investigation (23, 43) . This was also part of the guidelines of the 1997 NAEPP expert panel report II, which stated that "the importance of airway remodeling and the development of persistent airflow limitation need further exploration and may have significant implications for the treatment of asthma".

Remodeling of the airway mucosa and submucosa can be assessed by measurement of the reticular basement membrane (RBM) thickness (44) and extent of epithelial shedding (45). Also, collagen deposition (46) and microvascular proliferation (47) are quantifiable aspects of airway remodeling.

Figure 9: RBM

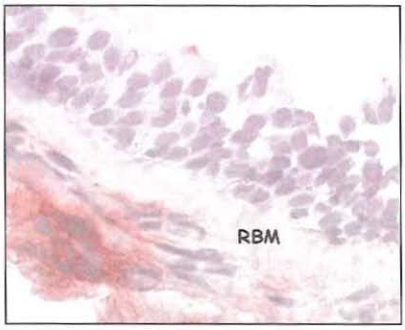
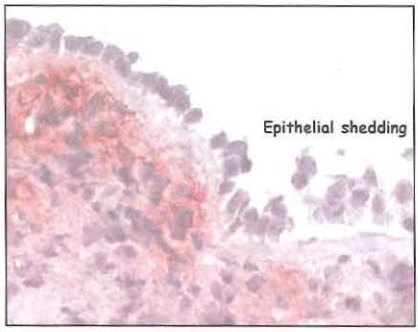


Figure 10: Epithelial desquamation



1.5 Establishing the diagnosis

Traditionally, the presence of asthma is suggested by a compatible history of cough, sputum, wheezing, chest tightness or breathlessness, particularly when the symptoms are variable. Physical examination is of little value in recognizing asthmatics. Conversely, pulmonary function measurements and measurement of the bronchial response to inhaled stimuli can help in identifying asthmatic subjects. Up till now, diagnosis and treatment decisions are mostly based on assessments of symptoms and simple measures of lung function. Only recently it has been put forward to include aspects of airway inflammation in the decision-making around diagnosis and therapy.

Lung function

Measurement of lung function, particularly the reversibility of bronchoconstriction, provides an easy to obtain direct assessment of airflow limitation. According to GINA guidelines, at least a 12 percent improvement in FEV_1, either spontaneously, or after inhalation of a bronchodilatator, or in response to a trial of anti-inflammatory therapy favors a diagnosis of asthma.

Bronchial hyperresponsiveness

Bronchial hyperresponsiveness (BHR) is one of the hallmarks of asthma and is often used as an indicator of asthma severity. Several studies have shown a clear relationship

between BHR and airway inflammation in symptomatic asthma (33, 48, 49), although there are clues that the relationship is not simple (32, 50). The degree of airway responsiveness can be assessed with a variety of inhaled stimuli, such as methacholine (MCh) or adenosine-5'-monophosphate (AMP). MCh induces airway constriction via direct stimulation of the muscarin receptors on airway smooth muscle cells. AMP, on the other hand, causes airway narrowing mainly through indirect mechanisms, in particular stimulation of mast cells and activation of neuronal reflexes in the lung (48). Since the presence of mast cells in the mucosa and airway smooth muscle cells is believed to play a predominant role in atopic asthma, the bronchial response to AMP, in addition to the response to MCh, may serve as indicator of the acute inflammatory process.

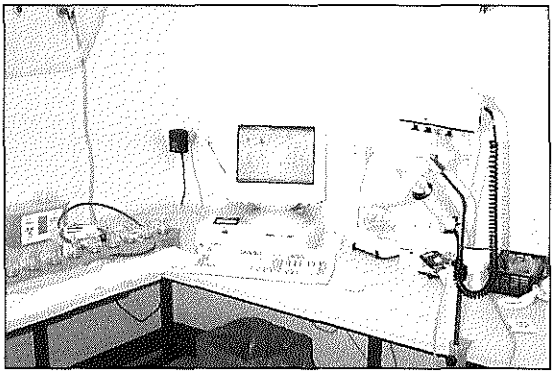


Figure 11: Lung function equipment with different doses of methacholine shown on the left

Nitric oxide

Nitric oxide synthase (NOS) is a newly identified enzyme system active in airway epithelial- and endothelial cells, macrophages, neutrophils, mast cells, autonomic

neurons, smooth muscle cells and fibroblasts. Nowadays, nitric oxide (NO) is known as a mediator of vasodilatation and bronchodilatation. The presence of an inducible form of NOS (iNOS) in human lungs suggests that increased production of NO, probably brought about by cytokines, may be relevant to the pathology of asthma (51). Many studies concerning atopic asthma demonstrate enhanced NO levels in exhaled air (52, 53). Furthermore, exhaled NO (eNO) levels are lowered by anti-inflammatory therapy, which offers opportunities to monitor compliance with- and effectiveness of treatment (54). The measurement of eNO can be performed repeatedly, even in children and patients with severe airflow obstruction, in whom invasive techniques are not feasible or desirable. Studies regarding eNO report a weak relationship between eNO levels and BHR (55, 56), indicating the complex interrelationships between the mechanisms involved. An increasing number of papers shows evidence that eNO is related to atopic asthma more than to non-atopic asthma or atopy per se. It was recently demonstrated that atopic asthmatic children had higher geometric mean eNO levels than non-atopic asthmatic children, atopic non-asthmatic children, or non-atopic non-asthmatic children, suggesting that both atopy and asthma are important in the context of elevated eNO levels (57). The usefulness and specificity of eNO values with respect to the monitoring of airway inflammation in atopic asthma are also still under investigation (58-62). It is proposed that airway acidification leads to non-enzymatic NO formation independent of inflammation, which may occur during acute severe asthma (63). Nevertheless, it is attractive to speculate that this simple, non-invasive test could be used to monitor the inflammatory status of the asthmatic airway during treatment with anti-inflammatory

medication (64-67). Additionally, exhaled NO may be useful as a diagnostic tool in asthma.

The measurement of exhaled NO has been subject to alteration. In 1997, the European Respiratory Society Task Force (68) published recommendations concerning the measurement of NO in exhaled air. Briefly, eNO can be measured with a chemiluminescence analyzer with a detection range of < 0.1 - 500,000 ppb. The measurement circuit consists of a mouthpiece connected to a two-way non-rebreathing valve through which subjects inhale ambient air or NO-free medical air, depending on the ambient air NO concentration. Subjects inhale to TLC and immediately exhale for as long as possible into a tube, with an in-line flow resistor to prevent contamination of exhaled air with air from the upper airways, which have a high NO content (69). Exhalation is performed at low flow, providing NO to diffuse properly from the lining epithelium to the bronchial lumen when air passes through the conducting airways, thereby amplifying the signal (70-72) A fine tube samples exhaled air from a side port situated directly after the mouthpiece to conduct the air to the analyzer continuously.

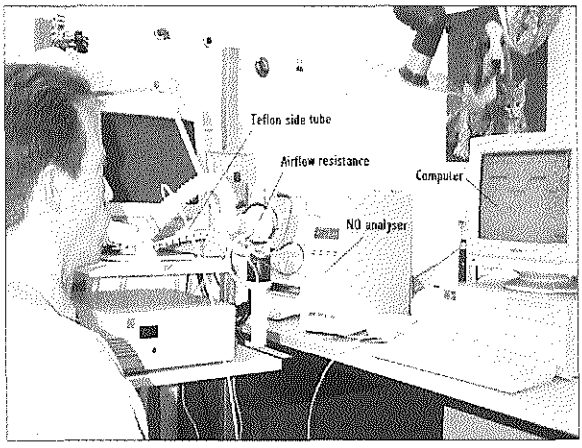


Figure 12: The author experiences an exhaled NO measurement

Hydrogen peroxide in exhaled air

Exhaled air condensate provides a noninvasive means of obtaining samples from the lower respiratory tract. Leukocytes involved in the asthma inflammatory cascade (predominantly eosinophils, mast cells and neutrophils) release mediators, including reactive oxygen species, i.e. superoxide anion that is dismutated to hydrogen peroxide (H_2O_2). H_2O_2 in exhaled air has therefore been proposed as a marker of airway inflammation (52, 73-75).

Induced sputum

Investigation of inflammatory mediators and cells in sputum induced by inhalation of nebulized hypertonic saline is increasingly used to monitor airway inflammation in asthma, and can be performed safely in subjects with moderate to severe asthma when carried out under carefully monitored conditions (52, 62, 76). It is suggested that analysis of induced sputum reveals information qualitatively similar to that obtained by analysis of bronchoalveolar lavage fluid or bronchial biopsy specimens (77). Sputum induction is appreciated not only for being noninvasive and repeatable (78) but also for yielding samples more concentrated and richer in airway secretions than those obtained by bronchoscopy (79).

Blood eosinophils

It has been proposed that determination of numbers of eosinophils in peripheral blood may also help to indicate the level of airway inflammation, thereby providing a surrogate, less invasive marker to monitor asthma. Indeed, within a population of atopic asthmatics, airway wall eosinophilia weakly correlates with eosinophilia in peripheral blood (34).

Flexible bronchoscopy

From all indices of airway inflammation, bronchial mucosal biopsy investigations still provide the golden standard (80, 81). How numbers and activation state of the different

cell types and mediators in the bronchial mucosa relate to less invasive markers of airway inflammation, including bronchial hyperresponsiveness, circulating eosinophils and eNO, is largely unclear (32-34).

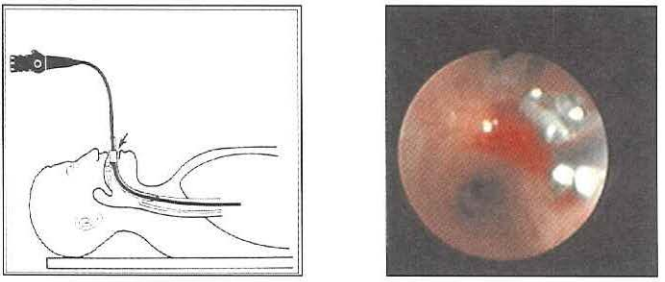


Figure 13: Bronchoscopy as a research tool for the study of asthma pathogenesis. A forceps taking a biopsy from a subcarina of one of the main bronchi is shown on the right.

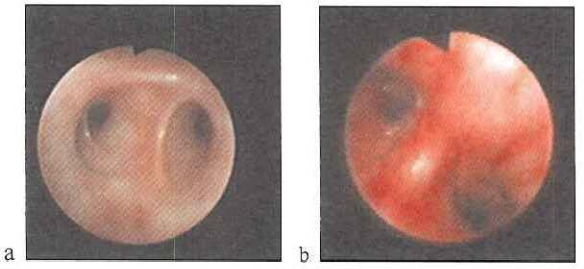


Figure 14: Normal (a) and inflamed (b) airways, with swelling of the mucosa, excessive mucus production and easy bleeding in the latter.

Analysis of bronchial biopsy specimens

Immunostained sections of bronchial biopsies can be analyzed with computer-assisted image analysis, a method which provides highly reproducible and reliable parameters of asthmatic airway inflammation (82). After setting of the reticular basement membrane and epithelial margins interactively, the program is primed to analyze the epithelium as well as an area 100 μm below the reticular basement membrane. Artefacts are excluded from analysis. The ratio of positive stained area divided by the total area analyzed is taken as measure for each immunohistochemical staining.

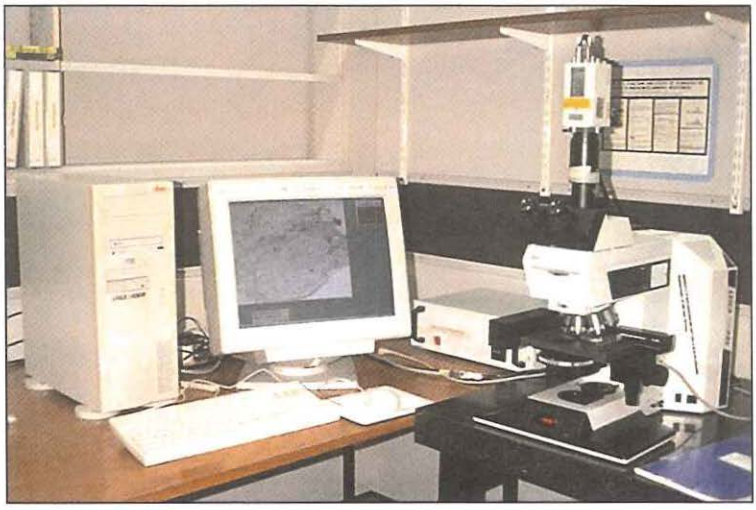


Figure 15: Image analysis

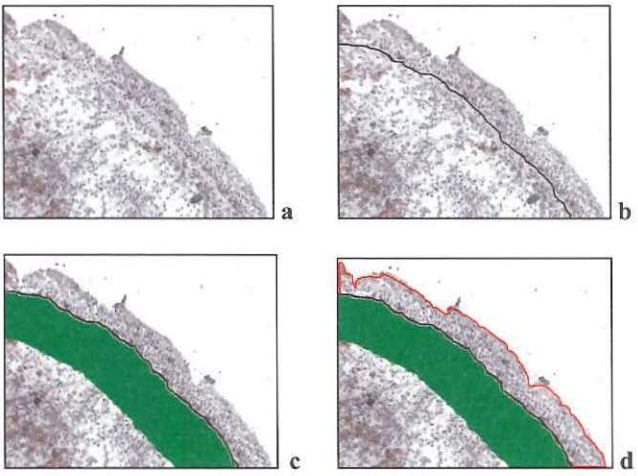


Figure 16: Interactively setting of the reticular basement membrane (black, b), 100 µm subepithelial zone (green area, c), and epithelial lining (red, d) in image analysis.

1.6 Treatment of asthma

Asthma is a chronic inflammatory disease of the airways involving a characteristic picture of airway infiltration with lymphocytes, eosinophils, and mast cells, subepithelial deposition of collagen, and hypertrophy and/or hyperplasia of smooth muscle cells, goblet cells and submucosal glands. The consequences of this chronic inflammatory process are still not understood in detail but include variable or persistent symptoms, bronchial hyperresponsiveness, and attacks of airflow limitation that may require emergency care or hospitalization and can even lead to death. Pathology studies have shown that antiinflammatory therapy can reverse or suppress airway inflammation (35, 83-85), whereas prospective controlled clinical trials have demonstrated that it can also diminish symptoms, reduce bronchial hyperreactivity, and reduce the frequency and severity of exacerbations (86-88) as well as the rate of decline in lung function (37). It is also highly likely, although it is not yet proven, that inhaled antiinflammatory therapy reduces the risk of asthma fatality and prevents, retards or even reduces airway wall remodeling (47, 89-92). These beneficial effects are easily shown in patients with moderate to severe asthma. Although benefits are also applicable to patients with mild asthma, it is less certain that the cost and harm of continuous antiinflammatory therapy are justified for the mildest forms of the disease (65, 85, 93, 94). For these patients, the most important issue that remains to be resolved is which factors lead to permanent airflow obstruction and what the effects of early, sustained treatment are on the possibility to reach complete sustained remission of asthma (89).

Inhaled corticosteroids have now become established as antiinflammatory therapy of choice in patients with persistent asthma (5). Inhaled corticosteroids largely avoid the adverse effects associated with oral steroids and are now also recommended in newly detected disease (94). Several different inhaled corticosteroids are available as therapeutic options for the treatment of asthma, including include fluticasone propionate, beclomethasone dipropionate, and budesonide.

Other therapies

Other drugs widely used to treat asthma include beta 2-agonists, theophylline, cromones, and anticholinergic agents. For acute asthma attacks, the inhaled beta 2-agonists are the most effective bronchodilators (95). Short- acting forms, such as salbutamol and terbutaline, give rapid relief; long-acting agents, i.e. salmeterol and formoterol, provide sustained relief and reduce nocturnal and exercise-induced asthma (96). Although some authors postulate that an effect on airway inflammation and remodeling is lacking (97), others have shown that, in addition to their bronchodilator action, long-acting β2-agonists may also bring about changes in airway inflammation and/or remodeling itself, including a decrease in mast cell mediator release (98) and in airway wall vascularity (47, 99).

Adverse effects are of minor importance when these drugs are used properly (100). The anticholinergic bronchodilators are more useful for treating COPD than chronic asthma. These drugs have virtually no side effects, and their onset is slower and their action

longer than that of inhaled beta 2-agonists. Finally, the antileukotrienes seem to provide some bronchodilation and have a minor effect on eosinophilia, with minimal side effects.

Combination therapy

The dose-response curve to inhaled corticosteroids is relatively flat, and there is a strong scientific rationale for adding long-acting inhaled beta2-agonists, which may be equivalent or preferable to increasing the dose of inhaled corticosteroids in patients with moderate-to-severe asthma (88, 101, 102).

Mild asthma

The anticipated natural history of the disease may influence treatment considerations. If mild asthma is destined to remain mild into old age, treatment is best determined by the symptoms of asthma at that time. On the other hand, if the chronic eosinophilic inflammatory process progresses over time to subepithelial fibrosis and irreversible airflow limitation, the early initiation and prolonged continuation of antiinflammatory therapy might be beneficial. Unfortunately, the ability to predict the outcome of mild asthma is limited at present. As yet, no studies have been performed that provide long-term physiologic and histologic follow-up over many years in a large cohort of individuals with mild asthma. At present, NAEPP guidelines indicate that patients with mild intermittent asthma are best treated with intermittent use of an inhaled beta-2-

selective adrenergic agonist, where antiinflammatory medications are only needed when asthmatic symptoms are reported more than twice a week (mild persistent asthma).

1.7 Growing out of asthma

Epidemiological work has shown that symptoms of atopic asthma often disappear in early adolescence (13). This apparent improvement eventually leads to cessation of treatment and discontinuation of routine check-ups at the out-patient clinic. The classification "clinical remission" is used to identify these subjects who are believed to have outgrown their asthma. Unfortunately, 30 to 80 % of these subjects in clinical remission experiences a relapse of symptoms later in life (2). The factors responsible for this high relapse rate are unknown. Several authors have demonstrated spirometry abnormalities and/or bronchial hyperresponsiveness in subjects during clinical remission of asthma (103-105). It is unclear whether these functional aberrations reflect ongoing airway inflammation or merely indicate structural changes of the airways as a late consequence of childhood asthma. Defining asthma as chronic inflammatory disease, persistent airway inflammation during clinical remission of atopic asthma could possibly account for the high relapse rate (106). On the other hand, from several studies it has emerged that structural changes, known as airway remodelling, finally lead to thickening of the airway wall (39, 41). A relationship between airway wall thickening and impaired airflow has been made plausible (107, 108). How the different aspects of airway wall thickening contribute to altered airway function is, however, incompletely understood (23, 43). If airway wall thickening is present in subjects during clinical remission of asthma, it could at least in part account for the functional abnormalities found during 'remission', but even so for the high relapse rate. In particular ongoing airway inflammation arises the question whether anti-inflammatory therapy should be continued

in subjects with apparently outgrown asthma. Monitoring airway inflammation, preferably without the use of invasive techniques, will then be needed to show the effect of treatment.

The discrepancy between the lack of symptoms and ongoing airway inflammation remains an other problem to be resolved. It is well-known that the correlation between symptom perception and other indices of asthmatic severity, such as the degree of airflow obstruction, is poor (109, 110). Poor perception of airway narrowing may, on its turn, itself lead to undertreatment of asthma (111, 112). Thus, the concept of blunted perception of dyspnea in subjects with apparently outgrown asthma is likely of great clinical relevance.

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Chapter

2

Aim of the study

"Everything hinges on the matter of evidence"

Carl Sagan

As stated in the introductory remarks, various questions arise from the epidemiological fact that a great deal of subjects with apparently outgrown asthma experiences a relapse of symptoms of asthma later in life. In the present thesis, the next questions will be addressed:

1 Are we able to produce convincing arguments of ongoing airway inflammation and/or remodeling in subjects who are believed to have outgrown their asthma?

To address this question, we measured spirometry values, bronchial responsiveness to different inhaled stimuli, and exhaled nitric oxide levels in adolescents in clinical remission of atopic asthma. Clinical remission was defined as complete absence of symptoms without the use of any medication in the year preceding the study. Chapter 3 describes the results of this study, wherein subjects in clinical remission were compared with subjects with symptomatic asthma and healthy controls. In Chapter 4 invasively obtained "proof" of ongoing airway inflammation and remodeling during clinical remission of atopic asthma is discussed. By means of flexible bronchoscopy, biopsies

were obtained from the airway walls in all subjects from the three study groups. A comparison of biopsy findings, including inflammatory cell type density and various indices of airway remodeling, was made between the groups. Also, data were compared with noninvasive markers of airway disease, such as exhaled nitric oxide levels and bronchial responsiveness to inhaled stimuli.

2 If persistent airway inflammation can be demonstrated during clinical remission, could blunted symptom perception explain the discrepancy between the lack of symptoms and ongoing disease?

This question will be dealt with in chapter 5, where a study is described in which we obtained "BORG" dyspnea scores from subjects in clinical remission and subjects with symptomatic asthma during MCh and AMP provocation.

3 Would subjects with subclinical airway inflammation and remodelling benefit from antiinflammatory treatment in the short-term?

In chapter 6, a double blind, longitudinal, placebo controlled study is described in which subjects in clinical remission of atopic asthma are treated for three months with either the salmeterol/flixotide propionate combination product (Seretide) or placebo. Again, invasive- as well as non-invasive indices were obtained from all subjects before and after treatment.

4 Asthma remission – does it exist?

This question will be dealt with in chapter 7. Whether "true" remission of asthma can be reached with or without the aid of prolonged anti-inflammatory treatment is as yet unknown. A review of relevant literature as well as a proposal to deal with subjects with apparently outgrown asthma is given.

Chapter

3

Adolescents in Clinical Remission of Atopic Asthma have Elevated Exhaled Nitric Oxide Levels and Bronchial Hyperresponsiveness

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ABSTRACT

Symptoms of atopic asthma often decrease or even seem to disappear around puberty. The aim of this study was to investigate whether this so-called clinical remission is accompanied by remission of airway inflammation, since symptoms relapse in a substantial proportion of subjects later in life.

To assess indicators of inflammation and/or structural damage of the airways, exhaled nitric oxide (eNO) and bronchial responsiveness to adenosine-5'-monophosphate (AMP) and methacholine (MCh) were determined in 21 subjects in clinical remission of atopic asthma. Clinical remission was defined as complete absence of symptoms of asthma without the use of any medication in the year preceding the study. Results were compared with those of 21 patients with current asthma and 18 healthy control subjects. We found significantly higher eNO values in the remission group than in healthy controls (geometric mean 18.9 ppb and 1.0 ppb, respectively; $p < 0.001$) whereas eNO values of the remission group and those of the subjects with current asthma (geometric mean 21.9 ppb) were similar ($p = 0.09$). The responsiveness to both AMP and MCh of subjects in clinical remission was significantly higher as compared with responsiveness of healthy controls, and lower than responsiveness of subjects with current asthma. A significant correlation could be established between eNO and responsiveness to AMP, but not between eNO and responsiveness to MCh. The results of this study are suggestive of persistent airway inflammation during clinical remission of atopic asthma. We speculate that subclinical inflammation is a risk factor for asthma relapse later in life, and that eNO

and responsiveness to both AMP and MCh can be used as different, non-invasive indices of the inflammatory process of the airways.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways, characterized by cellular infiltration, cellular activity and cell damage, but also by oedema, vascular leakage, and hypertrophy/hyperplasia of resident cells, as there are goblet cells and smooth muscles. Structural changes of the airway walls occur early in the course of the disease (1, 2). Epidemiological studies have shown that symptoms of atopic asthma often begin in early childhood and improve or seem to disappear around puberty (3, 4). However, a considerable proportion of asthmatics in "clinical remission" will have a relapse later in life (4). Several studies have shown spirometric abnormalities and bronchial hyperresponsiveness (BHR) to methacholine (MCh) or cold air challenge during clinical remission of asthma (5, 6). It is unknown whether these functional abnormalities, which are supposed to be indicative of asthma severity with respect to symptomatic asthma, reflect persistent airway inflammation or merely indicate residual airway damage. These considerations seem to be important, as it is reasonable to believe that persistent airway inflammation during clinical remission of atopic asthma has substantial impact on the risk of relapse at a later age. The question arises whether other available non-invasive physiological techniques can give information about the presence of an ongoing inflammatory process.

Nitric oxide synthase (NOS) is a newly identified enzyme system active in airway epithelial- and endothelial cells, macrophages, neutrophils, mast cells, autonomic neurons, smooth muscle cells, and fibroblasts. The existence of an inducible form of NOS (iNOS) in human lungs suggests that increased production of NO, probably induced by

cytokines, may be relevant to the pathology of asthma (7). Many studies concerning atopic asthma demonstrate elevated NO levels in exhaled air (8, 9). Furthermore, exhaled NO (eNO) levels are decreased by antiinflammatory therapy, which offers opportunities to monitor compliance with and effectiveness of treatment (10). The measurement of eNO can be performed repeatedly, even in children and patients with severe airflow obstruction, in whom invasive techniques are not feasible or desirable. Studies regarding eNO reported a weak relationship between eNO levels and BHR (11, 12), indicating the complexity of mechanisms involved in atopic asthma. The usefulness and specificity of eNO values with respect to the monitoring of airway inflammation in atopic asthma are still under investigation.

BHR is one of the hallmarks of asthma and is often used as an indicator of asthma severity. Several studies have shown a significant correlation between BHR and airway inflammation in symptomatic asthma (13-15). The degree of airway responsiveness can be assessed with a variety of inhaled stimuli, such as MCh or adenosine-5'-monophosphate (AMP). MCh induces airway constriction via direct stimulation of the muscarin receptors on airway smooth muscle cells. AMP, on the other hand, causes airway narrowing mainly through indirect mechanisms, in particular stimulation of mast cells and activation of neuronal reflexes in the lung (14). Since mast cells are believed to play a predominant role in atopic asthma, the bronchial response to AMP, in addition to the response to MCh, may serve as indicator of the inflammatory process.

The aim of this study was to measure eNO values and bronchial responsiveness to MCh and AMP, each reflecting distinct parts of the inflammatory process in the airways, in previously well-documented atopic asthmatic adolescents who were in clinical

remission for more than one year. Data were compared with data on adolescents with current asthma and healthy control subjects.

METHODS

Subjects

Adolescents, 18 - 25 years of age, with atopic asthma were selected from the Sophia Children's Hospital discharged patients files. Clinical remission was assumed if subjects reported complete absence of cough, wheezing and breathlessness at rest and on exertion and had not taken any medication in order to control asthmatic symptoms for at least 12 mo before to the study. Twenty-one eligible subjects in remission were included, and compared with 21 patients with asthma who had persistent symptoms at least once a month in the year preceding the study, and used inhaled $ß_2$-agonists on demand in order to relieve symptoms. All subjects had a history of wheezing and chest tightness and were previously diagnosed as having atopic asthma according to American Thoracic Society (ATS) criteria (16). In the past, all had a provocation dose/concentration of MCh or histamine producing a 20 % fall in FEV_1 of ≤ 150 µg (dosimeter method) or ≤ 8 $mg \cdot mL^{-1}$ (2-min tidal breathing method), and/or had an FEV_1 reversibility ≥ 12 % of predicted normal value. All had evidence of atopy defined by radio allergosorbent test (RAST) Class 2 or higher for at least one common airborne allergen. Participating subjects were lifelong nonsmokers, in stable clinical condition and did not take inhaled steroids, including nasal steroids, or anti-allergic medication such as cromoglycate and antihistamines for at least 1 yr before the study.

Eighteen healthy nonsmoking young adult volunteers were recruited via advertisement and served as control subjects. They had a negative personal and first-degree family history of asthma and atopy. Common exclusion criteria were an inability to perform lung

function tests reproducibly, perennial rhinitis, and other illnesses that may affect lung function. None of the subjects in the study reported symptoms of respiratory infection in the month prior to the study. The study was approved by the Medical Ethics Committee of the Erasmus University and University Hospital Rotterdam.

Study Design

We performed a cross-sectional study with three visits on separate days. At the first visit, subjects gave informed written consent and were asked about asthmatic symptoms and requirement for rescue medication during the past year, especially to avoid the inclusion of subjects with mild symptoms of asthma in the remission group. For the same reason, subjects were asked to complete Juniper's Quality of Life Questionnaire (17), which was verified by the investigator on hidden minor symptoms. Also, physical examination was performed and eNO and PD_{20} MCh determined. At Visit 2, scheduled at least 1 d after Visit 1, subjects underwent an AMP challenge test. At Visit 3, scheduled at least 3 d after Visit 2, FEV_1 and FEV_1 reversibility were tested. The sequence and intervals were chosen in order to avoid any influence of the AMP challenge on MHh responsiveness and/or on FEV_1 (18). The maximal time allowed between Visits 1 and 3 was 3 wk.

Spirometry

Short acting $ß_2$-agonists were not allowed within 8 hours before the test. Flow-volume curves were obtained using a Lilly-type pneumotachograph (Masterlab Jaeger, Würzberg, Germany). The best of three reproducible recordings of FEV_1 was expressed as percentage of predicted normal and used for analysis. Reversibility was tested by measuring FEV_1 before and 20 minutes after inhalation of 1 mg of terbutaline powder (Bricanyl turbuhaler, ASTRA, Lund, Sweden), and expressed as increase in percentage of predicted normal.

Nitric Oxide

Based on the recommendations of the European Respiratory Society Task Force (19), eNO was measured in exhaled air by means of chemiluminescence (model 280 nitric oxide analyzer, Sievers, Boulder, CO) with a sensitivity of 0.1 ppb and a detection range of < 0.1 - 500,000 ppb. The sampling flow was 0.2 L/min and the response time 0.2 s. Data were displayed continuously on a PC screen, and stored in a computer with a sample frequency of 20 Hz for later analysis. The analyzer was calibrated regularly according to the manufacturer's guidelines, employing certified calibration gases containing 0 ppb, 100 ppb and 9 ppm NO (Hoekloos, Barendrecht, the Netherlands). The measurement circuit consisted of a mouthpiece connected to a two-way nonrebreathing valve (Hans Rudolph, Kansas City, MO), through which subjects inhaled ambient air (if ambient NO was < 10 ppb) or NO-free medical air (if ambient NO was > 10 ppb) while seated, not wearing a noseclip. Subjects inhaled to TLC and immediately exhaled for as long as possible into a tube with an in-line flow resistor (20 cm H_2O L^{-1} s^{-1}, Hans Rudolph). This was done at a flow corresponding to 5 % of subject vital capacity per second, with the aid of a visual feedback display. A fine-bore Teflon tube continuously sampled exhaled air from a side port situated directly after the mouthpiece to the analyzer. Water vapor was absorbed by means of an NO-inert filter in the tube. Airflow was measured with a Lilly-type pneumotachograph (Masterlab Jaeger) positioned downstream of the resistor. An end-expiratory plateau of at least 10 s, where flow varied ± 10 % of the target flow, was the end point of the measurement. The test was done in triplicate and average eNO at the plateau calculated by means of custom-made software.

Methacholine and Adenosine-5'-MonoPhosphate Challenge

Challenge tests were performed at the same time of day (± 1 h) according to the dosimeter method validated by Birnie and coworkers (20). Short acting β_2-agonists were not allowed within 8 h before the test. Calibrated DeVilbiss 646 nebulizers (DeVilbiss Health Care, Somerset, PA) were filled with 3 ml of the appropriate solutions. Subjects inhaled four 5-µl volumes, using a French-Rosenthal dosimeter (Laboratory for Applied Immunology, Fairfax, VA). After recording baseline values, the challenge started with inhalation of 0.9 % NaCl. If a patient responded to saline or the lowest concentration of either MCh or AMP, they were assigned a PD_{20} value of half the starting dose. Inhalation provocation tests were performed using doubling concentrations of 0.15 to 78.4 mg/ml MCh bromide in phosphate-buffered saline (PBS) or 0.08 to 160 mg/ml AMP in normal saline (Sigma, St. Louis, MO). Provocative doses causing a 20 % fall in FEV_1 (PD_{20}) from baseline were calculated by means of linear interpolation of the logarithmic dose-response curve. If FEV_1 fell less than 20 % of the pre-challenge level at the highest dose administered, twice the highest dose was arbitrarily used as the PD_{20} value. An MCh PD_{20} value of 1,000 µg, corresponding approximately with 7.8 µmol cumulative, was considered the cutoff value for bronchial hyperresponsiveness to MCh.

Peak Expiratory Flow Rate (PEFR)

After being instructed by the investigator at visit 1, the patients recorded their peak expiratory flow rate (PEFR) with a Personal Best peakflow meter (Respironics, Nantes, France) twice daily at home during the period between Visit 1 and Visit 3. PEFR was

always recorded before bronchodilatation. Each measurement consisted of three attempts of which the highest value was used for further analysis.

Statistical Analysis

Because of their highly skewed distributions, PD_{20} and eNO values were analyzed after logarithmic transformation. Mean data were expressed as geometric mean $e^{\pm SEM(\ln X)}$. With respect to PD_{20} values, comparisons between groups were made by the Mann-Whitney test for unpaired samples. Correlation between different tests was made by the Spearman rank correlation test. The distributions of all other variables were not significantly different from a standard normal distribution. Hence, parametric techniques (Student *t* test, Pearson correlation coefficients) were applied. Mean values of these parameters were expressed as means ± SEM. A two-tailed p value of less than 0.05 was considered significant. Data were analyzed with the Statistical Package for the Social Science (SPSS, Chicago, IL) for Windows version 8.0.

RESULTS

Sixty subjects completed the study (21 subjects with current asthma, 21 subjects in clinical remission and 18 healthy controls). The male-to-female ratio was 15 to 6 in the asthmatic group, 18 to 3 in the remission group, and 10 to 8 in the control group. Age did not differ significantly between the groups (22 ± 2 yr in the asthmatic group, 21 ± 2 yr in the remission group and 24 ± 1 yr in the control group). Duration of remission in the remission group varied from 1 to 12 yr (median, 5 yr). All subjects of the asthma group and the remission group, and two subjects of the control group, had positive RAST tests. Results of eNO measurement, bronchial provocation tests and spirometry are summarized in Table 1.

TABLE 1: Exhaled NO and pulmonary function

	Asthmatics (n=21)	Clinical Remission (n=21)	Controls (n=18)
eNO (ppb)	$22e^{\pm 0.19}$ *	$14e^{\pm 0.15}$ *	$1e^{\pm 0.31}$
PD_{20} MCH (µg)	$94e^{\pm 0.37}$ *	$752e^{\pm 0.31}$ **	$4{,}954 \cdot e^{\pm 0}$
PD_{20} AMP (µg)	$1{,}110e^{\pm 0.37}$ *	$5{,}704e^{\pm 0.22}$ ***	$10{,}496e^{\pm 0}$
FEV_1 (% of predicted)	88 ± 12 *	93 ± 15 †	105 ± 13
Reversibility FEV_1 (%)	11 ± 1 *	7 ± 1 ††	4 ± 1
Diurnal PEFR variation (%)	13 ± 2	11 ± 1	8 ± 1

Abbreviations: PD_{20} MCH: provocative dose of methacholine causing a 20 % fall in FEV_1. PD_{20} AMP: provocative dose of adenosine-5'-monophosphate causing a 20 % fall in FEV_1. Reversibility FEV_1: change in FEV_1, expressed as increase in % of predicted normal value after administration of 1 mg terbutaline.
eNO, PD_{20} MCH and PD_{20} AMP are expressed as geometric mean $\cdot e^{\pm SEM(lnX)}$.
All other variables are expressed as mean ± SEM. * $p < 0.001$ versus healthy controls. ** $p < 0.001$ versus healthy controls and asthmatics. *** $p < 0.01$ versus healthy controls and asthmatics. † $p = 0.02$ versus healthy controls. †† $p < 0.02$ versus healthy controls and asthmatics

Exhaled NO Values

Geometric mean eNO values in the remission group were significantly higher than in healthy controls (mean, 18.9 and 1.0 ppb, respectively; $p < 0.001$), and slightly but not significantly lower than those of asthmatics (21.9 ppb; $p = 0.09$) (Table 1; Figure 1). Although there seemed to be a trend toward lower eNO values with longer duration of remission, a significant correlation between these variables could not be established.

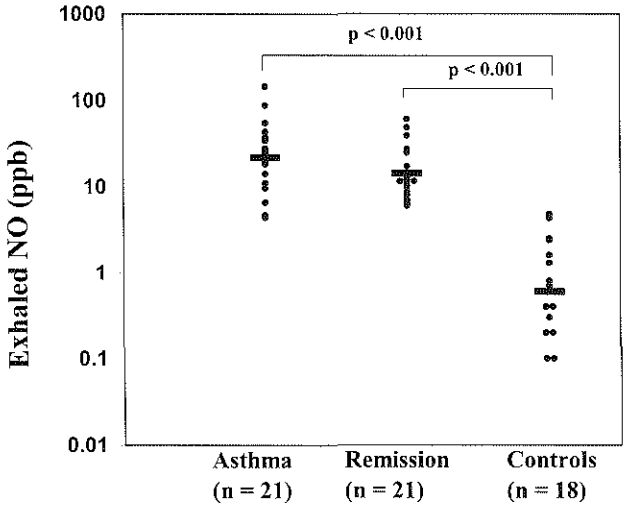

Figure 1: Exhaled NO values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Each dot represents one subject. *Horizontal bars* represent geometric mean values. The Y-axis shows NO values logarithmically.

Bronchial Challenge Tests

In the remission group, 11 of 21 subjects had a PD_{20} MCh of less than 1,000 μg, compared with none of the control subjects. Of the subjects with asthma, 19 of 21 showed a PD_{20} MCh value below this level. PD_{20} MCh values in the remission group were significantly lower than in the control group (geometric mean, 751 and 4,954 μg, respectively; $p < 0.001$), but higher than in the group of asthmatics (94 μg; $p < 0.001$) (Figure 2).

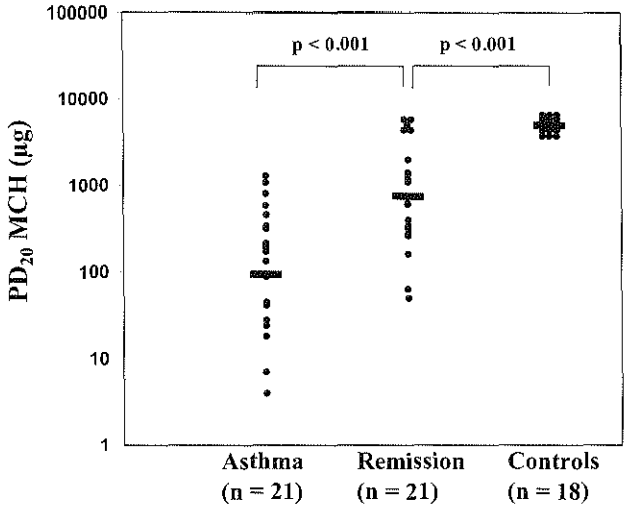

Figure 2: PD_{20} MCh (micrograms, not cumulative) values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Non-responders are arbitrarily given a value of twice the highest dose administered. *Horizontal bars* represent geometric mean values. The Y-axis shows PD_{20} MCh values logarithmically.

The AMP challenge showed significantly lower PD_{20} AMP values in the remission group than in the control group (geometric mean, 5,704 and 10,496 μg, respectively; $p < 0.01$), but higher values in the remission group than in asthmatics (1,110 μg, $p < 0.01$) (Figure 3).

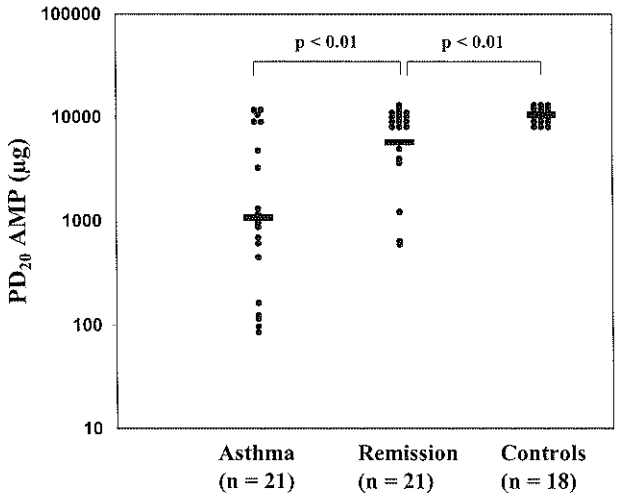

Figure 3: PD_{20} AMP (micrograms, not cumulative) values of currently asthmatic subjects, of subjects in clinical remission of atopic asthma, and of healthy controls. Non-responders are arbitrarily given a value of twice the highest dose administered. *Horizontal bars* represent geometric mean values. The Y-axis shows PD_{20} AMP values logarithmically.

In subjects in remission and those with asthma, PD_{20} MCh and PD_{20} AMP were highly correlated ($r = 0.72$, $p < 0.001$). There was a significant inverse correlation between PD_{20} AMP and eNO ($r = -0.51$; $p = 0.001$), while no correlation could be established between PD_{20} MCh and eNO ($r = -0.25$; $p = 0.1$). There was no significant relationship between formerly and currently assessed bronchial responsiveness, or between duration of remission and bronchial responsiveness.

Spirometry and PEFR

Baseline FEV_1 values, expressed as percentage of predicted normal value, were significantly reduced in subjects in remission compared to healthy control subjects (mean, 93 and 105 %, respectively; $p = 0.02$). No significant difference could be detected between subjects in remission and subjects with asthma (mean, 88 %). Reversibility in FEV_1 was significantly different ($p < 0.02$) between the three groups (mean, 11, 7, and 4 % in subjects with asthma, subjects in remission and healthy control subjects, respectively). Mean diurnal PEFR variation was 13% in subjects with asthma, 11% in subjects in remission, and 8% in control subjects, and showed no significant difference between the groups.

DISCUSSION

In this study, we demonstrated elevated levels of eNO and airway hyperresponsiveness to MCh and AMP in adolescents in long-standing clinical remission of atopic asthma. During remission, eNO levels were almost similar to those found in subjects with current asthma. There was a significant correlation between eNO values and AMP responsiveness, but not between eNO values and MCh responsiveness. A significant relationship between duration of remission and degree of abnormalities could not be established.

Studies regarding completely symptom-free subjects who had well-documented atopic asthma in the past are scarce (6). In our study, subjects were regarded to be in clinical remission when they reported complete absence of symptoms and were without treatment for at least one yr before the study. This definition was applied to avoid the inclusion of asthmatic subjects with mild symptoms in the remission group. The possibility that subjects with mild symptoms were included in other studies on asthma remission cannot be ruled out. In one of these studies, asthma was considered inactive when subjects reported no asthma attacks or use of medication during the preceding year, and were without frequent episodes of shortness of breath with wheezing (5). Following this definition of remission, the investigators reported bronchial hyperresponsiveness to MCh with or without airflow obstruction in subjects aged 18 - 61 years in remission.

Despite the relatively long median duration of remission, we found that subjects in clinical remission had significantly elevated eNO levels, as compared with eNO levels from healthy controls. Exhaled NO originates from several inflammatory cell types in the

airways, including epithelial- and endothelial cells and macrophages. Whether elevated eNO levels are caused by enhanced activity of inflammatory cells expressing NOS, potentially driven by inflammatory mediators or cytokines, and/or by enhanced diffusion through the airway wall due to structural damage remains to be resolved. An increasing number of papers shows evidence that eNO is related to atopic asthma more than to non-atopic asthma or atopy per se. It was recently demonstrated that atopic asthmatic children had higher geometric mean eNO levels than non-atopic asthmatic children, atopic non-asthmatic children, or non-atopic non-asthmatic children, suggesting that both atopy and asthma are important in the context of elevated eNO levels (21). In other studies performed recently, eNO levels were significantly higher in atopic subjects with asthma compared with levels from non-atopic asthmatic subjects (22, 23). Similar results were obtained in patients with rhinitis, whereas no difference was found in eNO levels between atopic and non-atopic control subjects (22). Horvath and Barnes found elevated eNO levels in atopic asymptomatic subjects, but suggested that this finding merely reflected an early stage of airway inflammation, possibly preceding the onset of asthmatic symptoms (24). In our study, two subjects in the control group had positive RAST test, but had no elevated eNO values. Thus, with reference to the suggestion of Horvath and Barnes, the presence of elevated eNO levels in our atopic remission group might well be due to subclinical airway inflammation. This is also supported by other studies in which a positive correlation was reported between eNO levels and markers of eosinophilic airway inflammation in induced sputum (8, 25). Others demonstrated an association between eNO and exposure to relevant allergens (26), or a reduction in eNO following antiinflammatory treatment (27). These study results provide sufficient indications that

airway inflammation is significantly associated with elevated eNO levels, thus providing a tool in monitoring disease activity. However, a range of normality needs to be established.

The eNO levels measured in this study tend to be somewhat lower than those measured in other studies (8, 28). This may be caused by technical factors. It has been shown that there is a marked flow-dependence of eNO values with lower values measured at high flow-rates and vice versa (29). We standardized the expiratory flow as 5% of vital capacity per second, which resulted in an expiratory flow of approximately 0.25 to 0.30 ml/min, which is higher than the flow used in some other studies. It is not clear whether such standardization of flow for lung volume is important when measuring eNO. Recently it was demonstrated that the flow-eNO relationship differed between asthmatic subjects and healthy individuals (30). This difference is, however, of minor importance compared to the differences we now report between the various groups.

We believe that responsiveness to AMP may serve as indicator of airway inflammation, although normal values for bronchial AMP responses have not been determined yet. It was demonstrated a few years ago that experimental instillation of AMP into an airway segment caused a prompt reduction in airway caliber, paralleled by a significant rise in prostaglandin D_2, histamine, and tryptase levels in the lavage fluid, suggestive of mast cell degranulation (31). Since mast cells are believed to play a predominant role in asthmatic airway inflammation, the response to AMP may reflect different aspects of inflammation as compared with the bronchial response to direct stimuli, such as methacholine or histamine. Results supporting this statement include a more pronounced improvement of AMP responsiveness following avoidance of allergen

(32), and following antiinflammatory therapy (33), as compared with effect on MCh- or histamine responsiveness. In recent years, it has been suggested that an exaggerated airway response to direct stimuli, like MCh, and abnormal FEV1 values, may exist or persist independently of "active" airway inflammation (34). Probably, these indices are also influenced by irreversible airway damage, caused by airway remodeling (35). Thus, being associated with the more slowly responding elements of inflammation within the airways. Our finding of a positive correlation between response to AMP and eNO levels, but not between responsiveness to MCh and eNO levels, is in agreement with this.

The clinical relevance of our findings is as yet unknown. Although one would expect the degree of abnormalities to be related to the duration of remission, a significant correlation could not be established. Probably, when subclinical airway inflammation is present during adolescence, it may persist for several years with a continuous risk to become clinically manifest again. Future longitudinal studies should assess the possible benefits of prolonged disease monitoring and antiinflammatory treatment during the asymptomatic phase. Also, the question arises whether or not indices associated with different parts of the inflammatory process, such as eNO and responsiveness to AMP and MCh, need to be included in the definition of asthma remission.

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Chapter

4

Airways Inflammation is Present during Clinical Remission of Atopic Asthma

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ABSTRACT

Symptoms of atopic asthma often disappear around puberty. However, asthmatic subjects in clinical remission will frequently have a relapse later in life. The aim of this study was to investigate whether subjects in clinical remission of atopic asthma have persistent airways inflammation and/or airways remodeling. Bronchial biopsies were obtained from subjects in clinical remission, asthmatic subjects, and healthy controls. The presence and/or activation state of eosinophils, mast cells, macrophages, T lymphocytes, interleukin (IL)-5, eotaxin and inducible nitric oxide synthase (iNOS) were analyzed. Results were compared with less invasive indicators of airways inflammation. Also aspects of airways remodeling were determined.

Eosinophils, T cells, mast cells, and IL-5 were significantly elevated in the airways mucosa of subjects in remission compared to controls. Also, blood eosinophil cell counts were significantly higher in subjects in clinical remission. Blood eosinophil cell counts, exhaled nitric oxide (eNO) levels, and bronchial response to adenosine-5'-monophosphate (AMP) correlated significantly with the quantity of tissue eosinophils. Significant airways' remodeling was found in subjects in clinical remission.

In conclusion, our study shows ongoing airways inflammation and airways remodeling in adolescents in clinical remission of atopic asthma. Subclinical airways inflammation may well determine the risk of an asthma relapse later in life.

INTRODUCTION

Symptoms of atopic asthma often begin in early childhood and mostly improve or even seem to disappear around puberty (1, 2). However, a considerable proportion of asthmatic subjects in clinical remission will have a relapse later in life (2). Several studies have shown spirometric abnormalities and bronchial hyperresponsiveness (BHR) to methacholine (MCh) or cold air challenge during clinical remission of asthma (3, 4). It is unknown whether these functional abnormalities, which are supposed to be indicative of asthma severity with respect to symptomatic asthma, reflect persistent activity of the airways inflammatory process or merely indicate structural changes of the airways as a consequence of childhood asthma. These structural changes, known as airways remodeling, are probably early events in the course of the disease which appear to progress. The process of remodeling leads to thickening of the airway wall (5-7). The exact physiologic consequences of airway wall thickening are, however, incompletely understood (8). If airway wall thickening is present in subjects in clinical remission of asthma, it could at least in part account for the functional abnormalities including bronchial hyperresponsiveness found during 'remission'. On its turn, ongoing 'active' airways inflammation could have substantial impact on the risk of a relapse later in life. As a consequence, subjects with subclinical airways inflammation could benefit from anti-inflammatory treatment (8-10). Recently we demonstrated elevated exhaled nitric oxide (eNO) levels and BHR to both MCh and adenosine-5'-monophosphate (AMP) during clinical remission of atopic asthma (11). With respect to eNO, increased expression of the inducible form of nitric oxide synthase (iNOS) is thought to underlie

these elevated eNO levels (12, 13). Studies regarding the precise relationship between iNOS expression and eNO are, however, scarce (12).

Proof of persistent airways inflammation and remodeling during clinical remission could be furnished from the analysis of bronchial biopsy specimens. Evidence has been accumulated to suggest that allergen-reactive type 2 T helper (Th2) cells, with eosinophils and mast cells as main effector cells, orchestrate asthmatic airways inflammation (14, 15). The presence of eosinophils in the airway wall is apparently the most reliable feature of bronchial asthma that seems to be related to asthma severity (16). Along with interleukin (IL)-5, a Th2 cytokine, and eotaxin, a chemoattractant for eosinophils, eosinophil-derived products such as major basic protein (MBP) have been investigated as markers of eosinophil participation in the pathogenesis of asthma (17). Also, elevated numbers of bronchial mast cells have been found in the airway mucosa of atopic and non-atopic asthmatic subjects (18, 19). Release of mediators from mast cells, such as granule-associated tryptase and chymase, leads to immediate bronchoconstriction and enhances airways inflammation (19).

In healthy airways, the inflammatory response to e.g. inhaled allergens is suppressed by alveolar macrophages. In asthma, however, a deviant function of alveolar macrophages may contribute to the allergic immune response in the airways (20).

How numbers and activation state of these different cell types in the bronchial mucosa relate to less invasive markers of airways inflammation, including bronchial hyperresponsiveness (BHR), circulating eosinophils and eNO, is largely unclear (21-23).

Remodeling of the airways mucosa and submucosa can be assessed by measurement of the reticular basement membrane (RBM) thickness (24) and extent of epithelial shedding (25). Also, collagen deposition (26) is a quantifiable aspect of airways remodeling.

To establish whether subjects in clinical remission of atopic asthma suffer from ongoing active airways inflammation and/or airways remodeling, we compared bronchial biopsy specimens of subjects with a long-standing clinical remission of atopic asthma with those of currently asthmatic subjects, and of healthy controls. Biopsy findings were compared to noninvasive markers of airways disease.

SUBJECTS, MATERIALS AND METHODS

Subjects

Adolescents with atopic asthma, 18 - 25 years of age, were selected from the Sophia Children's Hospital discharged patients files. Clinical remission of atopic asthma was defined as reported complete absence of asthmatic symptoms in subjects not taking any asthma medication for at least 12 months prior to the study. Eligible subjects were compared with patients with asthma who had persistent symptoms and used inhaled β_2-agonists on demand in order to relieve symptoms. All subjects were previously diagnosed as having atopic asthma according to ATS criteria (27). All subjects were lifelong non-smokers in stable clinical condition and did not take inhaled steroids or anti-allergic medication. Healthy non-smoking adult volunteers without a history of asthma served as controls.

The study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

Study design

We performed a cross-sectional study with three visits on separate days. Spirometry values, eNO, MCh- and AMP responsiveness were obtained according to methods we described earlier (11).

Blood eosinophils

Venous blood eosinophil numbers were counted by means of hemocytometry. Reference values ranged from 0.04 to 0.1 x 10^9 cells/liter.

Bronchoscopy

An experienced bronchoscopist (SEO) using an Olympus model BF IT 10 (Tokyo, Japan) performed flexible bronchoscopy. At least five bronchial biopsies were obtained from segmental divisions of the main bronchi.

Processing of bronchial biopsies

Bronchial biopsies were embedded in Tissue-Tek II OCT medium (Miles, Naperville, Illinois), snap frozen in liquid nitrogen, and stored at -80°C. Serial tissue sections (6 μm) were cut on a HM-560 cryostat (Microm, Heidelberg, Germany). At least two sections 120 μm apart from one biopsy specimen were placed on a poly-L-lysine-coated microscopic slide (Sigma Diagnostics, St.Louis, MO). Immunostaining was carried out with α-CD4 (T helper cells), α-CD8 (cytotoxic T cells), α-CD25 (activated cells) and α-CD69 (activated cells), α-CD68 (macrophages), α-MBP (Dako, Glostrup, Denmark), α-IL-5, α-eotaxin, α-tryptase, α-chymase (Chemicon Brunschwig Chemie, Amsterdam, the Netherlands), α-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and α-collagen III.

Binding of the antibodies was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Immunostained sections were analyzed with an image analysis system (Quantimed, Leica, Rijswijk, the Netherlands). With respect to the sub-epithelium, the program was set to analyze 100 μm below the reticular basement

membrane (RBM). The ratio of positive stained area divided by the total area analyzed was taken as measure for each immunohistochemical staining. RBM thickness was interactively measured at 20 μm intervals over a 1 mm RBM length (24). The occupancy of RBM with epithelium was plotted as ratio of occupied membrane length divided by the total membrane length.

Statistical analysis

Because of the skewed distribution of data, results are expressed as median value ± SEM. Comparisons between groups were made by the Mann-Whitney test for unpaired samples. Correlation between different indices was made by Spearman's rank correlation test. A two-tailed p-value of equal to or less than 0.05 was considered significant. Data were analyzed using Statistical Package for the Social Science (SPSS, Chicago, USA).

RESULTS

Fifty-four subjects completed the study (19 subjects with atopic asthma, 18 subjects in clinical remission of atopic asthma, and 17 healthy controls). Subjects characteristics are summarized in table 1. The median duration of clinical remission was 5 years.

TABLE 1: Subjects' characteristics

	Asthmatic subjects	Remission subjects	Healthy controls
N	19	18	17
Age (years)	22 ± 2	21 ± 2	24 ± 1
Sex (male : female)	13 : 6	15 : 3	9 : 8
FEV_1 (% of predicted)	88 ± 12	93 ± 15	105 ± 13
FEV_1 reversibility	11 ± 1	7 ± 1	4 ± 1

Variables are expressed as mean ± SEM.
Reversibility FEV_1: change in FEV_1, expressed as increase in % of predicted normal value after administration of 1 mg terbutaline

Immunohistochemistry results are summarized in table 2.

TABLE 2: Immunohistochemistry results

	Asthmatic subjects	Remission subjects	Healthy controls
Epithelium			
MBP	0.023 ± 0.0093*	0.0082 ± 0.0046*	0.0007 ± 0.0013
Tryptase	0.0068 ± 0.0020†	0.0119 ± 0.0029*	0.0025 ± 0.0009
Chymase	0.0100 ± 0.0035	0.013 ± 0.0030†	0.0067 ± 0.0016
CD4	0.001 ± 0.0004	0.0007 ± 0.0002	0.0009 ± 0.0013
CD8	0.025 ± 0.0045	0.018 ± 0.0068	0.0281 ± 0.0082
CD25	0.0093 ± 0.0050	0.0123 ± 0.0032	0.0058 ± 0.0039
CD69	0.0095 ± 0.0116	0.0076 ± 0.0182	0.0123 ± 0.0093
CD68	0.029 ± 0.0054	0.038 ± 0.0067	0.031 ± 0.019
IL-5	0.038 ± 0.0097	0.036 ± 0.018	0.017 ± 0.014
Eotaxin	0.001 ± 0.0013	0.0002 ± 0.0045	0.0002 ± 0.0005
INOS	0.12 ± 0.02††	0.15 ± 0.03	0.21 ± 0.03
Collagen III	0.078 ± 0.011	0.077 ± 0.024	0.062 ± 0.009
Sub-epithelium			
MBP	0.12 ± 0.026*	0.07 ± 0.024*	0.0042 ± 0.0086
Tryptase	0.037 ± 0.0082††	0.075 ± 0.012**	0.022 ± 0.010
Chymase	0.014 ± 0.0038	0.018 ± 0.0056†	0.0069 ± 0.0033
CD4	0.0043 ± 0.0040	0.0025 ± 0.0019	0.0041 ± 0.0027
CD8	0.022 ± 0.0040	0.014 ± 0.0033	0.019 ± 0.004
CD25	0.0094 ± 0.0126	0.0356 ± 0.0233†	0.0095 ± 0.0041
CD69	0.0062 ± 0.0198	0.0156 ± 0.0273	0.0085 ± 0.0063
CD68	0.058 ± 0.0069	0.069 ± 0.013	0.079 ± 0.032
IL-5	0.025 ± 0.0061	0.022 ± 0.0074**	0.0066 ± 0.0081
Eotaxin	0.0011 ± 0.0006†	0.0001 ± 0.0087	0.0001 ± 0.0003
INOS	0.041 ± 0.006	0.042 ± 0.014†	0.065 ± 0.016
Collagen III	0.16 ± 0.014	0.19 ± 0.016	0.17 ± 0.019

Variables are expressed as median density (ratio of positively stained area and total area) ± SEM. * $p \leq 0.001$ versus healthy controls, ** $p \leq 0.01$versus healthy controls, † $p \leq 0.05$ versus healthy controls, †† $p \leq 0.05$ versus remission subjects.

In both epithelium and sub-epithelium, MBP density was significantly higher in subjects in remission than in healthy controls ($p<0.001$ and $p=0.001$, respectively; figure 1 and 2).

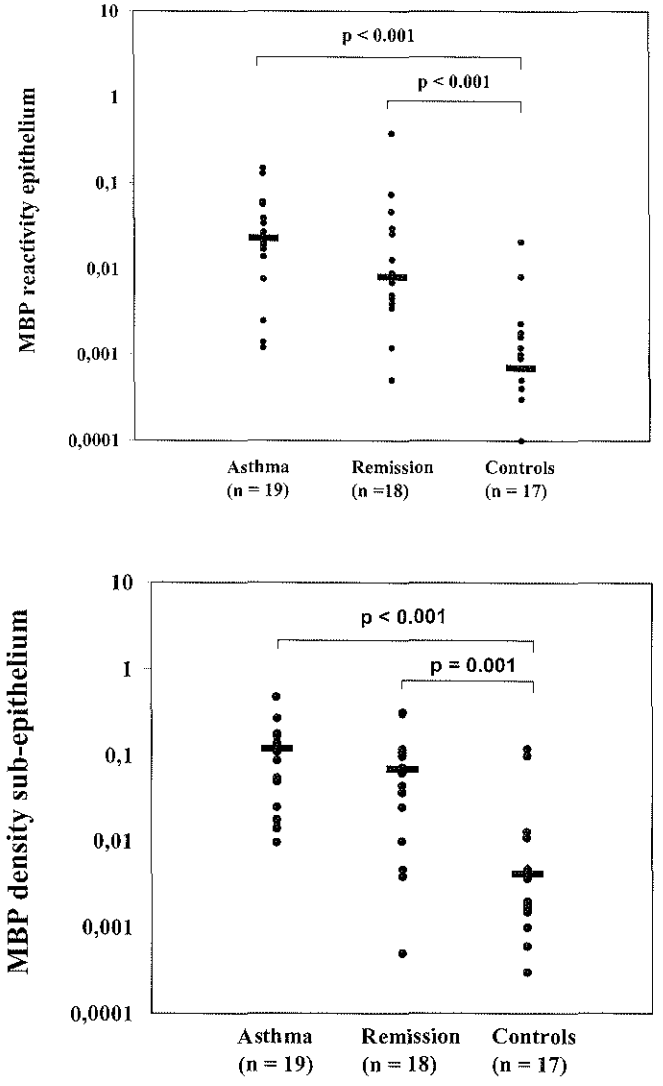

Figure 1: ratio of area of Major Basic Protein (MBP) staining and total area of epithelium (upper figure) and of sub-epithelium (lower figure) in bronchial biopsy specimens from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Each dot represents one subject. Horizontal bars represent median values. Y-axis shows values logarithmically.

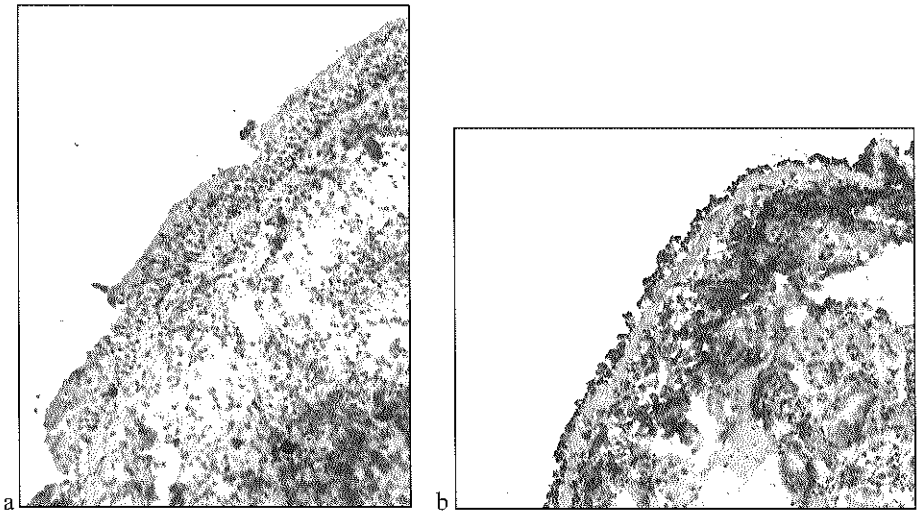

Figure 2: Bronchial biopsy specimen from a healthy control subject (a) and a subject in clinical remission of atopic asthma (b), immunostained with α-Major Basic Protein (MBP). Notice the increased MBP-positive area (the red stain) and epithelial shedding in the subject in clinical remission.

In bronchial epithelium of subjects in remission, there was more tryptase present than in that of healthy controls ($p=0.001$). In sub-epithelium, however, there was more tryptase detectable in subjects in remission than in control subjects and in patients with asthma ($p=0.008$ and $p=0.025$, respectively; figure 3 and 4).

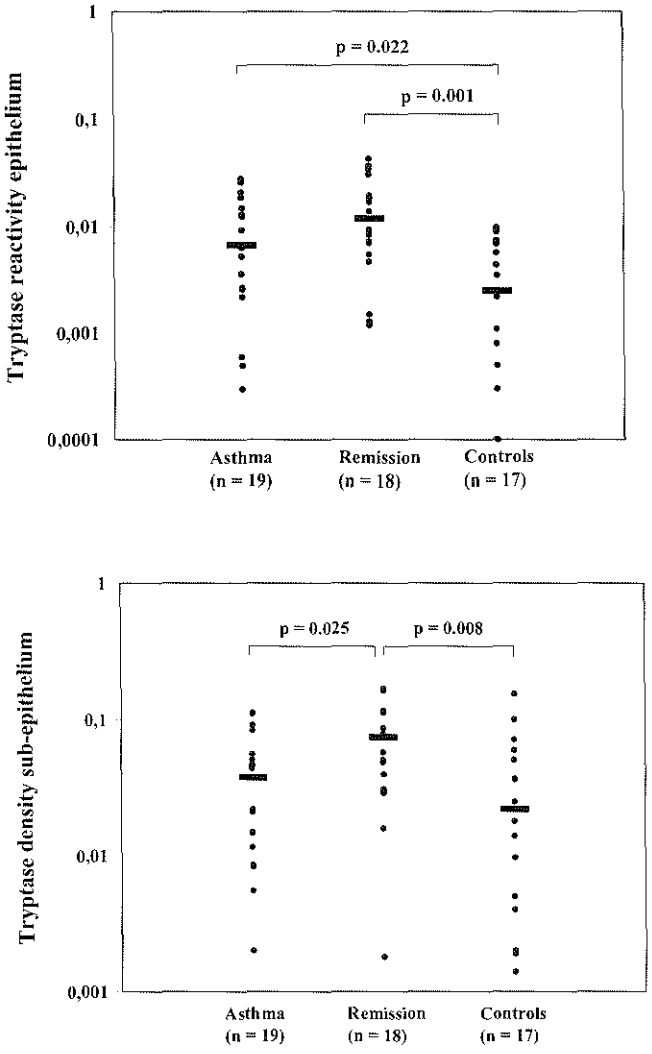

Figure 3: ratio of area of tryptase staining and total area of epithelium (upper figure) and sub-epithelium (lower figure) in bronchial biopsy specimens from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Each dot represents one subject. Horizontal bars represent median values. Y-axis shows values logarithmically.

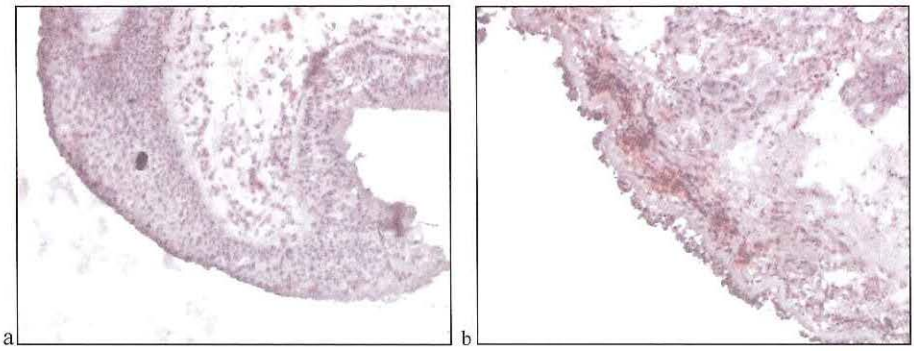

Figure 4: Bronchial biopsy specimen (α-tryptase) from a healthy control subject (a) and a subject in clinical remission of atopic asthma (b). Notice the increased tryptase-positive area (the red stain) and epithelial shedding in the subject in clinical remission.

Chymase density was higher in bronchial epithelium and sub-epithelium of subjects in remission than in that of healthy controls (p=0.044 and p=0.05, respectively).

There was no difference detectable in the presence of $CD4^+$ and $CD8^+$ cells in both epithelium and sub-epithelium among the experimental groups. However, there were more activated, $CD25^+$ cells present in the sub-epithelium of subjects in remission compared to control subjects (p=0.04). No such difference was detected for CD69.

$CD68^+$ cells were equally represented in sub-epithelium and epithelium in all three experimental groups.

In sub-epithelium, IL-5 density was significantly higher in subjects in remission than in healthy controls (p=0.006). Eotaxin density was significantly higher in patients with asthma than in healthy controls (p=0.05), while intermediate levels were detected in subjects in remission. In the epithelium, densities of IL-5 and eotaxin were similar in all three groups. Density of iNOS was significantly higher in sub-epithelium of healthy

subjects than in that of subjects in remission (p=0.05). In the epithelium, iNOS density was higher in healthy subjects than in asthmatic subjects (p=0.05). No difference in iNOS density could be detected between healthy control subjects and subjects in clinical remission.

With respect to indices of remodeling, RBM thickness differed significantly between subjects in remission and control subjects (10.9 ± 1.3 and 7.9 ± 1.0 μm, respectively; p<0.001). Current asthmatic subjects showed a median thickness of 11.5 ± 1.5 μm (Figure 5).

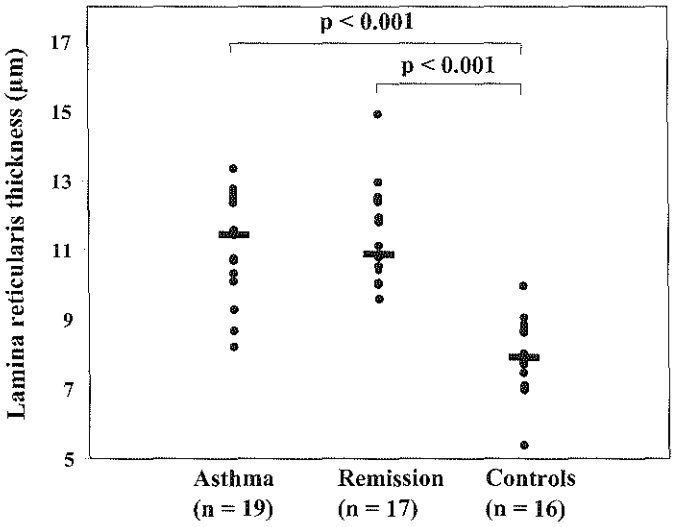

Figure 5: Thickness of the reticular basement membrane (RBM) in biopsy specimens from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Horizontal bars represent median values.

Furthermore, in subjects in remission the RBM occupancy with epithelium differed significantly from values obtained from control subjects (ratio 0.67 and 0.83, respectively; $p<0.003$), and from values from current asthmatic subjects (median ratio 0.50, $p<0.03$) (Figure 6).

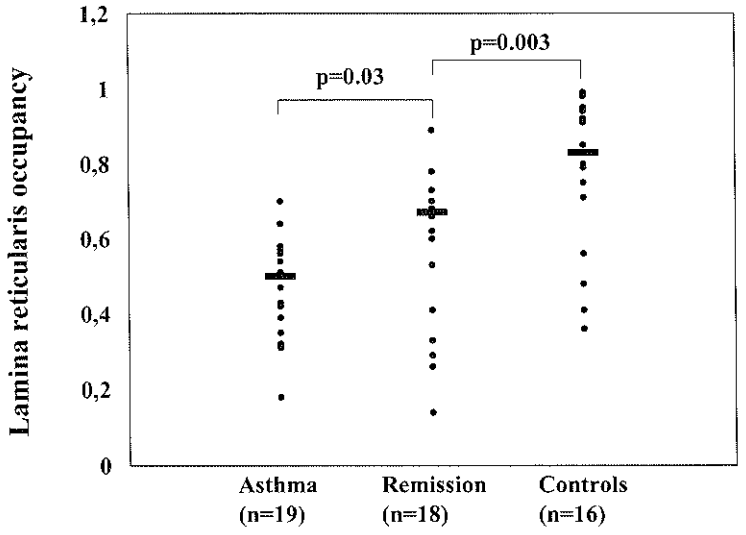

Figure 6: Occupancy of the reticular basement membrane (RBM) with epithelium in biopsy specimens from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Results are plotted as ratio of the length of occupied RBM divided by the total membrane length. Horizontal bars represent median values.

Collagen type III density in biopsy specimens was not significantly different between the groups. In peripheral blood, the number of eosinophils was significantly elevated in subjects in remission compared with control subjects ($0.22 \pm 0.17 \times 10^9$/l and $0.11 \pm 0.045 \times 10^9$/l, respectively; $p<0.001$; figure 7). Median eosinophil count in patients with asthma was $0.30 \pm 0.17 \times 10^9$/l.

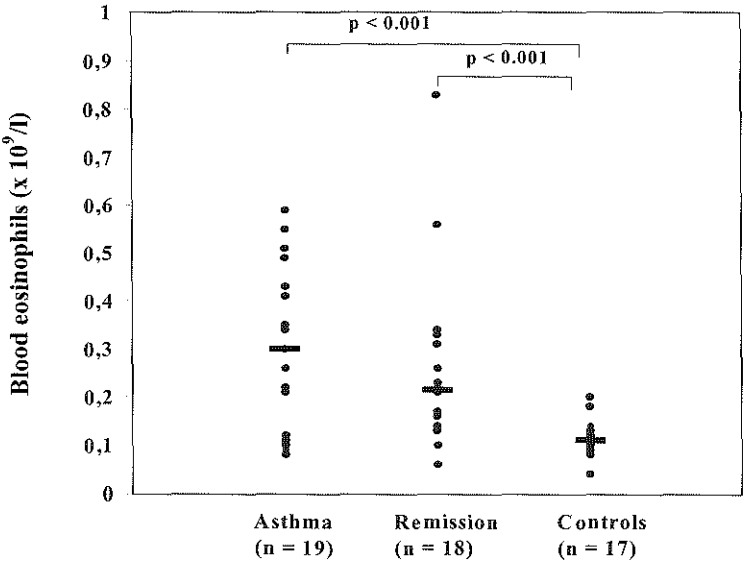

Figure 7: Eosinophil cell counts in blood samples from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Each dot represents one subject. Horizontal bars represent median values.

In subjects in remission and those with asthma, a significant positive correlation was found between blood eosinophil cell counts and MBP density in both bronchial epithelium and sub-epithelium ($r=0.41$, $p=0.017$ and $r=0.45$, $p=0.009$, respectively; figure 8a). A similar correlation was observed for eNO values (11) and MBP density in bronchial epithelium and sub-epithelium of subjects in remission and patients with asthma ($r=0.40$, $p=0.022$ and $r=0.35$, $p=0.043$, respectively; figure 8b).

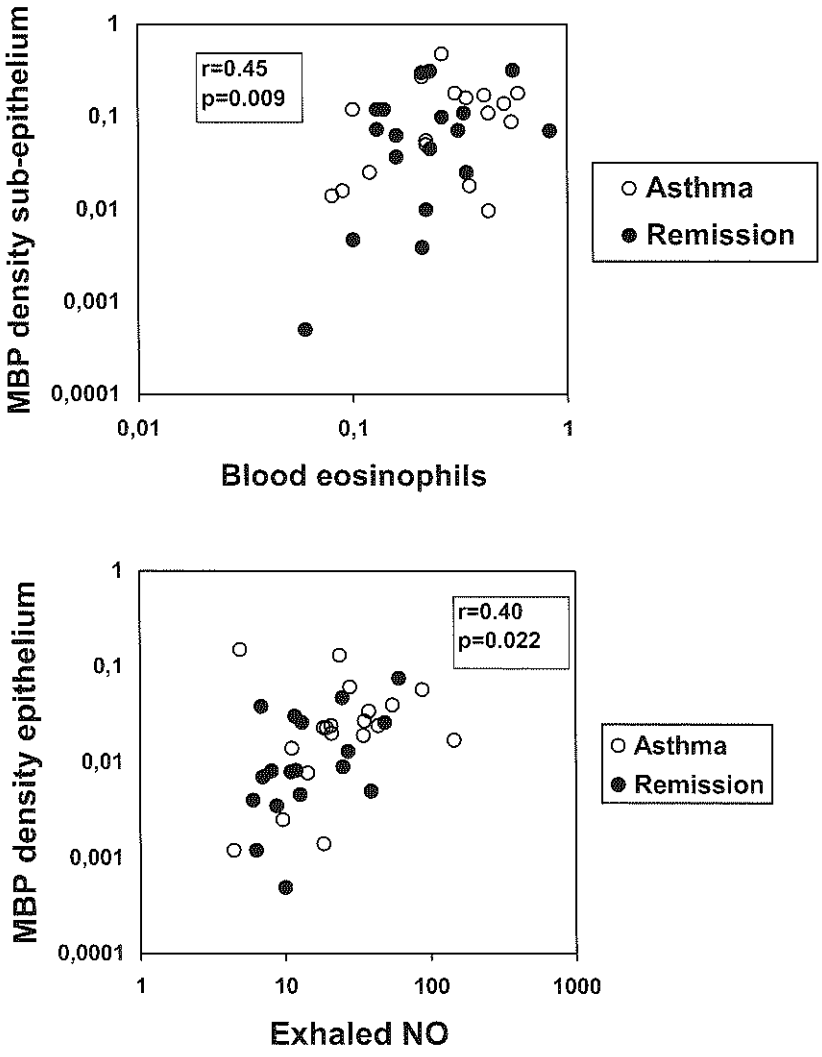

Figure 8: Correlation between blood eosinophil cell count and Major Basic Protein (MBP) density in sub-epithelium (r=0.45, p=0.009) (upper figure), and between exhaled Nitric Oxide (eNO) and MBP density in the bronchial epithelium (r=0.40, p=0.022) (lower figure) in the population of subjects in remission and currently asthmatic subjects. Each dot represents one subject.

PD_{20} AMP values correlated inversely with MBP density in sub-epithelium of subjects in remission and subjects with asthma (r=-0.37, p=0.032). In contrast, no significant correlation could be found between PD_{20} MCh values and MBP density in bronchial biopsy specimens. In subjects in remission and those with asthma, thickness of the RBM correlated with PD_{20} AMP (r=0.47, p=0.038), whereas the relationship with PD_{20} MCh was not significant (r=0.36, p=0.1).

DISCUSSION

In this study we demonstrated the presence of an ongoing active inflammatory process in the airways during long-lasting clinical remission of atopic asthma, as reflected by significantly increased levels of MBP, tryptase, chymase, CD25 and IL-5 in bronchial epithelium and/or sub-epithelium. In addition, eosinophil cell counts in blood samples were significantly elevated during clinical remission compared with those in healthy control subjects. Less invasive indicators of airways inflammation, such as blood eosinophils, eNO and PD_{20} AMP showed a significant correlation with MBP density in biopsy specimens from subjects in remission and patients with asthma. We also found evidence of airways remodeling during clinical remission. There was a significant correlation between RBM thickness and bronchial hyperresponsiveness to AMP.

Airways inflammation has been demonstrated in subjects with symptomatic asthma (28, 29). However, studies regarding the presence of inflammation in bronchial biopsy specimens from prolonged symptom-free subjects with a history of atopic asthma are lacking. Therefore, we applied a strict definition of clinical remission, where subjects were regarded in clinical remission if they reported complete absence of cough, chest discomfort or breathlessness for at least one year preceding the study, and did not use any medication in order to control asthma symptoms. In our study population, the median duration of remission was 5 years with only one subject that had been in remission for just one year. Not withstanding these strict inclusion criteria, we are the first to show direct evidence of substantial active airways inflammation, suggesting persistent disease in these otherwise asymptomatic subjects.

Increased numbers of eosinophils, one of the most important effector cells in atopic asthma, have been demonstrated in the bronchial mucosa of patients with asthma (17). We present similar findings in the bronchial mucosa of subjects in clinical remission. Also, elevated levels of tryptase- and chymase, constituents and markers of mast cells, were detected in the bronchial mucosa of subjects in remission compared to those of healthy controls. On the other hand, we found reduced tryptase density in the airway wall mucosa of subjects with asthma as compared with subjects in remission. This is suggestive of enhanced degranulation of mast cells in patients with asthma compared to subjects in clinical remission. Upon degranulation, mediators are diluted out into the tissue environment and rapidly degraded (30).

In the airway mucosa, there was no significant difference between the groups with respect to the presence of T cells. In previous studies, elevated T cell numbers where found in the airway mucosa, even in patients newly diagnosed with asthma (31). Others reported no increase in total numbers of T cells in bronchial mucosa specimens (32, 33), or just an increase in activated T cell numbers (34, 35). We found elevated levels of CD25 expression in bronchial sub-epithelium of subjects in remission compared to healthy subjects, which was not demonstrated in patients with asthma. The number of $CD69^+$ cells was not elevated. In our study, patients with asthma were in stable phase of disease and by definition the subjects in remission were without clinical symptoms all together. Therefore, disease activity should be regarded low in both groups. This may explain the absence of significantly elevated density of T cells. However, we did find increased levels of activated cells albeit not with an early marker of activation.

We found similar CD68 levels in all three groups. This may indicate that total macrophage numbers are relatively unimportant for asthma, and that a transformed and activated subset of these cells may be responsible for disease activity (20).

The relationship between markers of inflammation in bronchial biopsy specimens and other indices that presumably reflect distinct aspects of the inflammatory process, including peripheral blood eosinophils, eNO and the degree of BHR to various inhaled stimuli, is controversial. It has been proposed that determination of numbers of eosinophils in peripheral blood may help to indicate the level of airways inflammation (23). In the present study, blood eosinophil numbers correlated significantly with airway mucosal MBP density. Therefore, blood eosinophil cell numbers might serve as an indicator of eosinophilic airways inflammation in otherwise symptom free subjects with a history of atopic asthma.

A relationship between elevated cell numbers and/or cell activity in bronchial biopsy specimens and BHR has been demonstrated in some other studies (22, 36). Especially BHR with AMP as the inhaled stimulus has been proposed as a useful marker reflecting at least part of the inflammatory process in asthma (37). We found a significant correlation between PD_{20} AMP and MBP density in the airway wall mucosa in patients with current asthma and subjects in clinical remission of asthma. However, such a correlation could not be established between PD_{20} MCh values and MBP density in the airway mucosa. This is in agreement with other reports where MCh responsiveness appeared to be unrelated to indices of inflammation determined in bronchial biopsy specimens, and more to structural changes of the airways (21, 38). Previously, we reported that levels of eNO were significantly correlated with PD_{20} AMP, but not with

PD_{20} MCh (11). This is in agreement with finding that both PD_{20} AMP and eNO are related to the activity of cells known to play a role in the inflammatory process in asthma. In contrast, MCh acts directly on smooth muscle (SM) cells in the airway walls. Therefore, the response to MCh potentially reflects the structural changes found in asthma (39). This is supported by the fact that bronchial hyperreactivity to MCh may exist independently of active airways inflammation (40). The fact that MCh acts directly on SM cells may explain the absence of a correlation between BHR to MCh and RBM thickness. On the other hand, the positive correlation between BHR to AMP and RBM thickness may be explained by the presumed contribution of inflammatory mediators to the process of RBM remodeling.

Although epithelial desquamation may be an artifact of tissue sampling and not a true pathologic feature of the disease (41), we were able to demonstrate that the denudation of the RBM is much more progressive in patients with asthma than in subjects in remission. In subjects in remission, denudation is more pronounced than in healthy control subjects. We hypothesize that the severity of airways disease determines the susceptibility of the epithelium for physical stress. Hence, the differences we observed between the groups may be a reflection of the overall integrity of bronchial epithelium in the asthmatic bronchus compared to the bronchus of subjects in remission and to that of healthy control subjects.

Exhaled NO levels have already been shown to correlate with blood- and sputum eosinophilia in atopic children and adults with mild-intermittent asthma (42-44). Our study is the first to show a significant correlation between eNO levels and airway eosinophilia, as reflected by MBP density in the airway mucosa. Recently, eNO levels

were reported not to correlate with mucosal eosinophils (45). This difference may be explained by methodological factors, which include patient selection and –numbers.

Surprisingly, we found enhanced iNOS density in the airway wall mucosa from healthy controls compared with asthmatics and subjects in remission. This is not in agreement with the hypothesis of increased expression of iNOS in airway epithelium as the cause of elevated eNO (12, 46). We speculate that iNOS unrelated mechanisms, including enhanced diffusion of NO through damaged epithelium, or iNOS independent NO generation under conditions of airway acidity (47) may explain this finding in subjects with airways inflammation. Furthermore, other isoforms of NOS could account for elevated NO production. Following an increase in NO, a negative feedback mechanism might result in lower iNOS levels in the airway mucosa from asthmatic subjects.

In conclusion, our study shows ongoing airways inflammation and airways remodeling in adolescents in clinical remission of atopic asthma. We speculate that subclinical airways inflammation may well determine the risk of asthma relapse later in life. Furthermore, we hypothesize that subjects with evidence of airways inflammation during "remission" could possibly benefit from anti-inflammatory treatment in the short- and/or long-term. We propose that blood eosinophils, eNO and airway responsiveness to AMP, each reflecting distinct parts of the inflammatory process, may be useful in the long-term monitoring of asthmatic airways inflammation.

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Chapter

5

Dyspnea Perception during Clinical Remission of Atopic Asthma

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ABSTRACT

Symptoms of atopic asthma often disappear around puberty. We recently demonstrated that this clinical remission is accompanied with ongoing airways inflammation in most subjects. The discrepancy between lack of symptoms and persistent airway inflammation suggests blunted perception of symptoms. In the present study, young adults in clinical remission of atopic asthma assigned themselves a modified BORG score during methacholine and adenosine-5'-monophosphate induced bronchoconstriction. BORG scores of subjects in clinical remission were compared with those of symptomatic asthmatic subjects. We found a marked variation in BORG scores at 20% fall in FEV_1. Significant differences in BORG scores between remission patients and asthmatics could not be detected. We conclude that perception of dyspnea, induced with methacholine- and adenosine challenge, is similar in young adults in clinical remission of atopic asthma compared to that of patients with symptomatic asthma. Hence, blunted perception seems to be an unlikely explanation for the discrepancy between lack of symptoms and ongoing inflammation. Other factors, which include physical and psychological ones, may play a role in the apparent absence of symptoms, thereby potentially leading to undertreatment.

INTRODUCTION

In asthma, symptoms often decrease or even seem to disappear around puberty. Unfortunately, clinical remission is followed by a relapse later in life in a considerable proportion of subjects (1, 2). We recently found ongoing airways inflammation during clinical remission of atopic asthma (3, 4). Persistent airways inflammation during clinical remission would possibly explain the high relapse rate. The relationship between dyspnea perception and other indices of asthmatic severity, such as the degree of bronchoconstriction, is poor (5, 6). Conversely, impaired dyspnea perception is especially believed to play a role in severe asthma (7-9). Poor perception of airflow obstruction may lead to undertreatment of asthma (10). Especially in view of the high relapse rate of asthma later in life, diminished perception of dyspnea in subjects in clinical remission of asthma is of great clinical relevance. Therefore, the hypothesis of our study was that subjects in clinical remission of atopic asthma have blunted perception of dyspnea. Dyspnea scores, including visual analogue scale (VAS) scores and (modified) BORG scores, can be assessed during spontaneous asthma or during induced bronchoconstriction (11). The aim of the present study was to compare dyspnea scores during induced bronchoconstriction between young adults in clinical remission of asthma and currently symptomatic asthmatic subjects. Since the mechanism of bronchoconstriction differs between direct stimuli (methacholine, MCh) and indirect stimuli (adenosine-monophosphate, AMP), and may give rise to different perception, , MCh as well as AMP challenges were used in both groups.

SUBJECTS, MATERIALS AND METHODS

Subjects

Young adults with atopic asthma, 18 - 25 years of age, were selected from the Sophia Children's Hospital discharged patients files. Clinical remission of atopic asthma was defined as reported complete absence of cough, wheezing and breathlessness at rest and on exertion in subjects not taking any asthma medication for at least 12 months prior to the study. Approximately 80 percent of subjects in clinical remission showed elevated nitric oxide levels, eosinophilic airway inflammation, and hyperresponsiveness to both MCh and AMP (3, 4). All subjects responding with a 20 percent fall in FEV_1 during induced bronchoconstriction with MCh or AMP were considered eligible for the present study. Ten eligible subjects in remission were included, and compared with ten patients with asthma who had persistent symptoms, assessed with theSF-36 quality of life/symptom score form, at least once a month in the year preceding the study, and used inhaled β_2-agonists on demand in order to relieve symptoms. Subjects with current asthma could thus be defined as having 'mild persistent asthma' according to GINA guidelines. All subjects had a history of wheezing and chest tightness and were previously diagnosed as having asthma according to ATS criteria (12). All were atopic defined as radio allergosorbent test (RAST) class 2 or higher for at least one common airborne allergen. All subjects were lifelong non-smokers in stable clinical condition and did not take inhaled steroids, including nasal steroids, or anti-allergic medication like cromoglycate and antihistamines for at least one year prior to the study.

Common exclusion criteria were an inability to perform lung function tests reproducibly, and illnesses that may affect lung function. None of the subjects in the study reported symptoms of respiratory infection in the month prior to the study.
The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam.

Study design

We performed a cross-sectional study with two visits on separate days. At the first visit, subjects gave informed written consent and were asked about their asthmatic symptoms, history and requirement of rescue medication during the past year. Also, physical examination was performed, followed by determination of MCh responsiveness. At the second visit, scheduled at least 1 day after visit 1, subjects underwent an AMP challenge test. During the tests, subjects were asked to score their degree of breathlessness after each dose given according to a modified BORG scale. The sequence and intervals of the provocation tests were chosen in order to avoid any influence of the AMP challenge on MCh responsiveness (13).

Methacholine and Adenosine-5'-MonoPhosphate challenge

Challenge tests were performed at the same time of day (± 1 hour) according to the dosimeter method as described in detail earlier (3, 14). Short acting β_2-agonists were not allowed within 8 hours prior to the test. If a patient responded to saline or the lowest concentration of either MCh or AMP, they were assigned a PD_{20} value of half the starting dose. Inhalation provocation tests were performed using doubling concentrations of 0.15 to

78.4 mg/ml MCh bromide in PBS or 0.08 to 160 mg/ml AMP in normal saline (Sigma Chemical Co., St. Louis, MO). During MCh and AMP challenge, before each FEV_1 measurement, perception of dyspnea was assessed on a modified BORG scale. A perception score at 20 percent fall in FEV_1 (PS_{20}) was obtained by interpolation of the two last points on the perception/fall in FEV_1 curve (10).

Statistical analysis

Data are expressed as mean $PS_{20} \pm$ SEM. Comparisons between groups were made by Student's t-test for unpaired samples. A two-tailed p-value of less than 0.05 was considered significant. Correlation was expressed as partial correlation coefficient. Data were analyzed using Statistical Package for the Social Science (SPSS, Chicago, USA) for Windows™ version 9.0.

RESULTS

Twenty subjects could be assigned both a PS_{20} MCh value and a PS_{20} AMP value (10 subjects with currently symptomatic atopic asthma and 10 subjects in clinical remission of asthma).

Subjects' characteristics are summarized in table 1. The two groups were comparable with respect to age, FEV_1, and PD_{20} AMP. PD_{20} MCh in currently symptomatic asthmatic subjects was significantly lower than in subjects in clinical remission (p=0.034).

TABLE 1: Subject's characteristics

	Asthmatic subjects	Remission subjects
n	10	10
Age (range)	22.5 (19 – 25)	20.6 (19 – 24)
Sex (male : female)	7 : 3	10 : 0
FEV_1 (range)	90 ± 3 (80 – 106)	91 ± 4 (63 – 107)
PD_{20} MCh (range)	0.4 ± 0.3 (0.1 – 2.8)	2.3 ± 1.1 (0.1 – 9.9)
PD_{20} AMP (range)	5.3 ± 2.3 (0.6 – 27.3)	9.8 ± 2.6 (1.0 – 22.0)

All subjects, including the ones in clinical remission, experienced some degree of dyspnea at reaching a 20 percent decrease in FEV_1, even though there was a marked within-group variation in PS_{20} (for instance 0.5 to 9 in the remission group with respect to MCh provocation). Nevertheless, for both inhaled stimuli no significant difference

between currently asthmatic subjects and subjects in clinical remission with respect to PS_{20} could be detected (Figure 1 and 2).

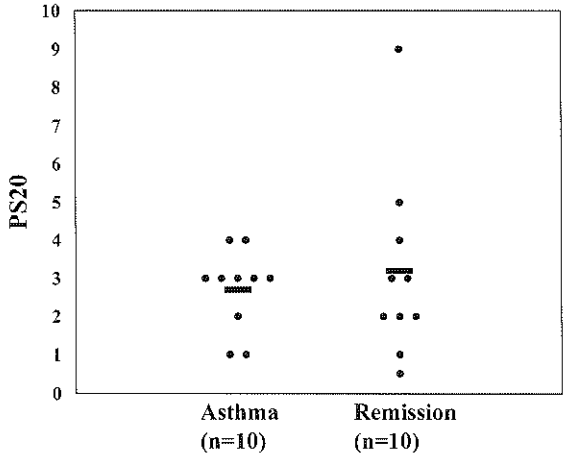

Figure 1: PS_{20} MCh values in subjects with asthma and subjects in clinical remission of atopic asthma. Each dot represents one subject. Horizontal bars represent median values.

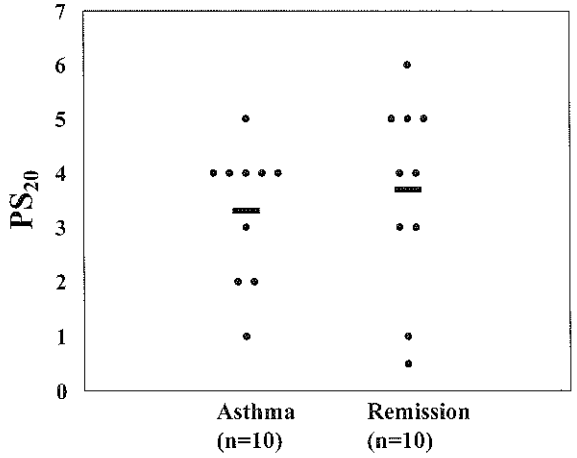

Figure 2: PS_{20} AMP values in subjects with asthma and subjects in clinical remission of atopic asthma. Each dot represents one subject. Horizontal bars represent median values.

Mean PS_{20} MCh was 2.7 ± 1.1 (range 1-4) and 3.2 ± 2.5 (range 0.5-9) for currently asthmatic subjects and subjects in remission, respectively, and 3.3 ± 1.3 (range 1-5) versus 3.7 ± 1.9 (range 0.5-6) with respect to AMP induced bronchoconstriction. We did not find a significant relationship between bronchial responsiveness, expressed as PD_{20}, and respiratory distress at reaching a 20 percent decrease in FEV_1 for both inhaled stimuli (r=0.23 and p=0.34 for MCh; r=0.06 and p=0.81 for AMP; figure 3 and 4).

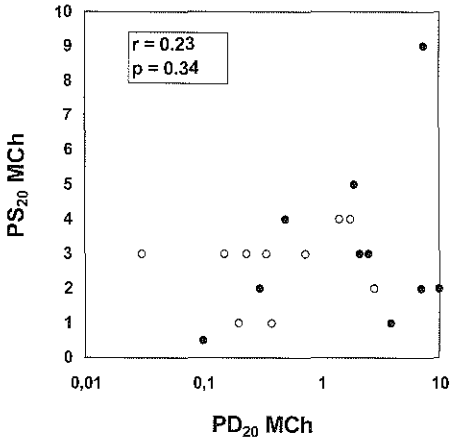

Figure 3: Partial correlation coefficients between PD_{20} and PS_{20} with respect to MCh challenge in currently asthmatic subjects (o) and subjects in clinical remission of atopic asthma (●).

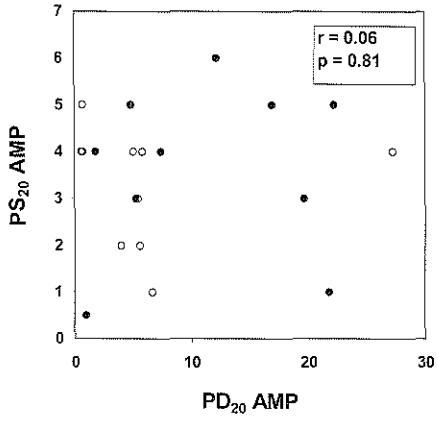

Figure 4: Partial correlation coefficients between PD_{20} and PS_{20} with respect to AMP challenge in currently asthmatic subjects (o) and subjects in clinical remission of atopic asthma (●).

DISCUSSION

In this study we did not find significant differences in dyspnea perception between currently asthmatic subjects and subjects in clinical remission of atopic asthma at 20 percent fall in FEV_1 with MCh and AMP induced bronchoconstriction. MCh responsiveness differed significantly between subjects in remission and currently symptomatic asthmatics, whereas AMP responsiveness did not. All subjects experienced some degree of dyspnea at reaching a 20 percent fall in FEV_1. Dyspnea scores varied from 'very, very slight dyspnea' to 'almost extreme dyspnea'. A significant correlation between dyspnea perception and degree of bronchial hyperresponsiveness could not be detected.

The finding that MCh-, but not AMP responsiveness differs significantly between subjects in clinical remission and currently asthmatic subjects, can be explained by a difference in mechanism of action. Where MCh responsiveness may be influenced by structural changes in the airways and active airway inflammation, AMP is believed to reflect only active airway inflammation, including increased numbers of mast cells. Thus, since currently asthmatic subjects may suffer from both more pronounced structural changes and more active inflammation, as compared with subjects in remission, MCh responsiveness may well separate the groups better than AMP responsiveness.

In 1994, Boulet and coworkers suggested that lack of symptoms may be insufficient as a tool to determine that asthma is in true remission (15). Indeed, there is a weak correlation between symptoms on the one hand and bronchial hyperresponsiveness and airways inflammation on the other. Several investigators have pointed to the possibility

of diminished perception of dyspnea in subjects with more severe grades of asthma, potentially leading to undertreatment in these subjects (7-9). Such an inverse relationship is not a consistent finding (10). Also in our study, there was no correlation between dyspnea perception and PD_{20}. However, our study population had a limited range of asthma severity.

Reduced dyspnea perception during clinical remission could possibly explain the absence of symptoms despite persistent airways inflammation and bronchial hyperresponsiveness (4) found in these subjects. Studies concerning symptom perception in asymptomatic asthmatics are scarce. In the present study we included subjects in clinical remission of atopic asthma supposedly having no grade of asthma at all. Given the ongoing airways inflammation we recently documented in a considerable proportion of those subjects (3, 4), it seemed of the greatest importance to show whether diminished perception of symptoms is present. The results of the present study do not confirm that blunted symptom perception explains the lack of symptoms. Subjects in clinical remission are obviously capable to detect airway narrowing at a similar level as compared with currently asthmatic subjects. On the other hand, others found a poor relationship between perception of spontaneous 'daily life' asthma and artificially induced bronchoconstriction (10, 11). It can therefore not be excluded that this is also true for subjects in clinical remission of asthma. Thus, it might still be possible that, as a result of biomechanical, pathophysiological or psychological factors, abnormal daily life fluctuations in airflow are not interpreted as asthma by subjects in clinical remission (6, 16). A fast decline in FEV_1 as a result of MCh or AMP induced bronchoconstriction is probably less liable to interindividual differences, and thus could explain the present

findings. Nevertheless, since dyspnea and other asthmatic symptoms seem to be an interplay between the degree of asthmatic inflammation and many other influences including physical and psychological factors (6, 7), we suggest that the absence of asthmatic symptoms in subjects regarded as ex-asthmatics should be interpreted with great care.

In conclusion, our study indicates that the lack of symptoms despite persistent bronchial hyperresponsiveness and ongoing airways inflammation during clinical remission of asthma seems not to be due to blunted symptom perception. Other factors, which include physical and psychological ones, may play a role in the apparent absence of symptoms in daily life. This is a potential factor leading to undertreatment.

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Chapter

6

Airway Inflammation during Clinical Remission of Atopic Asthma - Benefit from Anti-Inflammatory Treatment?

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Submitted

ABSTRACT

Symptoms of atopic asthma often disappear around puberty. In 30 to 80 % of the subjects with apparently outgrown asthma, however, symptoms will recur later in life. This high relapse rate is possibly due to ongoing airway inflammation and remodeling during clinical remission. We hypothesized that a-symptomatic subjects with ongoing airway inflammation and remodeling may benefit from anti-inflammatory treatment.

A double-blind, randomized placebo-controlled trial was conducted in 28 subjects in clinical remission of atopic asthma with bronchial hyperresponsiveness to methacholine (MCh) as non-invasive indicator of ongoing airway inflammation/remodeling. Intervention consisted of the salmeterol/fluticasone propionate combination product (SFC 50/250 µg bid via the Diskus™ inhaler) or placebo for three months. The change in lung function (FEV_1), bronchial response to MCh and adenosine monophosphate (AMP), the fraction of nitric oxide in exhaled air (FENO), and quality of life scores were measured. Also, bronchial biopsies were taken and cryo sections immunostained for eosinophils (major basic protein; MBP) and mast cells (tryptase and chymase) before and after treatment. The change in reticular basement membrane (RBM) thickness, one of the parameters of airway remodeling, was also determined.

SFC treatment improved PD_{20} MCh ($p=0.014$) as well as PD_{20} AMP ($p=0.011$), and reduced FENO ($p<0.001$) significantly. Furthermore, SFC treatment reduced tryptase in the subepithelium of bronchial biopsy specimens ($p=0.01$), and slightly reduced RBM thickness ($p=0.05$). However, eosinophils in (sub)epithelium were affected not significantly, as were chymase levels, blood eosinophils and quality of life scores.

In conclusion, we found that three months of treatment with combination therapy with fluticasone propionate and salmeterol improved airway hyperresponsiveness and FENO as non-invasive markers of airway inflammation, but had limited effect on inflammation in bronchial biopsies. Long-term clinical trials are needed to show benefits in terms of a reduced risk of relapse and/or of irreversible airflow obstruction later in life. If that proves to be the case, anti-inflammatory therapy during clinical remission of atopic asthma could be justified.

INTRODUCTION

Atopic asthma commonly starts in childhood and often seems to disappear in early adolescence (1, 2). Unfortunately, a substantial proportion of asthmatic adolescents with apparently outgrown asthma will have a relapse later in life, mostly before the age of 30 (2). Recently we and others found markers of airway inflammation and structural airway changes in airway biopsy specimens and bronchoalveolar lavage (BAL) fluid from young adults with a longstanding clinical remission of atopic asthma (3, 4). Ongoing inflammation and remodeling possibly contribute to irreversible airway narrowing (5) and bronchial hyperresponsiveness, thereby providing a basis for a future relapse of asthma.

It is unclear whether the long-term prognosis of subjects in clinical remission of asthma can be altered with prolonged anti-inflammatory therapy. There is ample evidence that inhaled steroids decrease airway inflammation (6), whereas the effect on remodeling is less evident (7, 8). An effect on subepithelial collagen deposition (9) and airway wall vascularity (10) was, however, found. Hence, the question arises whether subjects in clinical remission of atopic asthma could benefit from anti-inflammatory treatment (9, 11, 12). When it comes to "optimal" treatment of moderate to severe asthma, there is a strong scientific rationale for combining inhaled steroids with long-acting β2-agonists. (13, 14). Therefore, it was the aim of this study to investigate the effect of a short course of such asthma treatment on indices of airway inflammation and remodeling in young adults in longstanding clinical remission of atopic asthma.

SUBJECTS, STUDY DESIGN AND METHODS

Subjects

Young adults, 18 - 30 yrs of age, were selected from the Sophia Children's Hospital discharged atopic asthma patients files. Clinical remission of atopic asthma was defined as reported complete absence of asthmatic symptoms while not taking any asthma or allergy medication for at least 12 mo prior to the study. All subjects were previously diagnosed as having atopic asthma according to ATS criteria (15). All subjects were lifelong non-smokers in stable clinical condition. We conducted a double blind, parallel-group, randomized placebo-controlled trial in 28 subjects who were in clinical remission of atopic asthma for a median duration of 5 yrs, with a PD_{20} methacholine (MCh) cumulative less than or equal to 7.8 μmol at the beginning of the study.

The study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

Study design

All subjects underwent non-invasive and invasive measurements on 3 visits within 3 wks, followed by a 3-months' intervention period, after which all measurements were repeated on 3 visits. At the first visit, subjects gave written informed consent and were asked about asthmatic symptoms and requirement of rescue medication during the past yr, especially in order to avoid the inclusion of subjects with mild asthma. Also, quality of life scores were obtained. Thereafter, physical examination was performed and the fraction of NO in exhaled air (FENO) and PD_{20} MCh determined. At visit 2, scheduled at least 1 day after visit 1, subjects underwent an AMP challenge test. At visit 3, scheduled

at least 3 days after visit 2, FEV_1 and FEV_1 reversibility were tested. The sequence and intervals were chosen in order to avoid any influence of the AMP challenge on MCh responsiveness and/or on FEV_1 (16). All baseline measurements were used to obtain the subjects' characteristics (table 1). For further analysis on a specific variable, only subjects with pre- as well as post-treatment values (table 2 and 3) were used.

Spirometry values, MCh- and AMP responsiveness, FENO and blood eosinophils were obtained according to methods we described earlier (17). These are summarized briefly hereafter. Methods of obtaining quality of life scores as well as collecting and processing bronchial biopsies are described in more detail. Intervention consisted of the salmeterol/fluticasone propionate combination product (SFC 250/50 μg bid via the Diskus™ inhaler) or placebo for 3 mo ± 2 wks. Therapy adherence and adverse effects were recorded by the primary investigator. If more than 85 percent of the totally prescribed number of doses was emptied out of the device, therapy adherence was arbitrarily considered adequate.

Bronchial hyperresponsiveness

Challenge tests were performed according to the dosimeter method validated by Birnie and coworkers (18). Study medication was not allowed within 36 hrs prior to the test. After recording baseline values, the challenge started with inhalation of NaCl 0.9 %. Inhalation provocation tests were performed using doubling concentrations of 0.15 to 78.4 mg/ml MCh bromide in PBS or 0.08 to 160 mg/ml AMP in normal saline (Sigma Chemical Co., St. Louis, MO, USA). Cumulative provocative doses causing a 20 % fall in FEV_1 (PD_{20}) from baseline were calculated by means of linear interpolation of the logarithmic dose-response

curve. If FEV_1 fell less than 20 % of the pre-challenge level at the highest dose administered, twice the cumulative total inhaled dose was arbitrarily used as the PD_{20} value.

Spirometry

Study medication was not allowed within 36 hrs prior to the test. Flow-volume curves were obtained using a Lilly-type pneumotachograph (Masterlab Jaeger, Würzberg, Germany). The best of 3 reproducible recordings of FEV_1 was expressed as percentage of predicted normal and used for analysis. Reversibility was tested by measuring FEV_1 before and 20 minutes after inhalation of 1 mg of terbutaline powder (Bricanyl turbuhaler, ASTRA, Lund, Sweden), and expressed as increase in percentage of predicted normal.

FENO

FENO was measured in exhaled air by means of chemiluminescence (model 280 nitric oxide analyser, Sievers, Boulder, CO) according to recommendations of a European Respiratory Society Task Force (19). Subjects inhaled to TLC and immediately exhaled for as long as possible into a tube with an in-line flow resistor (20 cm H_2O/L/sec, Rudolph, MO). This was done at a flow corresponding to 5 % of the subjects' vital capacity per second.

Bronchoscopy

Premedication consisted of terbutaline 1 mg by metered dose inhalator and atropine 500 µg intramuscularly. After topical anesthesia with oxybuprocaine 10mg/ml to a

maximum of 20 ml, and xylocaine 10%, an Olympus model BF IT 10 (Tokyo, Japan) was passed by oral route. All bronchoscopies were performed by the same pulmonary physician (S.E.O.) At least 5 bronchial biopsies were obtained with the Olympus Swing Jaw forceps from segmental divisions of the main bronchi at both occasions.

Processing of bronchial biopsies

Bronchial biopsies were embedded in Tissue-Tek II OCT medium (Miles, Naperville, Illinois), snap frozen in liquid nitrogen, and stored at -80°C. Serial tissue sections (6 μm) were cut on a HM-560 cryostat (Microm, Heidelberg, Germany). At least two sections 120 μm apart from one biopsy specimen were placed on a poly-L-lysine-coated microscopic slide (Sigma Diagnostics, St.Louis, MO). Immunostaining was carried out with α-major basic protein (MBP, Sanbio BV, Uden, the Netherlands), α-tryptase and α-chymase (Chemicon Brunschwig Chemie, Amsterdam, the Netherlands). Binding of the antibodies was detected by the immuno-alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Immunostained sections were analyzed with an image analysis system (Quantimed, Leica, Rijswijk, the Netherlands). With respect to the sub-epithelium, the program was set to analyze 100 μm below the RBM. The ratio of positive stained area divided by the total area analyzed was taken as measure for each immunohistochemical staining. RBM thickness was interactively measured at 20 μm intervals over a 1 mm RBM length (20).

Quality of life

Quality of life scores were obtained with the aid of a modified RAND Health Insurance Study Questionnaire (21), the so-called RAND 36-item Health Survey, Dutch Version. RAND 36 consists of eight different categories of general health, that is to say physical functioning, social functioning, role limitations, emotional problems, mental health, pain, general health perception and health change. Items are scored 1 to 5 or 6. A higher score means better health. In each category, 100 is the maximum total score.

Statistical analysis

Lung function data (FEV_1, FEV_1 reversibility, PD_{20} MCh and PD_{20} AMP) and quality of life data (RAND 36) were parametrically analyzed using ANCOVA, where the baseline measurement of the outcome variable considered was included as covariant in comparing the two treatment groups (SFC and placebo). Within-subject changes from baseline of these variables were tested using the paired t-test for either treatment group. For the PD_{20} variables, the analyses were done after $\log_2$ transformation. PD_{20} MCh was used as primary variable. The other outcome variables were analyzed by comparing changes from baseline between the two treatment groups using the Mann-Whitney test. Within-subject changes from baseline of these other variables were tested using the Wilcoxon signed rank test for either treatment group. P-values below 0.05 were supposed to denote statistical significance.

RESULTS

Both the salmeterol/fluticasone propionate combination (SFC) group and the placebo group consisted of 14 subjects. Groups were well matched with respect to age, sex, FEV_1, FEV_1 reversibility, bronchial response to MCh and AMP and FENO (table 1). One subject in the placebo group refused the second bronchoscopy because of a negative experience with the first one. One other subject, also in the placebo group, could not undergo bronchoscopy because of a bleeding disorder. Therapy adherence was adequate since none of the subjects had used less than 85 percent of the prescribed total number of doses. Both treatments were well tolerated.

TABLE 1: **Subjects' characteristics**

	SFC	Placebo
n (male)	14 (11)	14 (9)
Age (yr)	19.6 (18 - 23)	20.4 (18 - 24)
FEV_1 (% pred)	94.1 (63 - 119)	101.2 (87 - 120)
FEV_1 reversibility (%)	9.9 (0 - 31)	6.9 (0 - 23)
PD_{20} MCh (µmol)	1.7 (0.1 - 6.2)	3.3 (0.3 - 7.4)
PD_{20} AMP (µmol)	24.0 (1.0 - 60.5)	23.1 (1.4 – 60.5)
FENO (ppb)	11.9 (7.2 – 22.0)	9.5 (4.0 – 23.4)

SFC: salmeterol/fluticasone propionate combination.All variables but FENO (median; range) are expressed as means (range). FENO: Fraction of nitric oxide in exhaled air.

SFC treatment for three months significantly improved PD_{20} MCh ($p=0.014$, table 2, fig 1) as well as PD_{20} AMP ($p=0.011$, table 2, fig 2), and reduced FENO ($p<0.001$, table 2, fig 3). Furthermore, SFC treatment reduced tryptase-positive cells in the bronchial subepithelium ($p=0.01$, table 3, fig 4 and 5), whereas MBP-positive cells in both the

epithelium and subepithelium decreased slightly, but not significantly (table 3, fig 6). Following SFC treatment, thickness of the RBM was reduced slightly (p=0.05, table 3, fig 7). Compared to placebo, FEV_1 and FEV_1 reversibility, blood eosinophils, chymase density and quality of life scores did not change as a result of three months of SFC treatment. Results are summarized in table 2 and 3.

TABLE 2: Results pre- and post-treatment – non-invasive measurements

	SFC	Placebo	Significance (p)
FEV_1 pre (% pred)	93.9 ± 4.6 (13)	101.5 ± 2.7 (13)	NS
FEV_1 post (% pred)	99.9 ± 2.7 (13)	101.7 ± 3.7 (13)	
FEV_1 reversibility pre (%)	10.5 ± 2.9 (13)	6.5 ± 1.8 (13)	NS
FEV_1 reversibility post (%)	4.7 ± 1.7 (13)	6.2 ± 1.2 (13)	
PD_{20} MCh pre (µmol)	1.7 ± 0.5 (14)	3.3 ± 0.7 (14)	0.014
PD_{20} MCh post (µmol)	19.8 ± 4.8 (14)	6.3 ± 2.9 (14)	
PD_{20} AMP pre (µmol)	24.0 ± 6.6 (14)	23.1 ± 5.7 (14)	0.011
PD_{20} AMP post (µmol)	44.8 ± 6.0 (14)	27.6 ± 6.9 (14)	
FENO pre (ppb)	13.0 ± 1.4 (12)	9.5 ± 1.5 (12)	< 0.001
FENO post (ppb)	4.9 ± 1.8 (12)	10.8 ± 2.2 (12)	
Quality of life pre	89.9 ± 4.2 (12)	84.5 ± 4.3(13)	NS
Quality of life post	88.2 ± 4.5 (12)	79.8 ± 3.9 (13)	

SFC: salmeterol/fluticasone propionate combination. Pre: pre-treatment. Post: post-treatment. FENO: Fraction of nitric oxide in exhaled air. All variables but FENO (median ± SEM) are expressed as means ± SEM. Number of subjects between brackets. NS: not significant.

TABLE 3: Results pre- and post-treatment –blood and biopsy measurements

	SFC	Placebo	Significance (p)
Blood eosinophils pre (x 10^9/l)	0.3 ± 0.05 (12)	0.3 ± 0.08 (12)	NS
Blood eos post (x 10^9/l)	0.4 ± 0.07 (12)	0.3 ± 0.08 (12)	
MBP subepithelium pre *	0.016 ± 0.005 (11)	0.008 ± 0.005 (11)	NS
MBP subepithelium post *	0.008 ± 0.008 (11)	0.009 ± 0.009 (11)	
MBP epithelium pre *	0.017 ± 0.003 (11)	0.014 ± 0.004 (11)	NS
MBP epithelium post *	0.006 ± 0.006 (11)	0.017 ± 0.008 (11)	
Tryptase subepithelium pre *	0.012 ± 0.004 (10)	0.005 ± 0.002 (11)	0.01
Tryptase subepithelium post *	0.004 ± 0.001 (10)	0.005 ± 0.005 (11)	
Tryptase epithelium pre *	0.003 ± 0.0008 (10)	0.001 ± 0.001 (11)	NS
Tryptase epithelium post *	0.0009 ± 0.0009 (10)	0.004 ± 0.001 (11)	
Chymase subepithelium pre *	0.002 ± 0.001 (7)	0.004 ± 0.001 (9)	NS
Chymase subepithelium post *	0.0009 ± 0.003 (7)	0.003 ± 0.001 (9)	
Chymase epithelium pre *	0.001 ± 0.0003 (7)	0.0009 ± 0.0009 (9)	NS
Chymase epithelium post *	0.001 ± 0.0005 (7)	0.001 ± 0.0009 (9)	
RBM thickness pre (μm)	9.7 ± 0.8 (8)	9.1 ± 0.4 (11)	0.05
RBM thickness post (μm)	8.7 ± 1.3 (8)	9.0 ± 1.1 (11)	

SFC: salmeterol/fluticasone propionate combination.
* The ratio of positive stained area divided by the total area analyzed was taken as measure for immunohistochemical stainings. Pre:pre-treatment. Post: post-treatment.
RBM: thickness of the reticular basement membrane (μm).
Values are expressed as medians ± SEM. Number of subjects between brackets. NS: not significant.

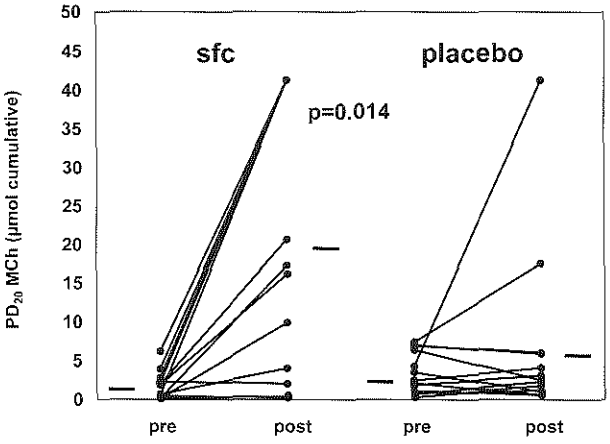

Figure 1: Cumulative PD_{20} MCh values pre- and post-treatment for three months with the salmeterol/fluticasone propionate combination product (sfc, n=14)) or placebo (n=14). Each line represents one subject. Non-responders are arbitrarily given a value of twice the highest cumulative dose given. Horizontal bars represent mean values.

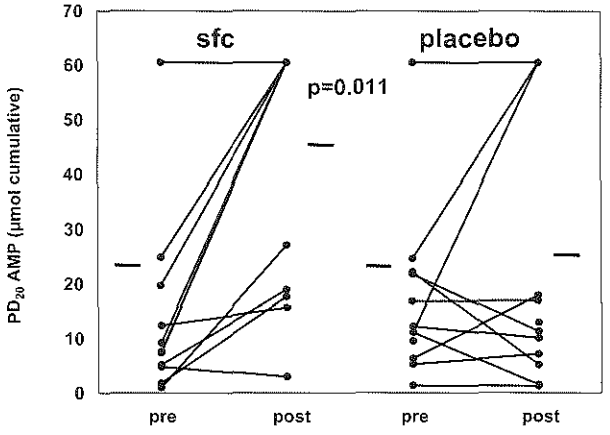

Figure 2: Cumulative PD_{20} AMP values pre- and post-treatment for three months with the salmeterol/fluticasone propionate combination product (sfc, n=14)) or placebo (n=14). Each line represents one subject. Non-responders are arbitrarily given a value of twice the highest cumulative dose given. Horizontal bars represent mean values.

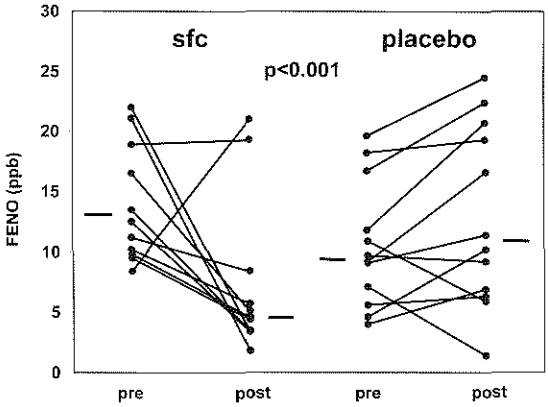

Figure 3: FENO pre- and post-treatment for three mo with the salmeterol/fluticasone propionate combination product (sfc, n=12) or placebo (n=12). Each line represents one subject. Horizontal bars represent median values.

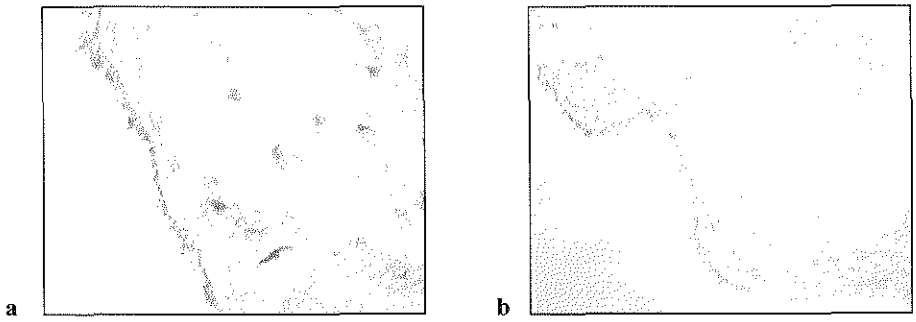

Figure 4: Example of a biopsy specimen pre-(4a) and post-treatment (4b) from a subject in clinical remission with tryptase staining (mast cells) in red.

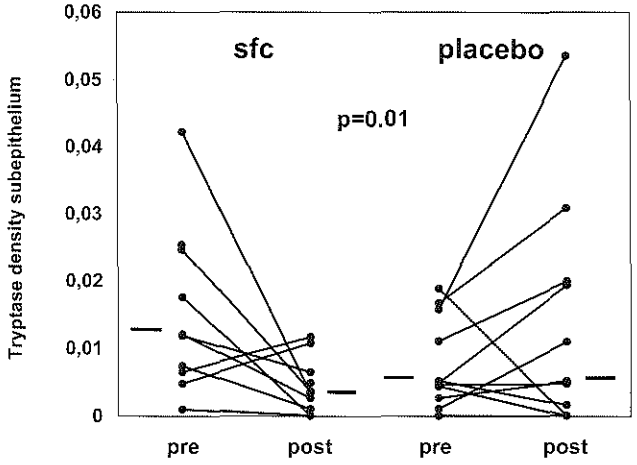

Figure 5: Tryptase density in the bronchial subepithelium pre- and post-treatment for three months with the salmeterol/fluticasone propionate combination product (sfc, n=10) or placebo (n=11). Each line represents one subject. Horizontal bars represent median values.

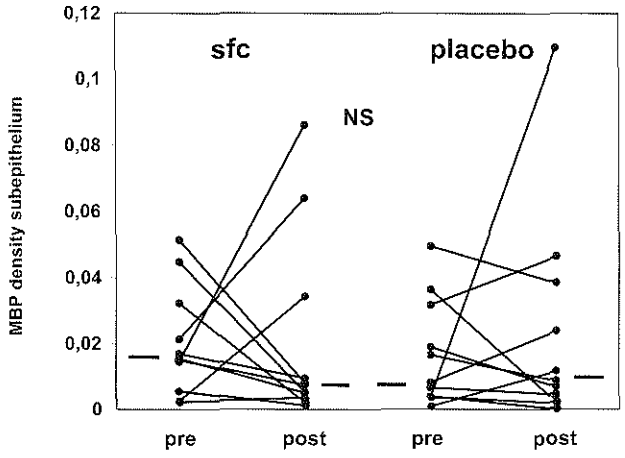

Figure 6: MBP density in the bronchial subepithelium pre- and post-treatment for three months with the salmeterol/fluticasone propionate combination product (sfc, n=11) or placebo (n=11). Each line represents one subject. Horizontal bars represent median values.
NS: not significant.

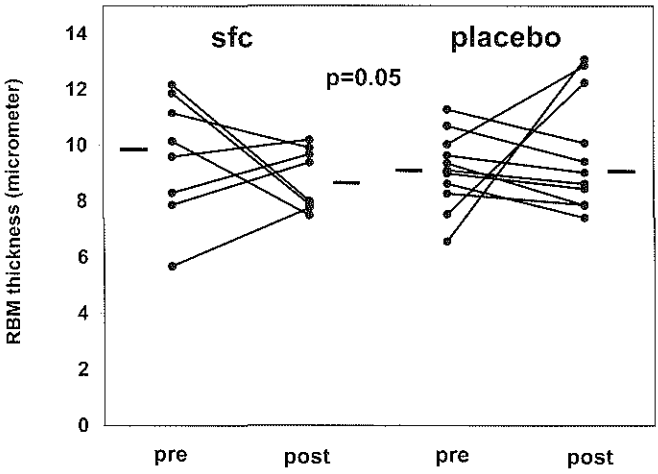

Figure 7: Thickness of the reticular basement membrane (RBM) pre- and post-treatment for three months with the salmeterol/fluticasone propionate combination product (sfc, n=8) or placebo (n=11). Each line represents one subject. Horizontal bars represent median values.
NS: not significant.

DISCUSSION

Many subjects in clinical remission of atopic asthma have ongoing airway inflammation and remodeling (3, 4), which may predispose for a relapse later in life. The present study shows that combined treatment with an inhaled steroid and a long-acting $ß_2$-agonist significantly reduced bronchial hyperresponsiveness and FENO levels, whereas invasively obtained markers of inflammation and remodeling remained largely unchanged.

Anti-inflammatory treatment normalized both MCh responsiveness and AMP responsiveness in a substantial proportion of the subjects in the present study. Where AMP responsiveness is believed to be related to active airway inflammation by inducing mast cell degranulation, MCh acts through direct mechanisms on the airway smooth muscle, thereby at least in part reflecting structural changes, i.e. airway remodeling, in the airways (22). We recently found a significant relationship between AMP responsiveness and eosinophilic airway inflammation (3). Hence, a normal response to AMP suggests the absence of clinically significant active inflammation (23). Normal MCh responsiveness, on the other hand, relates to the absence of manifest structural changes in the airways as well. In a study of Koh and colleagues, BHR to MCh was unchanged in subjects in clinical remission of asthma after nine months of treatment with inhaled budesonide (24). Whether this was due to more severe and irreversible airway changes, or a different kind of study medication as compared with our treatment, is unclear. Thus, next to FENO measurement, both MCh and AMP challenge might be useful in monitoring disease activity and response to treatment.

A highly significant reduction in FENO levels was seen. More specifically, 10 out of 12 subjects treated with SFC reached FENO values within the normal range after three months of treatment. It has been recently shown that FENO correlates with airway mucosal eosinophilia (3, 25), BAL eosinophils (26) and sputum eosinophils in atopic asthma (27). Therefore, FENO might be a useful tool in monitoring disease activity and effectiveness of treatment (28-30). Our data suggest that FENO might be a more rapidly responding marker of active inflammation than are tissue eosinophils.

With respect to markers of airway inflammation in tissue specimens, only tryptase levels improved significantly. Since a central role in the inflammatory process has been attributed to bronchial mast cells (31, 32), the demonstrated effect of anti-inflammatory therapy on these cells may be of clinical relevance. Changes in MBP density, reflecting eosinophil activity, showed a trend towards a reduction in the SFC group, but were not significant. We speculate that MBP changes might reach significance when more subjects would have been studied.

We found that the thickness of the reticular basement membrane (RBM), one of the characteristics of airway remodeling, was reduced in some subjects, but not in all. Overall changes were of borderline significance. It can be argued that these changes may be the result of reduction in RBM edema due to plasma leakage (12, 33, 34), which might also be influenced by the addition of a long acting ß2-agonist (LABA) to the study medication (10). Ward et al. recently demonstrated a significant effect of fluticasone propionate on thickness of the RBM after nine months of treatment in subjects with mild asthma (35). Other characteristics of airway remodeling, such as subepithelial collagen deposition and smooth muscle hyperplasia, presumably respond more slowly, if at all (8, 9). Therefore,

our intervention may have been too short to induce important changes in the RBM thickness.

The choice of salmeterol plus fluticasone propionate was based on the concept of optimal combination treatment as therapy of choice in moderate to severe asthma. In recent years, an additive or even synergistic effect of long acting β2-agonists (LABA) on airway inflammation (14, 36) and airway vascularity (10) has been documented. Also, LABA can improve well being by providing immediate relief. As we found no evidence of subjective improvement or better quality of life in this study, the added value of the long-acting $ß_2$-agonist in subjects with subclinical asthma remains conceptual. It was, however, not the aim of this study to elucidate which treatment would be best for the subjects in clinical remission.

The present study also demonstrates that the improvement in bronchial hyperresponsiveness, FENO values and mast cell activation is not accompanied by improved quality of life. We previously reported that the perception of acute onset airflow obstruction was intact in subjects in clinical remission of asthma (3). Presumably, slow changes in the degree of airway inflammation and remodeling are not well perceived by asthmatic subjects. Especially in patients with asthma in clinical remission or with mild asthma, this has important implications for the way we make treatment decisions, as the presence of symptoms is obviously not a sensitive indicator of ongoing disease.

The clinical implications of our study seem to be rather complicated. The effects of our intervention on hyperresponsiveness, FENO and tissue mast cells were significant and might be beneficial to the patient. Any effect on the long-term prognosis is, however,

unclear. Moreover, it is questionable whether subjects who have no subjective benefit from treatment will continue to take prescribed inhaled steroids for an unspecified time, since adherence with treatment is already a worry in patients with symptomatic asthma.

In conclusion, our data show that three months of combination therapy improved airway responsiveness and FENO as non-invasive markers of airway inflammation, but had limited effect on inflammation in bronchial biopsies, and did not affect quality of life scores. Long-term controlled clinical trials are therefore needed to show benefits in terms of a reduced risk of relapse and/or of irreversible airflow obstruction later in life. If that proves to be the case, anti-inflammatory therapy during clinical remission of atopic asthma could be justified.

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Chapter

7

Asthma Remission - Does it exist?

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ABSTRACT

Subjects that are believed to have grown out of asthma often develop symptoms again later in life. Ongoing airway inflammation may determine the risk on later relapse, although the mechanisms involved remain poorly understood. Additionally, patients with childhood asthma may develop irreversible airflow obstruction, i.e. airway remodeling, as a result of chronic airway inflammation. Recently both airway inflammation and remodeling could be demonstrated in bronchial biopsy specimens from young adults who considered themselves grown out of asthma. It is also shown that evidence of airway inflammation and remodeling can be obtained non-invasively, thereby providing the opportunity to monitor disease activity. If chronic airway inflammation and/or remodeling are consistent findings in asymptomatic subjects with a history of atopic asthma, the question arises whether natural history can be positively altered with prolonged anti-inflammatory therapy. Such benefit on the long-term prognosis is, however, as yet not shown. Since epidemiological work has demonstrated that a certain percentage of subjects with apparently outgrown atopic asthma remains asymptomatic, without needing therapy, for the rest of their lives, it can be argued that "asthma remission does exist". The question is whether this percentage can be increased with prolonged anti-inflammatory therapy and regular control.

INTRODUCTION

Symptoms of atopic asthma often disappear around puberty (1, 2). Unfortunately, 30 to 80% of subjects who apparently have grown out of asthma develops symptoms again later in life (3). As yet, the underlying risk factors are unknown. Martinez and coworkers (4) suggested that chronic airflow limitation may contribute to recurrence of symptoms in adult life, even after long periods of clinical remission and especially among active smokers. The mechanisms leading to this persistent airflow limitation are, however, incompletely understood. Recently some authors have suggested that ongoing airway inflammation is the principal cause of progressing airway abnormalities (5, 6) and, consequently, the high relapse rate (7, 8).

In this review certain issues will be discussed, such as the concept that ongoing airway inflammation and remodeling coincide during "remission" of asthma. Also the discrepancy between ongoing inflammation and the temporary absence of symptoms will be dealt with. Finally, the question whether the long-term prognosis of asymptomatic subjects with persistent inflammation can be altered with anti-inflammatory therapy and intensified control is addressed.

In the last paragraph, we make an effort to answer the question "will asthma ever go away" and to propose a strategy for subjects who believe to have grown out of asthma.

ONGOING AIRWAY INFLAMMATION DURING CLINICAL REMISSION

Few studies have looked at asymptomatic subjects with a history of asthma. In 1978 Kerrebijn et al (9). examined pulmonary function in 24 children who had not received treatment for over a year nor had any symptoms of their asthma. Although clinical recovery from asthma seemed to be apparent, increased bronchial smooth-muscle tone was still present in these children. In 1994, Boulet et al (10) demonstrated that most of a group of 30 ex-asthmatics considered to be in asthma remission showed a persistent increase in airway responsiveness to methacholine (MCh). It is, however, not clear whether these abnormalities were accompanied by ongoing airway inflammation or were solely due to structural changes as a late sequel of childhood asthma. To answer this question, we recently performed fiberoptic bronchoscopy in 18 subjects in clinical remission of asthma, 19 subjects with symptomatic asthma, and 17 healthy controls (7). Biopsies were taken from the subcarinae of the main bronchi. Immunohistochemical staining showed a significantly increased density of eosinophils (major basic protein staining) and mast cells (tryptase) in the bronchial mucosa of subjects in remission as compared with that from healthy controls (figure 1 and 2). The authors thus demonstrated the presence of ongoing inflammation despite the absence of symptoms.

Recently, Warke and colleagues performed bronchoalveolar lavage (BAL) in 35 normal children, 10 children with outgrown viral wheeze, and 25 children with outgrown asthma (8). They found elevated numbers of eosinophils in the BAL fluid of children who had apparently outgrown their asthma compared to controls ($p=0.002$, figure 3). There was no

relationship between the length of remission and degree of airways eosinophilia. They speculated that ongoing airway inflammation might be a risk factor for future relapse.

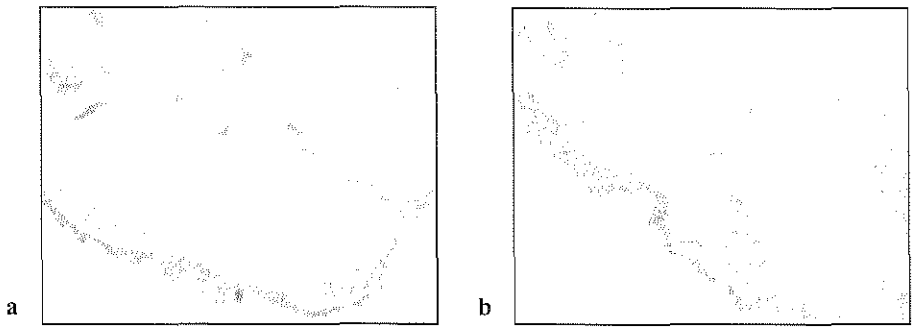

Figure 1: Major basic protein (MBP) density in a bronchial biopsy specimen from a subject in clinical remission of asthma (a) and from a healthy control (b). MBP is stained red.

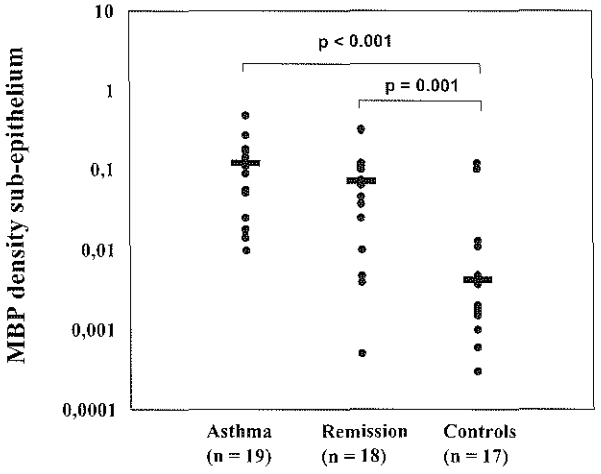

Figure 2: Ratio of area of major basic protein (MBP) staining and total area of sub-epithelium in bronchial biopsy specimens from currently asthmatic subjects, subjects in clinical remission of atopic asthma, and healthy controls. Each dot represents one subject. Horizontal bars represent median values.
Van den Toorn et al. 2001; Am J Respir Crit Care Med 164(11):2107-13.

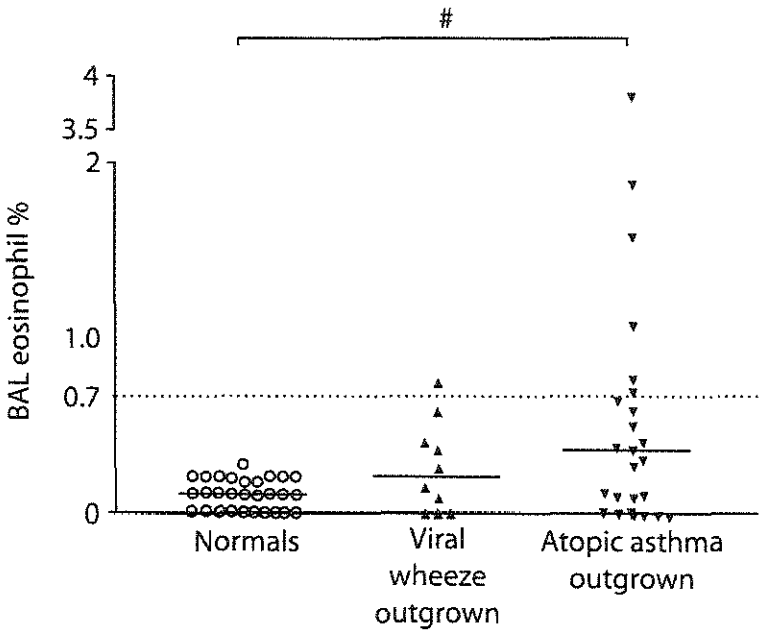

Figure 3: Bronchoalveolar lavage (BAL) fluid for healthy children, viral wheeze outgrown and atopic asthma outgrown. The value marked 0.7 (dashed line) corresponds to the previously defined upper limit of normal BAL fluid eosinophil %, and the solid lines represent median values. # p=0.002.
Warke et al. 2002; Eur Respir J 19(2):284-7. With permission of M.D. Shields.

AIRWAY REMODELING DURING CLINICAL REMISSION

The end result of asthmatic airway inflammation and its healing processes is an altered structure referred to as airway remodeling. This altered airway structure includes fibrosis and increased smooth muscle, mucus gland mass, vessel area, thickening of the reticular basement membrane (RBM) and epithelial desquamation (11). As a consequence of these structural changes, the airway wall in asthma is usually thickened, which may result in permanent airflow obstruction. Cokugras et al demonstrated thickening of the RBM even in 9-year old children with moderate asthma (12). Page and colleagues described recently how human mast cells from asthmatic patients induce human airway smooth muscle (HASM) cell proliferation. They proposed that mast cells fulfill a central role in the remodeling process (13), which was also suggested by others (14, 15).

Invasively obtained "proof" of airway remodeling during clinical remission of asthma is scarce. In our study, thickness of the RBM in airway biopsy specimens from subjects in clinical remission of asthma differed significantly from those from healthy controls (7). Also, occupancy of the RBM with epithelium differed between the study groups.

MONITORING DISEASE ACTIVITY DURING CLINICAL REMISSION

Since flexible bronchoscopy is not feasible as a routine monitoring tool, non-invasive markers that predict the degree of airway inflammation and/or remodeling are needed. Possible indices of airway inflammation include the level of sputum eosinophils, the presence of bronchial hyperresponsiveness (BHR) to inhaled stimuli, and exhaled nitric oxide (eNO) levels.

In 1995, Fahy demonstrated that analysis of induced sputum in subjects with asthma reveals information about eosinophil percentages qualitatively similar to those obtained by analysis of BAL fluid (16). A close relationship between sputum eosinophils and eosinophil percentage in BAL fluid could also be demonstrated in a study by Grootendorst and colleagues (17). With respect to treatment decisions, Green and Pavord could recently demonstrate that a management strategy directed at normalising the sputum eosinophil count reduced asthma exacerbations (2002 ATS poster A320), which finding for the first time provides "evidence" for the usefulness of monitoring airway inflammation.

BHR can be measured with different kind of stimuli. Provocation with adenosine 5'-monophosphate (AMP), as an indirect airway challenge, has been suggested to be a better marker of airway inflammation than direct challenges, such as provocation with methacholine (MCh) (18, 19). Where MCh directly induces smooth muscle contraction, AMP is thought to have its effect through the stimulation of bronchial mast cells. The presence of mast cells in airway smooth muscle cells has recently been shown to be a hallmark of asthma which is highly associated with disordered airway function (20). In a

study by Van den Berge and coworkers, PC_{20} MCh and PC_{20} AMP were measured in 120 patients with atopic asthma (21). In contrast to PC_{20} MCh, PC_{20} AMP could be predicted by the percentage of eosinophils in sputum. This is in agreement with our findings, where the density of mucosal eosinophils correlated significantly with BHR to AMP and with eNO levels, but not with BHR to MCh (7). Also when measuring the effect of allergen avoidance (22, 23) and of anti-inflammatory therapy (24-26) on airway inflammation, PC_{20} AMP has been proven to be a useful monitoring tool. On the other hand, Sont et al. could demonstrate a significant correlation between PC_{20} MCh and eosinophil number in the lamina propria (27).

The response to MCh may, thus, provide information about active inflammation as well as structural airway changes as a consequence of airway remodeling (28-30). In a study by Ward et al, it was shown that an early improvement in BHR to MCh with fluticasone treatment was associated with early changes in inflammation, but the more progressive and larger improvement was associated with later improvement in airway remodeling (31).

Recent articles on the subject have evidenced of a close relationship between eNO levels and exposure to relevant allergens (32, 33), or allergen avoidance (34) in atopic asthmatic subjects. Furthermore, eNO positively correlates with the level of blood- and sputum eosinophils in asthmatic subjects (35-37). The observation that eNO levels in atopic asthmatic subjects are closely related to the eosinophil percentage in BAL fluid (38) and in bronchial biopsy specimens (7, 39) from atopic asthmatic subjects indicates sufficiently that elevated eNO levels are highly associated with eosinophilic airway

inflammation, thereby providing a unique tool in monitoring disease activity. It also seems to be justified to categorise asthma activity with the aid of eNO levels (40, 41).

In conclusion, sputum eosinophils, eNO levels and BHR to AMP provide useful tools in monitoring disease activity, whereas BHR to MCh may also or merely reflect remodeling processes in the airways.

SYMPTOM PERCEPTION DURING CLINICAL REMISSION

Blunted symptom perception would provide a plausible explanation for the discrepancy between persistent airway inflammation and the absence of symptoms during clinical remission. Several studies have shown that the relationship between asthmatic symptoms and other indices of asthma severity is poor. Teeter et al. found asthma symptoms to correlate poorly with the level of airway obstruction (42). A low degree of "perceptiveness" for bronchoconstriction is especially demonstrated in subjects with severe asthma (43, 44). Bijl-Hofland hypothesized that either patients with more severe asthma show adaptation of "perceptiveness" for airway obstruction or, perhaps instead, that low perceptiveness leads to more severe asthma as a result of undertreatment (44). If, after years of symptomatic asthma, adaptation or loss of perceptiveness plays a role in subjects in clinical remission of asthma, it could possibly explain the discrepancy between the lack of symptoms and the ongoing airway inflammation in these subjects. In a study from our group, however, BORG-scores after a 20% fall in FEV_1 during MCh- and AMP induced bronchoconstriction were not different between subjects in clinical remission and subjects with symptomatic asthma (45), thereby making blunted dyspnea perception not a very likely explanation for the stated discrepancy. We suppose that the weight given by patients to breathlessness depends on many other factors, including age, where younger patients find it more troublesome than older ones (46). Besides, a slow change in airflow obstruction is possibly less perceived than a fast deterioration due to provocation with MCh or AMP. This was also shown by Rietveld, who demonstrated that patients with prolonged airway obstruction perceived symptoms less well and were more

vulnerable to negative effects of asthma than patients with acute onset airway obstruction (47).

RATIONALE FOR ANTI-INFLAMMATORY THERAPY DURING CLINICAL REMISSION OF ASTHMA

Since some studies have evidenced of ongoing airway inflammation during clinical remission of atopic asthma (7, 8), it has increasingly been a subject of debate that prolonged anti-inflammatory therapy should be prescribed to these patients. Since airway inflammation is considered the cornerstone of asthma (48), inhaled steroids are widely accepted as first-choice therapy, even in the mildest forms of disease (49). A down-regulating effect on several cell types in the bronchial mucosa (50, 51), sputum eosinophils (52, 53), exhaled NO (53, 54) and bronchial hyperresponsiveness (55) has been demonstrated. Airway remodeling aspects such as collagen deposition and thickness of the RBM have also been shown to respond to some degree to anti-inflammatory therapy (56, 57). If airway remodeling corresponds with progressive airway obstruction, a smaller decline in lung function with anti-inflammatory therapy could possibly be reached, as shown by Grol et al. (58). Several authors have focussed their attention to the important concept that with the aid of inhaled corticosteroids natural history and final outcome of asthma might be altered (59-61). Koh et al. studied the effect of inhaled budesonide on BHR to MCh of subjects in long-standing clinical remission of asthma (62). After nine months of therapy no significant change on BHR was seen in this study. It was suggested that the mechanisms underlying BHR in this clinical setting could be different from those in symptomatic asthma. As BHR to MCh is in the first place thought to reflect structural changes in the airways, the absence of any effect of anti-inflammatory therapy on this parameter could be due to irreversible remodeling of the airways in the

subjects studied. On the other hand, De Kluijver et al demonstrated that asymptomatic worsening of airway inflammation in subjects with mild asthma could be prevented by inhaled steroids (63). In a study from our group (64), a significant reduction in bronchial hyperresponsiveness, exhaled nitric oxide levels, bronchial mast cells and thickness of the reticular basement membrane was seen after three months of combination therapy with fluticasone proprionate plus salmeterol in subjects in clinical remission of atopic asthma. However, bronchial eosinophils, blood eosinophils, and quality of life were unchanged.

Thus, it is still unclear whether subjects, who seem to have grown out of asthma, but show evidence of ongoing disease, would benefit from prolonged anti-inflammatory therapy in the long-term. Since several study results indicate that inhaled steroids can positively influence progressive airflow limitation and long-term prognosis in symptomatic asthmatics, this therapeutic modality should presumably also be (re) considered in subjects with subclinical airway inflammation. Whether this approach is really able to alter the natural history in subjects in clinical remission of asthma is as yet not known. Besides, therapy adherence in asymptomatic young adults will presumably be a major problem. Long-term controlled clinical trials are therefore needed to show benefits in terms of a reduced risk of relapse and/or of irreversible airflow obstruction later in life.

DOES ASTHMA EVER GO AWAY?

There is ample evidence from the literature and our own studies that most young adults who consider themselves grown out of asthma just appear to be asymptomatic. The question remains whether true remission can be achieved, with or without the aid of anti-inflammatory therapy. It may be that the absence of any signs of airway inflammation and airway remodeling is representative of true remission. This was only true for 10 or 15 percent of our population of asymptomatic subjects with a history of atopic asthma. Epidemiological data show that a small percentage of young adults in clinical remission remains asymptomatic for the rest of their lives. These may very well be the subjects in true remission. It is largely unknown whether "additional remissions" can be achieved with prolonged anti-inflammatory treatment, and, if so, when this treatment can safely be stopped. Bahceciler and colleagues recently investigated which parameters would be useful in deciding whether inhaled corticosteroids therapy could be stopped. They demonstrated that only the duration of clinical remission prior to cessation of treatment had proven to be a useful parameter (65). In addition to their finding, Sont et al have shown that a better control of airway inflammation can be achieved when airway hyperresponsiveness is used as additional guide (66). Thus, long-term control at the outpatient clinic with non-invasive measurements reflecting airway inflammation and remodeling is presumably needed to decide whether anti-inflammatory therapy should be started, continued or stopped.

We propose that adolescents who seem to have grown out of asthma should be monitored for years after symptoms have disappeared, thereby using non-invasive measurements of

airway inflammation and remodeling, such as exhaled nitric oxide levels, sputum eosinophils and the response to MCh and/or AMP. Treatment might be safely withheld in subjects without signs of airway inflammation or remodeling. In all other patients continuation of anti-inflammatory therapy has the theoretical potential to positively alter the prognosis of atopic asthma. Long-term studies are needed to examine the efficacy and safety of treatment on asymptomatic subjects with chronic airway inflammation.

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Chapter

8

Conclusions and recommendations for future research

With reference to the questions, postulated in chapter 2, it can be concluded that:

1 Ongoing airway inflammation and remodeling can be demonstrated invasively and non-invasively in the majority of subjects who are believed to have grown out of asthma.

2 Blunted symptom perception is not a likely explanation for the discrepancy between ongoing airway inflammation and absence of symptoms in subjects in clinical remission of atopic asthma.

3 Anti-inflammatory treatment in subjects with subclinical airway inflammation reduces bronchial hyperresponsiveness, exhaled NO levels, and bronchial mast cells, but the effect on long-term prognosis is as yet not clear. Even so, the value of prolonged monitoring of airway inflammation and remodeling during clinical remission of asthma has to be elucidated.

4 True asthma remission seems to be a rare phenomenon.

FUTURE RESEARCH

Long-term controlled clinical trials on prolonged anti-inflammatory therapy in subjects in clinical remission of asthma are needed to show benefits in terms of a reduced risk of relapse and/or of irreversible airflow obstruction later in life. If that proves to be the case, anti-inflammatory therapy during clinical remission of atopic asthma could be justified.

Chapter

9

Summary

In this thesis, the presence of ongoing airway inflammation in subjects in clinical remission of atopic asthma is demonstrated. Next to a discussion of non-invasive and invasive methods which can be used to produce evidence of airway inflammation, the concept of blunted dyspnea perception to explain the discrepancy between absence of symptoms and ongoing airway pathology in subjects with apparently outgrown asthma is evaluated. Furthermore, the desirability to treat a-symptomatic subjects with ongoing disease with anti-inflammatory therapy is dealt with. Finally, an answer is sought to the question whether true remission in asthma is possible.

In Chapter 1, epidemiology and classification of atopic asthma are reviewed. Although the presence and extent of symptoms of asthma are the basis of all classification systems, it is emphasised that airway inflammation is the hallmark of atopic asthma. An overview of non-invasive and invasive methods to evaluate airway inflammation in asthma, with the bronchial biopsy histology as golden standard, is provided. Newer indices, such as exhaled nitric oxide (eNO), are described in more detail. Next, treatment guidelines of atopic asthma and possible future developments are discussed. It is concluded that

asthma is dealt with. Although a great part of patients and doctors believe that most subjects with asthma will grow out of asthma in early adolescence, a considerable proportion of subjects in clinical remission of asthma will have a relapse later in life. It is suggested that ongoing airway inflammation and remodeling are the basis for this continuing risk on relapsing symptoms. The question is raised whether ongoing airway abnormalities can indeed be demonstrated during clinical remission, and, if so, whether this ongoing inflammation should be treated with anti-inflammatory therapy.

In Chapter 2, the aims of the studies are presented. This thesis aims to produce evidence of persistent airway inflammation and remodeling in young adults who seem to have outgrown their asthma. For this purpose, non-invasive as well as invasive methods are evaluated. Besides, symptom perception was measured to find an explanation for the discrepancy between the absence of symptoms and ongoing airway inflammation. Finally, we examined whether subjects with subclinical airway inflammation could benefit from anti-inflammatory treatment.

In Chapter 3, non-invasive evidence of persistent airway inflammation during clinical remission of atopic asthma is demonstrated, as reflected by an elevated fraction of exhaled nitric oxide (FENO) levels and bronchial hyperresponsiveness to adenosine monophosphate (AMP) and methacholine (MCh). With respect to eNO, we found no overlap between values of 21 subjects in clinical remission of atopic asthma and those of 18 healthy controls. Values of FENO in subjects in remission were similar to those of 21 subjects with current asthma. A significantly higher AMP- as well as MCh

responsiveness was present in the remission group as compared with the control group. Furthermore, AMP responsiveness showed a significant inverse relationship with FENO levels, probably suggesting that both indices are associated with active elements of airway inflammation, whereas responsiveness to MCh may also reflect airway remodeling.

In Chapter 4, invasive "proof" of persistent airway inflammation and airway remodeling in the majority of subjects in clinical remission of atopic asthma complements the findings of chapter 3. By means of flexible bronchoscopy, bronchial biopsies were taken from 19 subjects with current atopic asthma, 18 subjects in clinical remission of atopic asthma, and 17 healthy controls. Immunohistochemical staining of biopsy specimens demonstrated an elevated density of eosinophils, T lymphocytes, mast cells, and IL-5 in the airways mucosa of subjects in remission compared to controls. Also, blood eosinophil cell counts were significantly elevated in subjects in clinical remission. Blood eosinophil cell counts, FENO levels, and bronchial response to AMP correlate significantly with the density of tissue eosinophils, indicating that these measurements could be used to monitor airway inflammation with minimal burden to the patient.
MCh responsiveness did not show a relationship with the density of eosinophils in the airway mucosa. Since MCh is believed to act directly on smooth muscle cells in the airways, whereas AMP acts via activation of bronchial mast cells, it was concluded that AMP- and MCh responsiveness probably relate to different aspects of the inflammatory process.

Surprisingly, inducible nitric oxide synthase (iNOS) activity in the airway mucosa from healthy controls was higher than in subjects with asthma and subjects in remission. It could thus well be that iNOS-unrelated mechanisms, including augmented diffusion of NO through damaged epithelium, or iNOS independent NO generation under conditions of airway acidity may explain this finding. Furthermore, other isoforms of NOS could be responsible for elevated NO production.

In conclusion, both ongoing airway inflammation and remodeling were demonstrated during clinical remission of atopic asthma, and can be monitored with non-invasive measurements.

In Chapter 5, dyspnea perception was investigated in subjects in clinical remission of atopic asthma and subjects with current asthma. The hypothesis was that blunted symptom perception in subjects in clinical remission of asthma could explain the discrepancy between the lack of symptoms and persistent airway inflammation. Twenty subjects with bronchial hyperresponsiveness to MCh or AMP assigned themselves a perception score of dyspnea on a modified BORG scale at reaching a 20% fall in FEV_1 (PS_{20}) during MCh- or AMP challenge. Dyspnea perception was highly variable but did not differ significantly between both groups, nor between challenge tests. It was concluded that subjects in clinical remission are not different from currently asthmatic subjects with respect to their perception acute changes in FEV_1. Perception of symptoms in daily life might, however, be different from these results, since not only physical, but also emotional and social factors are believed to play a role in the subjective feeling of discomfort, and we can not exclude that daily life fluctuations in airflow limitation are

not well perceived by subjects in clinical remission of asthma. Therefore, the absence of symptoms in subjects with a past history of asthma does not mean that airway inflammation is gone.

In Chapter 6, we assessed the effect of anti-inflammatory therapy in subjects in clinical remission of atopic asthma. It was hypothesized that if airway inflammation and/or remodeling are present during clinical remission despite a lack of symptoms, this could well respond to the use of inhaled steroids. A double-blind, randomized, placebo controlled trial with the salmeterol/fluticasone propionate combination product (SFC 50/250 µg bid via the Diskus™ inhaler) was conducted in 28 subjects in clinical remission with proven hyperresponsiveness to MCh as non-invasive indicator of ongoing disease. SFC treatment for three months improved PD_{20} MCh as well as PD_{20} AMP, and normalized FENO in most subjects. SFC treatment also reduced mast cells in subepithelium, had a minimal effect on the thickness of the reticular basement membrane (RBM), but did not alter tissue eosinophils, blood eosinophils, and quality of life. The added value of the long acting $ß_2$ agonist (salmeterol) could not be extracted from this study. It was suggested that airway hyperresponsiveness and FENO could represent fast responding elements of airway pathology, whereas tissue markers of inflammation and remodeling need more time than three months to respond. Based on the results of this study, the use of prolonged anti-inflammatory therapy during clinical remission of asthma is not justified. However, it encourages further investigation with long-term studies. Whether inhaled steroids might prevent a relapse of asthma later in

life thus remains to be proven. Furthermore, major problems with therapy adherence in subjects without symptoms can be anticipated.

In Chapter 7, an answer is sought to the question whether real asthma remission exists. The majority of subjects with childhood asthma seems to grow out of asthma in early adolescence. The appellation "clinical remission" is used in this context to define the absence of symptoms without therapy. Current classification guidelines do not mention this sizeable group of formerly asthmatic subjects who are at risk to develop symptoms again later in life, mostly before the age of 30. More specifically, epidemiological studies have shown that in about 30 to 80 % of subjects in clinical remission of atopic asthma the absence of symptoms is only temporary. We postulate that this is due to persistent airway inflammation and remodeling. In about one fifth of the subjects in clinical remission in our study, significant airway inflammation and/or remodeling could not be demonstrated. Are these the subjects in true remission? If so, can we heighten this percentage with prolonged anti-inflammatory therapy? In Chapter 7 a review of the literature on asthma remission is given, thereby trying to find an answer to this questions.

Chapter

10

Samenvatting

Allergisch astma ontstaat meestal op jonge leeftijd, en wordt gekarakteriseerd door een chronisch ontstekingsproces in de luchtwegen. Veel patiënten laten rond de puberteit een aanzienlijke vermindering van klachten zien, waarbij een groot deel zelfs geheel klachtenvrij wordt en veelal geen medicatie meer hoeft te gebruiken. Dit noemen we "klinische remissie". In deze fase worden patiënten uit de medische controle ontslagen, en wordt er veelal gesproken van "over astma heen groeien". Uit een aantal studies is echter gebleken dat ongeveer de helft van deze ex-patiënten op latere leeftijd, meestal al voor het dertigste levensjaar, een recidief krijgt. Tijdens het "goede" interval zijn door sommige auteurs longfunctieafwijkingen vastgesteld, zoals verhoogde luchtwegprikkelbaarheid en luchtwegobstructie. Het is echter onduidelijk of deze afwijkingen verband houden met persisterende ontstekingsverschijnselen, dan wel met structurele veranderingen in de luchtwegen als uiting van "restschade" ten gevolge van astma op de kinderleeftijd. Tevens bestaat er onduidelijkheid over de relatie tussen de aard en uitgebreidheid van de ontstekingsverschijnselen en de prognose van astma. Het doel van ons onderzoek was na te gaan of bij personen met allergisch astma, die in klinische remissie zijn gekomen, al dan niet een ontstekingsproces in de luchtwegen

bestaat, en of, indien dit het geval is, dergelijke personen gebaat zijn bij het gebruik van ontstekingsremmende medicijnen. Tevens wordt er gezocht naar een verklaring voor de discrepantie tussen de afwezigheid van klachten en de aanwezigheid van het ontstekingsproces.

In hoofdstuk 1 worden de epidemiologische aspecten en classificatie van allergisch astma besproken. Hoewel de aanwezigheid en uitgebreidheid van astmatische symptomen een belangrijk uitgangspunt lijken te vormen voor alle classificatiesystemen, wordt (chronische) luchtwegontsteking toch als het kenmerkende element van allergisch- en niet allergisch astma gezien.
Een overzicht van niet-invasieve en invasieve methoden om deze luchtwegontsteking te bestuderen, met luchtwegbiopten als gouden standaard, wordt in dit hoofdstuk gegeven. Nieuwere methoden, zoals het meten van de hoeveelheid stikstofoxide (NO) in uitgeademde lucht, worden in detail beschreven. Het hoofdstuk wordt vervolgd met een uiteenzetting van de mogelijkheden van medicamenteuze therapie bij allergisch astma. Geconcludeerd wordt dat tot op heden inhalatiesteroïden nog altijd de therapie van eerste keus vormen. Als laatste wordt het gegeven van "over je astma heen groeien" nader uitgewerkt. Het feit dat een groot percentage van patiënten, die menen over hun astma te zijn heen gegroeid, op latere leeftijd weer klachten krijgt, geeft al aan dat de afwezigheid van symptomen blijkbaar niet de afwezigheid van ziekte impliceert.

In hoofdstuk 2 worden de verschillende studies uiteengezet die als doel hebben de aanwezigheid van luchtwegontsteking en/of remodeling aan te tonen bij personen die van

mening zijn over hun astma te zijn heen gegroeid. Voor dit doel worden zowel niet invasieve als invasieve methoden geëvalueerd. Daarnaast wordt er onderzocht of de verklaring voor de discrepantie tussen het gebrek aan klachten en de aanwezige luchtwegontsteking kan worden gezocht in veranderde symptoomperceptie. Als laatste wordt gekeken of personen met niet-manifeste luchtwegontsteking voordeel kunnen hebben bij het gebruik van ontstekingsremmende therapie.

In hoofdstuk 3 werd bij patiënten in klinische remissie van allergisch astma, bij patiënten met actief astma en bij gezonden met niet-invasieve methoden gezocht naar persisterende luchtwegontsteking aangedragen. Hierbij werd gebruik gemaakt van NO in uitgeademde lucht en luchtweggevoeligheid voor adenosine monofosfaat (AMP) en metacholine (MCh). Met betrekking tot de uitgeademde hoeveelheid NO vonden wij geen overlap tussen meetwaarden van 21 personen in klinische remissie en die van 18 gezonde controlepersonen. Meetwaarden van personen in remissie bleken nauwelijks verschillend te zijn van die van 21 personen met symptomatisch astma.
Tevens werd er een verhoogde luchtweggevoeligheid voor zowel AMP als MCh in de remissie groep gevonden, indien vergeleken met de controle groep. Daarnaast bestond er een duidelijke omgekeerde relatie tussen de reactie op AMP en hoogte van de uitgeademde NO hoeveelheid, hetgeen mogelijk impliceert dat beide variabelen gerelateerd zijn aan luchtwegontsteking, terwijl de reactie op MCh mede of vooral beïnvloed kan zijn door structurele afwijkingen.

In hoofdstuk 4 wordt invasief onderzoek naar het bestaan van luchtwegontsteking en structurele afwijkingen bij de personen in klinische remissie van allergisch astma beschreven. Met behulp van flexibele bronchoscopie werden luchtwegmucosa biopten verkregen van 19 personen met symptomatisch astma, 18 personen in klinische remissie, en 17 gezonde controles. Immunohistochemische kleuring van bioptmateriaal liet een verhoogde dichtheid van eosinofielen, T lymfocyten, mestcellen, en IL-5 in het luchtwegslijmvlies van personen in klinische remissie zien, wanneer werd vergeleken met gezonde controles. Tevens was de hoeveelheid eosinofielen in het bloed verhoogd. Er bleek een significant verband te zijn tussen eosinofielen aantal in het bloed, uitgeademde hoeveelheid NO, en reactie op AMP enerzijds, en de mate van eosinofieleninfiltratie in het luchtwegslijmvlies anderzijds. Dit betekent dat dergelijke meetmethoden waarschijnlijk gebruikt kunnen worden om de aanwezigheid van luchtwegontsteking te controleren en te vervolgen, met minimale belasting voor de patiënt.

De reactie op MCh vertoonde echter geen relatie met de eosinofielen dichtheid in luchtwegslijmvlies. Daar MCh een direct effect uitoefent op de gladde spieren in de luchtwegen, terwijl AMP met name mestcel-degranulatie bewerkstelligt, kan worden geconcludeerd dat de reactie op AMP en MCh aan verschillende aspecten van het ontstekingsproces gerelateerd zijn. Hierbij lijkt de reactie op MCh onafhankelijk van actieve luchtwegontsteking verhoogd te kunnen zijn.

Een opvallende bevinding was tevens dat er een verhoogde activiteit van het induceerbare stikstof oxide synthase (iNOS) in de luchtwegmucosa van gezonde controles aanwezig bleek te zijn, wanneer wordt vergeleken met de situatie in personen met symptomatisch

astma en personen in klinische remissie van astma. Dit was niet in overeenstemming met de aanname dat verhoogde iNOS expressie in de luchtwegmucosa de bron vormt van de verhoogde hoeveelheid NO in uitgeademde lucht van personen met astma. Het is dus mogelijk dat niet aan iNOS gerelateerde mechanismen, waaronder toegenomen diffusie van NO door beschadigd epitheel of veranderingen in de luchtwegzuurgraad, verantwoordelijk zijn voor dit onverwachte resultaat. Daarnaast kunnen andere vormen van NOS een bijdrage leveren aan de verhoogde NO productie.
Concluderend, zowel actieve luchtwegontsteking als structurele veranderingen zijn aantoonbaar bij personen in klinische remissie van atopisch astma, en kunnen worden aangetoond middels niet-invasieve meetmethoden. De recente bevindingen van diverse auteurs, waaronder verminderde longfunctie en verhoogde luchtwegprikkelbaarheid tijdens het klinische remissie kunnen gerelateerd zijn aan zowel actieve ontsteking als structurele veranderingen in de luchtwegen.

In hoofdstuk 5 werd de perceptie van kortademigheid onderzocht bij personen in klinische remissie en bij personen met symptomatisch astma. De hypothese is dat verminderde symptoomperceptie bij personen in klinische remissie de discrepantie tussen het gebrek aan klachten en de persisterende luchtwegontsteking kan verklaren. Aan 20 personen in klinische remissie, met verhoogde gevoeligheid voor MCh of AMP, werd gevraagd een kortademigheidscore toe te kennen bij het bereiken van een 20 procent daling in FEV_1 tijdens MCh- of AMP provocatie. Kortademigheidperceptie bleek grote variatie tussen personen te vertonen maar was niet duidelijk verschillend tussen personen in klinische remissie en personen met symptomatisch astma voor zowel MCh als AMP.

Geconcludeerd werd dat personen in klinische remissie niet verschillen van personen met symptomatisch astma met betrekking tot de perceptie van een snelle opgelegde daling in FEV_1. De perceptie van astmatische symptomen in het dagelijks leven zou echter anders kunnen zijn, daar niet alleen lichamelijke, maar ook psychische en sociale factoren van invloed kunnen zijn op de beleving van symptomen. Er kan in ieder geval worden gesuggereerd dat de afwezigheid van symptomen bij personen met een voorgeschiedenis van astma niet hoeft te betekenen dat het ziekteproces in de luchtwegen is verdwenen.

In hoofdstuk 6 werd het effect van astmabehandeling onderzocht bij personen in klinische remissie van astma. Hierbij werd uitgegaan van de hypothese dat, indien luchtwegontsteking en/of remodeling aanwezig blijven tijdens klinische remissie, subklinische ontstekingsverschijnselen kunnen verminderen door het gebruik van inhalatiesteroïden. Een dubbelblinde, gerandomiseerde, placebo gecontroleerde studie werd daarom verricht met het salmeterol/fluticasone propionate combinatie product (SFC 50/250 µg 2dd via de Diskus™ inhaler) bij 28 personen in klinische remissie met bewezen hyperreactiviteit voor MCh als niet-invasief kenmerk van luchtwegpathologie. SFC behandeling gedurende drie maanden verminderde in deze studie de hyperreactiviteit voor MCh en AMP, en normaliseerde de uitgeademde hoeveelheid NO bij de meeste personen in het onderzoek.
Tevens werd er na drie maanden een duidelijke vermindering van mestcellen in het luchtwegslijmvlies, een geringe regressie van de dikte van de reticulaire basaalmembraan (RBM), maar geen significante vermindering van weefsel eosinofielen, bloed eosinofielen, en kwaliteit van leven gezien. De toegevoegde

waarde van een lang werkend β_2-mimeticum kan uit de studieresultaten niet geëxtraheerd worden. Er werk gesuggereerd dat luchtwegreactiviteit en uitgeademde hoeveelheid NO mogelijk snel responderende elementen van luchtwegpathologie zijn, terwijl weefsel eosinofielen en andere invasieve kenmerken wellicht meer dan drie maanden nodig hebben om te reageren.Geconcludeerd werd dat de bevindingen van deze studie het (opnieuw) voorschrijven van ontstekingsremmende medicijnen aan personen in klinische remissie van astma niet rechtvaardigen. Wel moedigen deze resultaten aan tot lange-termijn studies. De vraag of inhalatiesteroïden een recidief van astma op latere leeftijd kunnen voorkomen blijft dus nog onbeantwoord. Daarnaast zullen problemen met de therapietrouw bij personen zonder klachten dergelijk lange-termijn onderzoek niet vereenvoudigen.

In hoofdstuk 7 wordt benadrukt dat een meerderheid van de jeugdige patiënten met astma in de vroege pubertijd over het astma heen lijkt te groeien. De term "klinische remissie" wordt in deze context gebruikt als aanduiding voor de afwezigheid van symptomen zonder het gebruik van inhalatie medicatie gedurende tenminste 1 jaar.
Binnen de huidige classificatiesystemen is deze omvangrijke groep van volledig klachtenvrije astmapatiënten in het geheel niet terug te vinden, ondanks het feit dat dezen vaak op latere leeftijd een recidief van astmatische klachten krijgen, over het algemeen zelfs al voor het dertigste levensjaar. Epidemiologische studies hebben aangetoond dat bij 30 tot 80% van personen in klinische remissie de afwezigheid van symptomen slechts tijdelijk is. Er wordt naar voren gebracht dat persisterende luchtwegontsteking dit aanzienlijke risico op een recidief zou kunnen veroorzaken. In ongeveer 20 procent van

de personen in klinische remissie in ons onderzoek konden geen luchtwegontsteking en/of structurele afwijkingen meer worden aangetoond. Zijn dit dan de personen in echte remissie? En zo ja, kunnen we dit lage percentage dan verhogen middels langdurige ontstekingsremmende behandeling? In hoofdstuk 7 wordt een overzicht van de beschikbare literatuur over astma remissie gegeven, waarbij getracht wordt een antwoord op deze vragen te geven.

Chapter

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List of abbreviations

AMP	adenosine-5'-monophosphate
ATS	American Thoracic Society
BHR	bronchial hyperresponsiveness
CRF	clinical research file
eNO	exhaled nitric oxide
FENO	fraction of exhaled nitric oxide
FEV_1	forced expiratory volume s^{-1}
HASM	human airway smooth muscle
IL-5	interleukin-5
iNOS	inducible nitric oxide synthase
MBP	major basic protein
MCh	methacholine
PD_{20}	provocative dose resulting in 20 percent decrease in FEV_1
PEFR	peak expiratory flow rate
PS_{20}	perception score at reaching a 20 percent decrease in FEV_1
RAST	radioallergoimmunosorbent test
RBM	reticular basement membrane

SFC	salmeterol/fluticasone propionate combination
Th2	type 2 T-helper
TLC	total lung capacity

Chapter

12

Dankwoord

Collega G. T. Verhoeven zei ooit: "het is eenvoudiger om meerdere marathons uit te lopen dan éénmaal te promoveren". Daar deze uitspraak na maar liefst vijf marathons tot stand is gekomen, moeten we wel aannemen dat het voltooien van een proefschrift een uitputtende bezigheid is, die met een wazige blik op de finish en een wisselend gevoel van verzuring gepaard kan gaan. In het zicht van- en vooral na het bereiken van de finish slinkt het pijngevoel echter snel, en telt uitsluitend nog het resultaat. Ook het volbrengen van slechts twee marathons heeft de schrijver van dit proefschrift de overtuiging gegeven dat ook het "finishgevoel" bij het promotieonderzoek een gevoel van voldoening moet zijn. De blessures zijn echter van behoorlijke omvang en geven soms een nieuwe dimensie aan het begrip "beroepsgerelateerde aandoeningen". Naast de alom bekende muisarm was er bij mij sprake van een "microfilm-nystagmus" (na het beoordelen van ongeveer 2000 patiëntendossiers op voorbij rollende microfilm) en "image analysis hallucinaties" (het herkennen van een basaalmembraan in de trapleuning). Gelukkig waren dergelijke ongemakken van tijdelijke aard en hebben zij er niet toe geleid dat voor de finish moest worden uitgestapt. Dit proefschrift zou echter niet tot stand zijn gekomen zonder de hulp van velen die nu snel doorlezen of ze daadwerkelijk met name worden genoemd. In zijn algemeenheid verdienen het laboratorium longziekten in de hoogbouw,

de afdeling longfunctie en de mensen van het archief van het Sophia Kinderziekenhuis, de verpleegkundigen in de bronchoscopiekamer, Glaxo SmithKline B.V., en natuurlijk alle proefpersonen mijn speciale dank. Aan enkele personen wil ik echter enige persoonlijke woorden wijden.

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Dr S.E. Overbeek, beste Shelley, onze onderzoeksrelatie heeft een intens karakter gekend. Je historische uitspraken tijdens de uitvoering van een bronchoscopie ("ik zie niks!" of "wat is dat bloederig!") zal ik (en zullen de proefpersonen) niet snel vergeten. We zullen het wel nooit eens worden wie van ons tweeën het opstapbankje bij de bronchoscopie harder nodig heeft. Je enorme inzet en belangstelling voor het onderzoek, maar ook voor het thuisfront, zijn bewonderenswaardig. Bedankt voor al je hulp!

Dr J-B Prins, beste Jan-Bas, op het moment dat ik dit schrijf ben je al niet meer werkzaam in de Erasmus Universiteit. Aan jou verliest het laboratorium een persoon met

vele kwaliteiten. Je bijzonder kritische noot en schijnbare onbekendheid met het woordje "goed" hebben onze samenwerking echter niet altijd tot een gemakkelijke gemaakt. Na een bezoek aan je kamer op de 22e vroeg ik me vaak af: "is er nog leven na(ast) het promotieonderzoek?". Gelukkig heb je buiten het werk laten zien ook over een andere en gezellige persoonlijkheid te beschikken. Daarnaast heb je op velerlei wijze, waaronder je vakkundige begeleiding bij het maken van voordrachten, je waarde voor dit onderzoek bewezen, waarvoor ik je veel dank verschuldigd ben.

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Bij de eenzame uren in het archief in het Sophia Kinderziekenhuis heeft Maarten mij altijd met woord en daad terzijde gestaan.

Paul Mulder dank ik voor het feit dat hij mij heeft bijgestaan in de soms moeilijk te doorgronden brij van de statistiek. De uiting "ieder zijn vak" heb ik hier altijd voor ogen gehouden.

Van het Sint Fransiscus Gasthuis wil ik niet nalaten Arie Rietveld te noemen die mijn interesse voor de intensive care geneeskunde tot grote hoogten heeft weten te doen stijgen. Arie, het steeds weer kopiëren van "Up to Date" heeft me weliswaar enige flessen wijn gekost, maar weegt ruimschoots op tegen de vele plezierige en leerzame momenten die ik op de IC hebben gehad. Ook dank ik je voor je min of meer subtiele pogingen om mijn echtgenote te overtuigen van het nut van een eigen snowboard. Daarnaast wil ik van hetzelfde gasthuis Arjan Rudolphus bedanken voor zijn zachte drang om mij met links te laten scopiëren, en Youke Tan alsnog mijn verontschuldigingen aanbieden voor het feit dat ik een (oude) scoop ondanks bijtring door een patiënte aan flarden heb laten bijten.

Tevens is uiteraard een woord van dank voor de vele proefpersonen die aan het onderzoek hebben meegewerkt op zijn plaats. Al varieerde de reactie na een

bronchoscopie nog van "piece of cake" tot "never nooit meer", na de provocatietesten waren sommigen zelfs niet meer in staat om hun reactie te geven (omdat ik het nodig vond de FEV_1 tot onder 40% van de beginwaarde te laten dalen). De bereidwilligheid om het gehele onderzoek te doorlopen was bij vrijwel alle deelnemers daarom indrukwekkend. Het feit dat een aantal van hen de in het vooruitzicht gestelde riante vergoeding al ruimschoots hadden uitgegeven doet hier niets aan af.

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Chapter

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Curriculum Vitae

Leon Michiel van den Toorn werd geboren op 16 februari 1966 te Amsterdam. Het diploma O-VWO behaalde hij in 1984 aan het Bonhoeffer College te Castricum. In 1989 voltooide hij de studie medische biologie aan de Vrije Universiteit te Amsterdam. Tijdens deze studie liep hij onderzoeksstages op de afdeling fysiologie (Dr G.C. van den Bos) en interne geneeskunde (Prof. Dr. R.O.B. Gans). In 1987 was hij tevens begonnen met de studie geneeskunde aan dezelfde universiteit, hetgeen resulteerde in een met succes afgelegd artsexamen in 1994. Vervolgens was hij gedurende twee jaar werkzaam als AGNIO interne geneeskunde, longziekten en cardiologie in het Spaarne Ziekenhuis te Heemstede. Alhier werd zijn belangstelling voor de longziekten gewekt, hetgeen een AGNIO-schap longziekten in het Academisch Ziekenhuis te Rotterdam ten gevolge had. Op 1 april 1997 werd tevens een start gemaakt met voorliggend promotieonderzoek (promotoren Prof. Dr. H.C. Hoogsteden en Prof. Dr. J.C. de Jongste) op de afdelingen longziekten (Erasmus MC Rotterdam) en kindergeneeskunde/subafdeling kinderlongziekten (Erasmus MC locatie Sophia). Vanaf 1 juli 2000 tot 1 maart 2002 heeft hij als AGIO longziekten zijn vooropleiding interne geneeskunde in het St. Fransiscus Gasthuis te Rotterdam doorlopen (opleiders Dr. Tjen en Prof. Dr. H.C. Hoogsteden).

Sinds 1 maart 2002 is hij voor de vervolgopleiding longziekten weer werkzaam in het Erasmus MC.

Chapter

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