Airway inflammation in asthma : from concept to the clinic

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Chapter

General introduction and aims of the studies

1. Definition of asthma

Asthma is a serious public health problem in countries throughout the world. It is one of the most common chronic diseases worldwide (1). The current Global Initiative for Asthma (GINA) guidelines provide the generally accepted definition for asthma:

Asthma is 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, particular at night or in the early morning. These episodes are usually associated with widespread, but variable airflow obstruction that is often reversible either spontaneously or with treatment (1).

For most patients with asthma this means that they are having a life-long chronic disease. Asthma may affect children and adults of all ages, but in the majority of patients the first symptoms start at young age. The fundamental causes of asthma are still not known. Symptoms of breathlessness and wheezing occur often following exposure to allergens. 90% of the children with asthma are allergic to common airborne allergens such as house dust mite (HDM), animals, fungi or pollen. Inhaler therapy with bronchodilators may improve these symptoms, but in most patients continuous daily anti-inflammatory therapy is needed to control symptoms. Although major improvements in asthma medical treatment have taken place in the past decades, the disease may be severe and sometimes fatal and can still not be cured (1).

2. Burden of asthma

In children, the prevalence of asthma symptoms varies widely between countries from 1.6% to even 36.8% (2). However, when an objective measurement as airway hyperresponsiveness is also taken into account, the prevalence rates drop to 0-11.1% (1). Although fewer data are available for adults, similar prevalence rates have been described (3). The highest rates in Europe have been reported in the UK (2).

Despite the large variation in asthma occurrence between countries, the reported increase in prevalence of asthma during the eighties and nineties was very consistent word-wide (4). In the UK, the prevalence of asthma at age 16 increased from 3.8% in 1974 to 6.5% in 1986. In the same study, the prevalence of eczema and hay fever doubled in same period, suggesting that the increase in asthma was part of a general increase in atopic disease (5). Although improved diagnosis may be an explanation for this, it cannot explain the rise of asthma, since populations were studied with the same methods on many occasions several years apart. The reasons for the differences in prevalence and the recent increase are still poorly understood. There are now several reports suggesting that the tide has turned. Asthma prevalence seems to be decreasing or at least no longer increasing (6-10). A recent study in the Netherlands demonstrated that wheeze in Dutch children has decreased from 13.4% in 1989 to 9.1% in 2001 (11). Interestingly, the study

reported that the use of asthma medication has increased. Possibly, better asthma control may partly explain the concurrently decreasing trend in the prevalence of asthma.

Even with improvements in treatment, patients may still die of asthma. Although mortality data are unreliable in some countries, they may provide an indication of the burden of asthma. Recently, standardised mortality rates (SMR) were published for males (1.54 (1.10–2.09)) and for females (1.91 (1.44–2.49)) (12). Asthma morbidity and mortality seems to increase with socioeconomic deprivation and ethnicity (13).

Asthma may have considerable impact on physical, emotional, social and economic aspects of lives of patients (14). Despite the availability of effective therapies, asthma is not optimally controlled in many patients (15). Sleep disturbance is reported in one third of the children with asthma, whereas 60% report absence from school and activity restrictions (16). Disability adjusted life years (DALYs) is a measure of the burden of disease that assesses the years of healthy life lost due to disease or illness. The number of DALYs lost due to asthma worldwide has been estimated to be about 15 million/year. This makes asthma the 25th leading cause of DALYs lost worldwide in 2001 (17).

3. Risk factors for asthma

Asthma is a disease that may have multiple causes (1). The risk factors that might contribute to the development of asthma can be divided in two main factors: host factors and environmental exposure. These two factors may interact with each other both in the induction and subsequent expression of the disease (18) (Figure 1).

3.1. Host factors

One of the most important risk factors for asthma is heritability. If the mother and/or father have the disease, it is much more likely for the child to become allergic and asthmatic (18). Although multiple genes have been demonstrated to be related to this disorder, *the* asthma gene has not been identified yet and probably never will (19). The ADAM33 gene on chromosome 20p12 is such a gene that has been linked with asthma (20). It has been associated with asthma and bronchial hyperresponsiveness (21). Furthermore, polymorphisms of the ADAM33 are related to accelerated lung function decline (22). Atopy is another important host factor that predisposes individuals to develop this disease. It has been estimated that around one third of the asthma cases may be attributable to atopy (23). Finally, having (asymptomatic) airway hyperresponsiveness is an increased risk of becoming asthmatic (24).

3.2. Environmental exposures

In predisposed individuals, many environmental factors have been identified to increase the risk of developing asthma. An important determinant for the risk on asthma is exposure to house dust mite (25). An Australian study showed that in regions where HDM levels were high, more children were sensitised to HDM, and that subsequently

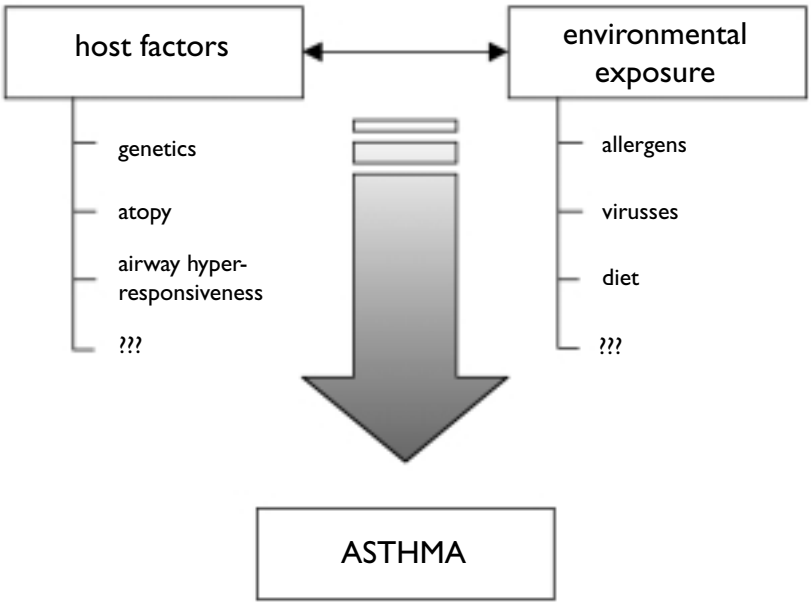

Figure 1. Causes of asthma.
A multi causal pathway may lead to the development of asthma. Several factors have been related with an increased risk of the disease.

these children had significantly more wheeze (26). The relationship with exposure to pets and asthma seems to be complex. Whereas dog ownership is not associated with sensitisation, the presence of a cat in the home may affect the risk on asthma (27). Exposure to cat dander may both increase as well as decrease the risk on sensitisation (27-30). It has been suggested that these conflicting results may be explained by the fact that the dose-response relationship with cat allergen is bell-shaped or that the timing of exposure (early or current pet ownership) is important or that high levels of other allergens influence sensitisation (31). Furthermore, these results have also been explained by the atopic status of the mother (32).

Respiratory syncytial virus (RSV) is a common cause of virus infection of the human respiratory tract during the first two years of life (33). It has been suggested that RSV infection may also predispose some children to the development of asthma (34). This is based on many observations that children who wheeze with RSV-induced bronchiolitis are more likely to develop into allergic asthmatics. In the Tucson Children's Respiratory study, one of the longest running respiratory cohort studies, RSV infection before the age of 3 was associated with an increased risk of wheezing by the age of 6 and 11. Remarkably, at the age of 13, RSV infection was no longer a risk factor for wheezing (35). It is still unknown whether RSV is a causal factor for asthma or targets children who are predisposed to asthma.
Growing up on a farm may be protective against the development of atopy (36-39).

However, exposures in the stables and farm in the first year of life seem to be crucial for this protective effect (40). It may also explain the absence of this association in New Zealand children (41). In contrast to central European farms where cattle are usually kept in stables built near the farmhouse, in New Zealand animals on large farm holdings stay outdoors throughout the year (42). The protective effect of living on a farm may result from elevated exposure to endotoxin, which is an intrinsic part of the outer membrane of gram-negative bacteria, since exposure to endotoxin is inversely related to the occurrence of asthma (43).

Diet is known to have a large effect on risk of many different diseases. Also relations between diet and the risk on asthma have been found. Consumption of fruit and vegetables, in particularly vitamin C, has been associated with a decreased prevalence of wheeze and asthma and may lead to a better lung function (44-46). In addition the consumption of full cream milk and butter has been suggested to reduce the risk of asthma (47).

4. Primary prevention of asthma

In view of the high prevalence of asthma and the fact that asthma cannot be cured, primary prevention must be considered (48). Several randomised controlled trials (RCTs) have attempted to reduce the risk on asthma. Studies investigating the effect of house dust mite avoidance by mattress covers have shown disappointing results (49-51). On the other hand, RCTs using multiple interventions have shown more effect. In the Isle of Wight Prevention Study, the risk on asthma and allergic sensitization was significantly reduced after an intervention on HDM avoidance combined with food restrictions (52). Moreover, these results were still significant after 8 years of follow-up (53). Similar results have been obtained in the Canadian Asthma Primary Prevention study after two and seven years of follow-up (54;55). The intervention in this study included reduced exposure to indoor allergens, avoidance of environmental tobacco smoke (ETS), encouragement of breast-feeding, and delayed introduction of other foods during the first 12 months of life.

Interesting data for asthma prevention come from intervention studies using probiotics (56). The term probiotics is referring to living or inactivated organisms that may exert beneficial effects on health when ingested. The most commonly used probiotics are lactobacilli and bifidobacteria. The proposed rationale for using probiotics against allergies and asthma is based on the relationship between the composition of intestinal flora and the presence of allergies. The gut of infants born in poor areas of developing countries, where allergy prevalence is low, is colonized earlier by lactobacilli compared to the gut of infants born in developed countries (57). A prospective study demonstrated that allergic children in Estonia and Sweden were less often colonized with lactobacilli, as compared with non-allergic children (58). Finish and Swedish studies also showed that differences in the neonatal gut microflora precede the development of

atopy, suggesting a crucial role of the balance of intestinal bacteria for the maturation of human immunity (59;60). The effect of probiotics on the development of asthma and allergies has been tested in clinical studies. Kalliomäki and coworkers have shown that *Lactobacillus* GG given prenatally to mothers and postnatally to their infants reduces the frequency of atopic eczema in a randomized placebo-controlled trial (61). Furthermore, the preventive effect of probiotics was persistent even at 4 years of age (62). Moreover, even in infants, who manifested atopic eczema, probiotics may counteract the allergic inflammation and thereby prevent further allergic disease (63). However, these studies have not yet been able to demonstrate positive effects of probiotics on the development of asthma.

Asthma is a multi-causal disease. As described above, several risk factors have been associated with the development of asthma. Nevertheless, the disease is complex and up to now can neither be cured nor be prevented. More understanding of the underlying pathology might lead to better knowledge and thereby treatment of asthma.

5. Airway inflammation

Inflammation of the airways is the main pathological characteristic of asthma. The inflammatory process can be separated into acute inflammation, chronic inflammation and airway remodelling. These pathologic mechanisms may lead to specific and overlapping clinical consequences for patients with asthma for example bronchoconstriction (acute), exacerbations (chronic), and persistent airflow obstruction (remodelling) (64). However, the link between these inflammatory features and clinical expression of the disease is often weak (Figure 2).

5.1. Acute inflammation

After inhalation of allergens, the acute allergic reaction is initiated when an allergen interacts with IgE that is bound to mast cells and basophils (65). These high-affinity IgE receptor (FcεRI) bearing cells release following activation preformed mediators, membrane-derived lipids, cytokines and chemokines (66). The release of the pro-inflammatory mediators such as histamine induces bronchoconstriction, mucus secretion and vasodilatation (64). The narrowing of the airway lumen is further increased via the induced microvascular leakage and edema (67). Antigen-presenting cells such as dendritic cells are also crucial in the allergic reaction. Dendritic cells can take up allergen and following the presentation of allergen to T cells, induce proliferation of naïve T cells (68). In the "late-phase" reaction inflammatory cells, such as eosinophils, CD4+ cells, basophils, neutrophils and macrophages are recruited and activated (64). The activation of T cells leads to the release of T helper cell, type 2 (Th2)-like cytokines that include IL-4, IL-5, IL-9, IL-13 (69).

The recruitment of cells into the airway wall is dependent on cytokines such as IL-5. IL-5 plays an important role in the mobilisation of eosinophils from the bone marrow (70).

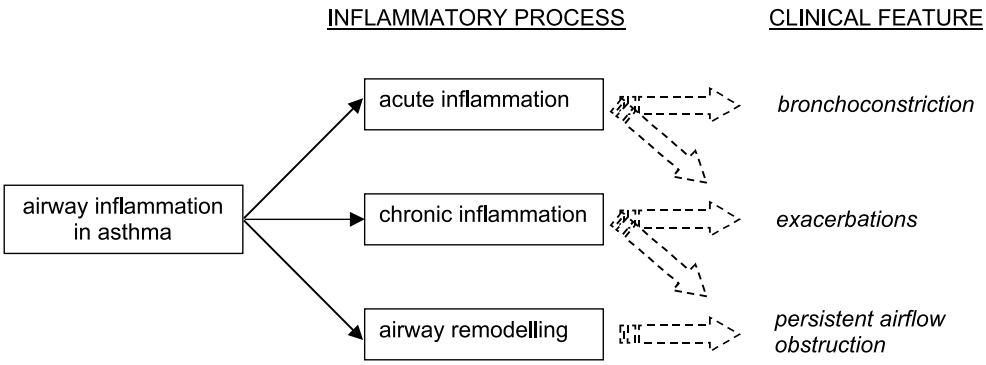

Figure 2. Airway inflammation in asthma.
Examples of clinical features that may result from a typical inflammatory process. These features are overlapping.

The Koch postulates of causation might be useful to confirm the central role of IL-5 in asthma. Indeed, elevated levels of IL-5 have been demonstrated in patients with asthma and have been related with severity (71). Second, administration of IL-5 to animals and humans has been associated with increased numbers of eosinophils, however its effects on airway hyperresponsiveness are unclear (72;73). This has led to the following questions: **Would exogenous IL-5 lead to airway inflammation in asthma? Is the route of IL-5 production crucial to its effect on the airways in patients with asthma? (Chapter 2)**

The fundamental importance of IgE in the pathogenesis of asthma has been clearly identified (74). Burrows *et al.* were the first who showed a strong association between serum IgE levels and self-reported asthma (75). Since then many epidemiological studies have demonstrated the relationship between asthma and IgE (65;76). Furthermore, high levels of circulating IgE have been shown to correlate with the risk of emergency room admissions in patients with asthma (77). Like other antibodies, IgE has two identical light and heavy chains. IgE has a very short half-life (<1 day) and is present in very low concentration in the circulation (78). Cross-linking of IgE molecules on high-affinity IgE receptors triggers the release of mediators by mast cells and basophils (79). Therefore, these cells are highly sensitive to allergens even when the concentration of IgE is very low. IgE production requires at least two distinct signals. The first signal is IL-4 (produced by T-cells, mast cells, basophils and eosinophils) and IL-13 (produced in addition by natural killer cells). The second signal is delivered by the interaction of CD40L on T cells with CD40 on B cells. The combination of these signals causes class switching to IgE and B cell proliferation (80). Recently, the first monoclonal antibody against IgE has been approved by Food and Drug Administration (FDA) in the US for the treatment of patients with severe allergic asthma. This antibody recognizes IgE at the same site as the high-affinity receptor for IgE (FcϵRI), but does not provoke histamine release from IgE-sensitized mast cells (81).
The acute symptoms of breathlessness and wheezing, which occur following exposure to

allergens are the clear clinical features of this acute inflammatory process. This has led to the following question: **Is treatment with anti-IgE improving both the clinical features and the inflammatory process in patients with asthma? (Chapter 7)**

5.2. Chronic inflammation

All cells of the airways, including eosinophils, T cells, mast cells, macrophages, and epithelial cells are involved in the chronic inflammation of asthma (82). Although eosinophilic inflammation plays a central role in the disease, it is not specific to asthma (83). Moreover, a sub-type of non-eosinophilic asthma has been described (84). The number of bronchial eosinophils has been associated with the severity of asthma (85). Eosinophils are potentially harmful in asthma through the release of highly toxic products (MBP, ECP, EDN and oxygen free radicals). The role of IL-5 in the observed eosinophilia in asthma is important: IL-5 regulates the development and differentiation of eosinophils, stimulates the release of eosinophils from the bone marrow into the peripheral circulation and is involved in the activation and survival of eosinophils (86). Interestingly, a study investigating the role of anti-IL-5 challenged the central place of eosinophils in asthma. In this study, a monoclonal antibody against IL-5 reduced the levels of eosinophils in blood and sputum of patients with asthma. However, it had no effect on early and late allergen response or airway hyperresponsiveness (87). Therefore, it may be questionable whether eosinophils and airway hyperresponsiveness are causally related in asthma.

T cells are likely to play a role in controlling the chronic inflammation through the release of Th2-cytokines (88). The majority of T cells are CD4+ cells, whereas CD8+ cells are less frequently identified, even during exacerbations of asthma (89). The frequency of cytokine producing CD4+ and CD8+ cells is similar and is increased as compared with normals (90;91). Interestingly, following glucocorticoid withdrawal, eosinophils are elevated in all patients, whereas increases in airway T cells (both CD4+ and CD8+) were found in only those who developed an exacerbation (92).

There is growing interest for the role of CD8+ T-cells in asthma. A cross-sectional relationship between CD8+ T-cells and the outcome of asthma has been observed in patients with fatal asthma (93). Furthermore, increased cytokine production of sputum CD8+ T-cells has been shown to be related with disease severity in patients with asthma (93). Antigen specific CD8+ T-cells in the lungs demonstrate a high cytotoxic activity and proliferate rapidly (94). These specific effector/memory T cells can be rapidly activated by antigens, like allergens and viruses (95). In the host response to virus infections, CD8+ T-cells may initiate eosinophil recruitment (95). Indeed, in mouse models CD8+ T-cells appear to be essential for the influx of eosinophils into the lung and the development of airway hyperresponsiveness during respiratory virus infections (96). Furthermore, CD8+ T-cells are required for the development of airway hyperresponsiveness following allergic sensitization (97) leading to increased inflammation (98;99). Although an important role for CD8+ T-cells in asthma seems likely, the association of CD8+ T-cells with asthma severity has only been demonstrated in

cross-sectional studies. This has led to the following question: **Are CD8 cells related to the outcome of asthma in a longitudinal study? (Chapter 5)**

5.3. Airway wall remodelling

The structural changes in the airways of asthmatics, which are referred to as airway wall remodelling, include thickening of the reticular basement membrane, increased airway smooth muscle, epithelial shedding, altered deposition of extracellular matrix (ECM) proteins. Since this process begins early in the development of asthma, remodelling may occur in parallel or could even be required for the development of chronic inflammation (100).

The basement membrane of the surface epithelium is composed of the basal lamina and lamina reticularis. Thickening of the latter is an early characteristic feature of patients with asthma (101). Epithelial reticular basement membrane thickening has been demonstrated in children with asthma, although in symptomatic infants with reversible airflow obstruction, it could not be found (102). In adults, following treatment with inhaled steroids, the wall thickness was reduced, however it remained elevated as compared to controls (103). Interestingly, a thicker airway wall as assessed by computed tomography (CT) appears to be related to reduced airway hyperresponsiveness (104). This suggests that the increased airway wall thickness, as observed in asthma, is protective for airway hyperresponsiveness. In some (105;106), but not all studies (107), the thickness of the sub-epithelial reticular layer was inversely associated with the level of lung function in asthma. The clinical features of airway remodeling may be the irreversible airway obstruction and decline in lung function, which is observed in patients with asthma. However, until now there are no prospective studies, which have shown a relationship between the thickness of the sub-epithelial reticular layer and lung function decline in patients with asthma. This has led to the following question: **Is the thickness of the sub-epithelial reticular layer related to the outcome of asthma in a prospective follow-up study? (Chapter 5)**

6. Asthma management

The GINA guidelines propose a six-part asthma management program (1). The *first* part involves patient education. In this way, patients will be able to achieve control of asthma by adjusting medication according to a management plan.

In the *second* part, the assessment and monitoring of asthma severity is based on symptoms and lung function (1). In order to quantify asthma symptoms, several symptom scores and quality of life questionnaires have been developed (108;109). The relationship between symptoms and other clinical measures is poor. Interestingly, a factor analysis has shown that this is primarily due to the fact that asthma health status has 4 distinct components: 1: asthma-specific quality of life, 2: airway calibre, 3: daytime symptoms and daytime $ß_2$-agonist use and 4: night-time symptoms and night-time $ß_2$-

agonist use (110). Measurements of lung function are recommended and overcome the problems of poor perception or under-reporting of symptoms by patients. Lung function measurements, in particularly, the degree of reversibility to bronchodilators such as $ß_2$-agonists, give insight in airflow limitation, whereas measuring the variability of lung function provides an assessment of airway hyperresponsiveness (1).

Peak expiratory flow (PEF) meters are useful for regular home monitoring of lung function. PEF is mostly being recorded two times a day. The measurements can be analysed and summarised in different ways: PEF-level (mean daily PEF, expressed as a percentage of predicted or of personal best) and PEF-variability (amplitude as percentage of mean value) (111) for the diagnosis of asthma, although its usage may be limited due to poor sensitivity of the measurement (112-114). It has been suggested that PEF-variability may provide additional information when used in conjunction with other clinical parameters for asthma patients, who are already on treatment (115). Indeed, the effectiveness of treatment appeared to be associated with the level of PEF-variablity (116). However, PEF-variability may fail to detect an asthma exacerbation (117).Therefore, the minimum morning PEF over 1 week, expressed as a percentage of recent best (Min%Max) has been suggested as an alternative, simpler index of PEF-variability (118;119). The are few studies, which have shown contribution of using PEF-variability to classify asthma control. This has led to the following question: **What is the value of including PEF-variability in addition to symptoms and $ß_2$-agonist use, in predicting the development of poor asthma control? (Chapter 6)**

The *third* part of asthma management involves avoidance of exposure to risk factors. The effectiveness of the reduction of allergen levels was shown in studies, which are performed at high altitude where allergens levels are low (120;121). Although the use of impermeable mattress cover seems to be ineffective, several other measures can be taken (122;123).

Pharmacological treatment (part *four* of the asthma management program) is needed in most patients. Treatment of asthma aims to reverse and prevent symptoms and airflow limitation (1). Medication can be divided into controllers and relievers. Controller medications (inhaled glucocorticosteroids, long-acting $ß_2$-agonists, and leukotriene modifiers) have to be used daily for long-term to maintain control of persistent asthma. At this moment, glucocorticosteroids are considered to be the most effective controller medications (1). In RCTs, inhaled steroids have shown to improve airway hyperresponsiveness and sputum eosinophils even in patients with mild asthma (124;125). Furthermore, in studies using bronchial biopsies, reduction in the thickness of the sub-epithelial reticular layer and a decrease in mast cells and eosinophils could be demonstrated (126;127). Reliever medications (short-acting $ß_2$-agonists and anticholinergics) are needed to act quickly in reducing bronchoconstriction.

Despite optimal treatment, exacerbations of asthma may occur. Part *five* of the asthma management plan concerns the managing of exacerbations. Exacerbations are often

related to a viral infection (128). An increase in symptoms usually precedes the worsening in lung function (129). Therefore, symptoms may be a sensitive marker for the early onset of an exacerbation. The severity of an exacerbation is difficult to define. Therapies include repetitive step-wise administration of short-acting $ß_2$-agonists, systemic glucocorticosteroids or even oxygen supplementation (1).

Finally, provision of regular follow-up care is needed in order to ensure that asthma control is maintained. Even in patients who are in clinical remission of asthma airway inflammation may still be present and treatment with inhaled steroids can be effective (130-132).

6.1. Limitations of asthma management

The assessment and monitoring of asthma control in the current guidelines is based on symptoms and lung function. Although this approach leads to good control of asthma in many patients, there is still room for improvement. As mentioned before, the burden of asthma is high. Large surveys have shown that the level of asthma control falls short of the goals for asthma management (15;133). Furthermore, asthma prognosis can be poor. Long-term follow-up has shown that patients with asthma have an accelerated decline in lung function (FEV_1) as compared to controls (134-136). Moreover, in adult patients with severe asthma, irreversible airway obstruction is common (137).

6.2. Improving asthma management

Several approaches are being undertaken to increase asthma control. First, asthma management may be improved by optimizing the use of current available treatment. Poor compliance to asthma medication has been repeatedly reported (138). Monitoring medication adherence might improve compliance and thereby asthma control (139). Interestingly, the internet appears to be a useful tool to reach this goal (140;141). It has also been hypothesized that patients do not perceive the need for daily therapy and therefore are not taking their medication. Based on this hypothesis, the efficacy of as-needed corticosteroids has recently been investigated in mild intermittent asthma (142).

Second, new drugs for asthma are being developed. These therapies include anti-inflammatory drugs (such as phosphodiesterase 4 (PDE4) inhibitors (143)) or "anti-allergic" drugs directed against specific components of allergic inflammation (such as omalizumab). Omalizumab, which is a humanized monoclonal antibody against IgE, is the first monoclonal antibody drug developed for the treatment of moderate-to-severe asthmatics to receive approval by the FDA in the US. The first clinical studies with intravenous anti-IgE have shown that both early (EAR) and late (LAR) asthmatic response to inhaled allergen are attenuated in patients with asthma (144;145). A recent double-blind, placebo-controlled study confirmed the effectiveness of anti-IgE treatment in inadequately controlled severe persistent asthma by showing a reduction in exacerbation rate and emergency room visits and an improvement in quality of life and morning peak expiratory flow rate (146). Although anti-IgE treatment has been shown to reduce IgE positive cells in bronchial mucosa of patients with asthma, a direct

association with its clinical effect has not been made (147). This has led to the following question: **Can the clinical effect of anti-IgE be explained by a reduction in allergen-induced airway inflammation? (Chapter 7).**

Third, the measurement of non-invasive (more direct) markers of inflammation might be beneficial for asthma management. The use of airway hyperresponsiveness (AHR), inflammatory markers in sputum and exhaled nitric oxide (NO) in the management of asthma will be discussed in the next paragraph.

7. Monitoring inflammation

Airway hyperresponsiveness can be defined as an increase in sensitivity to a wide variety of airway narrowing stimuli (148). The degree of AHR is related to asthma severity and airway inflammation (149;150). Furthermore, repeated measurements of AHR seem to reflect the changes in asthma control in response to treatment (151). Interestingly, asthma management based on reducing AHR on top of improving symptoms and lung function leads to more effective asthma control (152). Compared with conventional management, the AHR strategy resulted in fewer exacerbations, improved FEV_1 and a greater reduction in thickness of the sub-epithelial reticular layer.

The methods for sputum induction and processing have been recently standardized (153;154). The number of eosinophils in sputum is associated with asthma severity (155). Furthermore, sputum eosinophils already increase before the onset of an exacerbation (156). Following inhaled steroid treatment eosinophils in sputum significantly decrease (157). Asthma management based on minimising eosinophils in sputum has also been investigated. Patients in the "sputum management group" had fewer exacerbations than the patients who received the current standard treatment (158). Other inflammatory markers in sputum for monitoring airway inflammation in patients with asthma have been less thoroughly examined. Microvascular leakage, which is also an important characteristic of airways inflammation in asthma (67), has not often been measured to monitor inflammation in asthma. This has led to the following question: **Can induced sputum be applied to measure microvascular leakage in patients with asthma? (Chapter 4).**

Measurement of markers in exhaled air is non-invasive. In addition, exhaled NO has also been proposed as a marker for disease severity in asthma (159;160). Patients with asthma have increased levels of exhaled NO as compared to normals (161). Inhaled steroids reduce the levels of exhaled NO in patients with asthma (162) in a dose-dependent way (163). Moreover, increases in exhaled NO are associated with a worsening in asthma control (164). It was recently demonstrated that patients with asthma can be successfully treated based on the levels of exhaled NO (165;166).

One or more of the above-described non-invasive markers of inflammation will probably be implemented in future asthma guidelines. However, a comparative analysis is required before any of these markers can be recommended in the monitoring of asthma therapy. This has led to the following question: **What are the treatment-induced changes in airway hyperresponsiveness, sputum eosinophils and exhaled NO in one comparative study? (Chapter 3).**

8. Aims of the studies

In summary, this thesis addresses three different aspects of airway inflammation in asthma: role of inflammatory mediators in airway inflammation, monitoring of airway inflammation and asthma management of airway inflammation. The above-mentioned questions have been addressed in six studies relating to airway inflammation and asthma management in asthma.

Role of inflammatory mediators in airway inflammation
Chapter 2. Would exogenous IL-5 lead to airway inflammation in asthma? Is the route of IL-5 production crucial to its effect on the airways in patients with asthma? In this chapter the effects of IL-5 administered intravenously or by inhalation to patients with mild asthma was investigated on eosinophil counts in blood and in sputum, and on airway hyperresponsiveness.

Monitoring of airway inflammation
Chapter 3. What are the corticosteroid-induced changes in airway hyperresponsiveness, sputum eosinophils and exhaled NO in a comparative study? Twenty-five patients with asthma were treated for 4 weeks with inhaled steroids or placebo. Before, during and after treatment airway hyperresponsiveness, sputum eosinophils and exhaled NO were measured.

Chapter 4. Can induced sputum be applied to measure microvascular leakage in patients with asthma? This chapter examines the levels of albumin, fibrinogen, ceruloplasmin and alpha-2-macroglobulin as markers of leakage in induced sputum before and after a substance P challenge in patients with asthma. Inhaled NKA was used as a control challenge in this randomised, placebo-controlled, crossover study.

Chapter 5. Are CD8+ T cells related to the outcome of asthma in a follow-up study? Is the thickness of the sub-epithelial reticular layer related to the outcome of asthma in a longitudinal study? In this chapter, we aimed to investigate the prognostic significance of airway inflammation and remodelling on the decline in lung function in asthma. In 32 patients with asthma the relationship between bronchial eosinophils, CD8+ T cell, the thickness of the sub-epithelial layer, and the annual decline in lung function after 7½ years of follow-up was determined.

Asthma management

Chapter 6. What is the value of including PEF-variability in addition to symptoms and $ß_2$-agonist use, in predicting the development of poor asthma control? In a prospective study, we examined in 75 patients with asthma the value of including PEF-variability in addition to symptoms and $ß_2$-agonist use, in predicting the development of poor asthma control.

Chapter 7. Can the clinical effect of anti-IgE be explained by a reduction in allergen-induced airway inflammation? In a randomized, double-blind, placebo-controlled study, the effect of anti-IgE on allergen-induced airway inflammation in bronchial biopsies and on the expression FcεRI receptors and IgE+ cells was investigated in 25 patients with asthma. Furthermore, the effect of anti-IgE treatment on peak flow, airway hyperresponsiveness and sputum was determined.

Summary and conclusion

Chapter 8. A summary of the main results of the different studies is given in this chapter. In addition, implications of these findings are discussed.

Chapter 9. In this chapter, a summary of this thesis is given in Dutch.

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Chapter 2

Evidence for systemic rather than pulmonary effects of interleukin-5 administration in asthma

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Thorax 2001; 56: 935-940

Abstract

Background Interleukin-5 (IL-5) plays an important role in mobilisation of eosinophils from the bone marrow and in their subsequent terminal differentiation. We investigated whether IL-5 could induce pulmonary eosinophilia and bronchial hyperresponsiveness (BHR) independently of these effects by examining the effects of inhaled and intravenous administration of IL-5.
Methods Nine mild asthmatics received in random order inhaled (15μg) or intravenous (2μg) IL-5 or placebo in a double blind, crossover study. Blood samples were taken prior to and at 1/2, 1, 2, 3, 4, 5, 24 and 72 hours following IL-5 or placebo, and PC_{20} (methacholine) and eosinophil counts in induced sputum were determined.
Results Serum IL-5 was markedly increased 30 min after intravenous IL-5 ($p=0.002$), and sputum IL-5 increased 4 and 24 hours after inhaled IL-5 ($p<0.05$). Serum eotaxin was elevated 24 hours after intravenous but not inhaled IL-5 or placebo. Blood eosinophils were markedly reduced from 0.5 – 2 hours following intravenous IL-5 ($p<0.05$), followed by an increase at 3, 4, 5 and 72 hours ($p<0.05$). Sputum eosinophils rose significantly in all three groups at 24 hours but there were no between-group differences. Bronchial responsiveness (PC_{20}) was not affected by IL-5.
Conclusion The effects of IL-5 appear to be mainly in the circulation, inducing peripheral mobilisation of eosinophils to the circulation, without effect on eosinophil mobilisation in the lungs and on bronchial responsiveness.

Introduction

Chronic asthma is characterised by an inflammation of the bronchi with the presence of eosinophils and CD4+ T-helper cells (1,2). T-helper type 2 (Th_2) cells are the predominant source of the cytokine IL-5 and are elevated in the asthmatic airways (2). IL-5 plays an important role in the mobilisation of eosinophils from the bone marrow, and in the terminal differentiation and maintenance of mature eosinophils (3). IL-5 levels have been measured in the circulation of asthmatics (4), and rises during asthma exacerbations (1). Additionally, increased IL-5 mRNA expression is seen in bronchial mucosa and bronchoalveolar lavage CD4+ T cells in asthma (2,5), and IL-5 protein levels increase following allergen challenge (6). A positive correlation between asthma severity and IL-5 mRNA levels in bronchial biopsies has also been demonstrated (7).

In guinea pig and rodent models, airway administration of IL-5 induces an increase in airway eosinophils without demonstrable change in bronchial responsivess (8,9). Airway and blood eosinophilia has also been demonstrated in IL-5 transgenic mice, but without bronchial hyperresponsiveness (10-12). IL-5 administration to the airways of asthmatic subjects, however, has been associated with both peripheral blood eosinophilia, airway eosinophilia and bronchial hyperresponsiveness (13;14).

These conflicting observations suggest a complex co-operative role for IL-5 in the accumulation of circulating and airway eosinophils and their possible subsequent contribution to bronchial hyperresponsiveness. Since IL-5 also appears to have important effects on the bone marrow (9,15,16), we questioned whether the route of IL-5 production was crucial to its effects on the airways. We hypothesised that IL-5 confined entirely to the lungs and airways would have little systemic effect on eosinophil mobilisation, while IL-5 administered to the circulation might possess this primary effect. Further, we sought to establish whether pulmonary or systemic IL5 could induce specific pulmonary effects consistent with asthma. We therefore investigated the effects of IL-5 administered intravenously or by inhalation to patients with mild asthma on eosinophil counts in blood and in sputum, and on airway hyperresponsiveness.

Methods

Subjects

Nine non-smoking, atopic patients with mild persistent asthma (5 women, age range 25-41 years) participated in this study (Table 1). All subjects had a history of episodic chest tightness, and wheezing and symptoms were controlled by on-demand usage of short-acting inhaled $ß_2$-agonists alone. All subjects were free of respiratory tract infection symptoms for at least 2 weeks prior to study commencement. Atopy was determined by positive skin-prick responses (>3 mm) to extracts of 6 common aeroallergens (Vivodiagnost, ALK, Benelux). Baseline forced expiratory volume in 1 second (FEV_1) was >70% of predicted (17) and bronchial hyperresponsiveness (PC_{20} < 8 mg/ml

Table 1. Subject characteristics of mild asthma patients

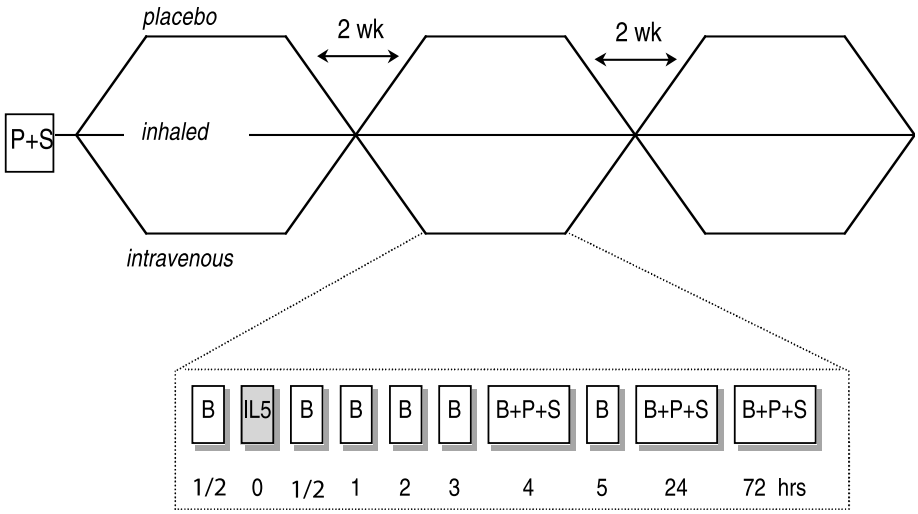
Subject	Sex	Age (years)	FEV_1 (% pred)	PC_{20} (mg/ml)	Sputum Eosinophils (%)
1	M	35	94	0.15	2.3
2	F	26	85.6	0.19	0.5
3	F	26	79	0.37	6.3
4	F	27	95	0.42	1.1
5	F	36	97	1.44	1.0
6	F	25	117	2.79	0.0
7	M	29	106	3.73	5.9
8	M	28	97	6.68	0.5
9	M	41	85	8.00	0.2
		30.3 ± 5.6*	95.1 ± 3.8*	1.15 ± 2.21#	1.00 (0-6.3)†

* mean-SEM; # geometric mean-SD in doubling doses; † median (range).

methacholine), were determined by standardised technique (18). The protocol was approved by the Ethics Committee of the Royal Brompton Hospital and all participants gave written informed consent.

IL-5 administration

Recombinant human IL-5 (Genzyme, Boston, MA) was reconstituted in 0.9% NaCl (2ml) and administered at a dose of 2 μg intravenously via a slow infusion in a forearm vein



IL5 = administration of inhaled(15 μg), intravenous (2 μg) IL-5 or placebo
B = blood sample
P = PC_{20} methacholine
S = sputum induction

Figure 1. Study design. Subjects were treated in random order with inhaled IL-5, intravenous IL-5 or placebo, in a cross-over design at 2 week intervals. Specimen sampling was performed at time-points noted.

over a period of 5 mins. This intravenous dose was calculated according to the levels of circulating IL-5 measured in circulating blood of patients with asthma during an exacerbation (1). IL-5 (15µg) was inhaled over 5 minutes via a nebulizer (MECIC-AID, Pagham, Sussex, UK) from a mouthpiece using a one-way exhaust valve (LC Plus, PARI, Surrey, UK). To ensure complete inhalation of the IL-5, the chamber was then refilled with 2ml 0.9% NaCl and the nebulisation repeated.

Lung function and methacholine challenge

Baseline FEV_1 was recorded from the best of three attempts using a dry wedge spirometer (Vitalogragh, Buckingham, UK) (17). Spirometry was performed prior to and hourly after the administration of IL-5 or placebo until the start of methacholine challenge at 4h. Methacholine challenges were performed according to a standardised methodology (18). After a nebulized saline challenge, doubling doses of methacholine (0.06-32 mg/ml) were administered via a dosimeter (Mefar, Bovezzo, Italy) with an output of 100µl. A total of five inhalations of each concentration was administered (inhalation time one sec and breath-holding time six sec). FEV_1 was measured 2 and 3 min after each dose. The test was discontinued if FEV_1 as compared with the control inhalation (0.9% NaCl) decreased by 20% or more. The PC_{20} was calculated by log-linear interpolation of the last two data-points.

Peripheral blood eosinophil counts

Peripheral blood eosinophils were identified by the combination of peroxidase staining and side scatter and enumerated using the automated Advia 120 Hematology System (Bayer, Newbury, Berks, UK).

Sputum induction

All subjects received 200µg salbutamol following the methacholine challenge. After recovery, sputum was induced according to a previously-described method (19). Hypertonic saline aerosols (NaCl 3.5%) were nebulised at room temperature via an ultrasonic nebuliser (DeVilbiss Ultraneb 2000) at maximum output. Subjects inhaled the aerosols during 3 x 5-minute intervals. Between each interval, or as soon as the subjects started coughing, they were instructed to wash their mouth and blow their nose in order to minimise salivary contamination. The induced sputum was collected into a 50-ml tube, kept at 4°C and processed within two hours.

Sputum processing

The whole sputum sample was diluted with 2 ml Hank's balanced salt solution (HBSS) containing 0.25% dithiotreitol (DTT; Sigma Chemicals, Poole, UK). The sample was gently mixed at room temperature, the volume determined and the sample further diluted with HBSS to 10 ml. The sample was centrifuged (350g, 10 min), supernatant was removed and the cell pellet resuspended in 1 ml HBSS. Total cell counts were determined on a haemacytometer slide. Cytospin slides were prepared (600 rpm, 6 min; Shandon, Runcorn, UK) and stained with May-Grunwald-Giemsa. Differential cell counts were performed by a blinded observer with 300 non-squamous cells counted on each of

two slides. To correct for the variable salivary contamination, differential cell counts were expressed as a percentage of 300 nucleated cells, excluding squamous epithelial cells. An adequate sample was defined if there was < 80% squamous cell contamination. Supernatants were stored at –80ºC.

IL-5 and eotaxin assays

Serum IL-5 and eotaxin and sputum IL-5 levels were measured by ELISA according to the manufacturer's instructions (Pharmingen, Cambridge, UK). Briefly, purified rat anti-human monoclonal antibodies were incubated at 2 µg/ml in coating solution (0.1M $NaHCO_3$ (BDH) pH 8.2 at 4 °C) overnight. Plates were washed then blocked with 10% FCS for 2h at room temperature. Sample supernatants were added to each plate in duplicate. Cytokine standards (R&D Systems, Abingdon, Oxon, UK) were diluted in 10% (v/v) FCS/PBS/Tween and added in duplicate. Plates were then incubated at 4º C overnight. Biotinylated rat anti-human antibody (R&D) was added and incubated at room temperature for 45 mins then washed before the addition of avidin-peroxidase. Plates were incubated at room temperature for 30mins before washing with PBS/Tween and (ABTS - Sigma) substrate. Plates developed for approximately 10 minutes before measurement on a plate reader (Anthos Labtec, Austria) at 405nm. The detection limit of this assay was 35 pg/ml.

Data analysis

PC_{20} was log-transformed before statistical analysis and reported as geometric mean (± SD). IL-5 levels and blood and sputum eosinophils were not normally distributed and were therefore log-transformed prior to analysis and reported as median (range), for eosinophils, and geometric mean (± SD), for IL-5 levels. For between group analyses, analysis of variance (ANOVA) for repeated measures was applied. Within group changes in PC_{20}, sputum and blood eosinophils, serum and sputum supernatant IL-5 were analyzed using the Student's paired t-test. Mean differences between treatments with 95% confidence intervals for these effect estimates are presented for serum IL-5 and blood eosinophil counts. All statistical analyses were performed using the SPSS program. P-values < 0.05 were considered as statistically significant.

Results

Serum and sputum IL-5 levels

Serum IL-5 levels was rapidly cleared from the circulation and returned to baseline levels between 1 and 72 hours. Serum IL-5 concentrations in atopic asthmatics increased significantly 0.5 hours following intravenous IL-5 administration (387.4 ± 162.1 pg/ml), compared with baseline (14.0 ± 12.8 pg/ml, p=0.002), with rapid clearance, returning to baseline levels between 1 and 72 hours (Figure 2). The levels of serum IL-5 at 1, 2, 3, 4 and 5 hours after intravenous IL-5 were 16.8 ± 5.9, 14.2 ± 8.4, 5.7 ± 3.5, 8.1 ± 3.9, and 3.7 ± 2.0 pg/ml respectively, indicating that by one hour the levels had returned towards baseline values. In order to determine whether there was any difference in the kinetics

of clearance of IL-5 between asthmatic and non-asthmatic volunteers, we also studied 3 non-asthmatic non-atopic volunteers. After 2 µg intravenous injection, serum IL-5 measured in non-asthmatic subjects was 3.6 ± 2.5 pg/ml at baseline and increased to 54.2 ± 24.6 pg/ml 0.5 hours following intravenous IL-5 (mean difference –60.53, 95% CI –107.7 - -13.3, p<0.05; Figure 2). These data indicate that there is no difference in the clearance in IL-5 between normal and asthmatic volunteers, although the asthmatics had higher levels of baseline serum IL-5 than non-asthmatics. There was a trend to elevation of serum IL-5 half an hour following inhaled IL-5 administration (18.18 ± 12.4 pg/ml to 51.14 ± 100.56 pg/ml) in the asthmatic subjects, but serum IL-5 was not affected by placebo. There was a significant increase in the IL-5 concentration measured in sputum supernatant from baseline at 4 and 24 hours following inhaled IL-5 (baseline: 49.0 ± 16.44, 4 hours: 2,067 ± 1,091 and 24 hours: 152.3 ± 42.8 pg/ml; p=0.04 and p=0.02,

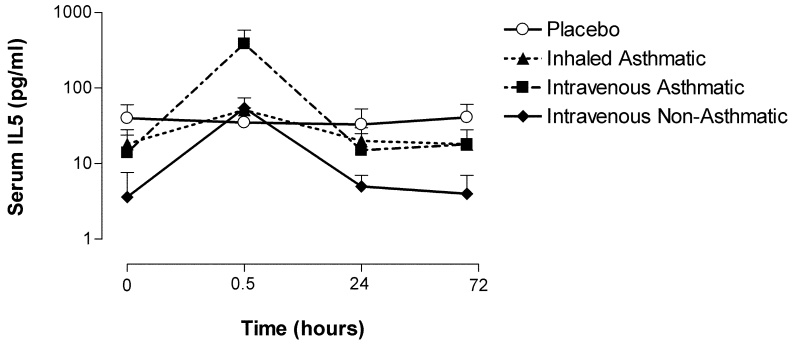


Figure 2A. Serum

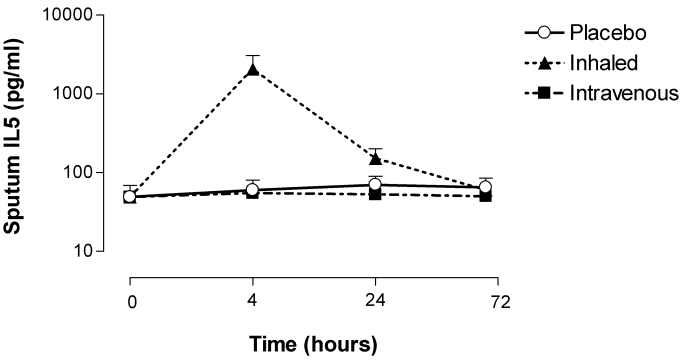


Figure 2B. Sputum

Figure 2.

A. Mean IL-5 serum levels. Serum IL-5 levels rose markedly following intravenous IL-5 but were not significantly elevated by inhaled IL-5 or placebo in patients with mild asthma. In addition, in normal volunteers, the administration of intravenous IL-5 led to a peak of serum IL-5 levels at 0.5 hr similar to that in asthma patients.

B. Mean IL-5 levels in sputum supernatants. Sputum IL-5 levels rose 4 hours following inhaled IL-5 and remained elevated at 24 hours but, were not affected by intravenous IL-5 or placebo.

respectively), but no changes in sputum supernatant IL-5 following placebo or intravenous IL-5.

Serum eotaxin

Serum eotaxin levels were 207.5 ± 80.2 ng/ml at baseline, 248.1 ± 93.6 at 0.5 hours, 232.5 ± 101 at 4 hours and 301.4 ± 113.6 at 24 hours following intravenous IL-5. There was a trend to increase in serum eotaxin following intravenous IL-5 with a 43.0 ± 23.3% increase at 24 hours compared to baseline (Figure 3). Comparative eotaxin levels following inhaled IL-5 was 12.0 ± 23.8 and after placebo 1.5 ± 6.8. No correlation was observed between peak IL-5 levels (0.5 hours) and eotaxin levels at 0.5 or 24 hours following IL-5, nor was there correlation between eotaxin levels and peripheral blood lymphocyte or eosinophil numbers.

Effect of IL-5 on peripheral blood cells

Blood eosinophils were reduced 30 minutes (mean difference 0.15, 95% CI 0.05 – 0.26, $p<0.01$) and 2 hours (0.13, 0.02 – 0.24, $p<0.05$) following the administration of intravenous IL-5 compared with baseline (Figure 4). The decrease in eosinophils 0.5 hours after intravenous administration was significantly different from changes following inhaled IL-5 and placebo ($p<0.03$). The increase in blood eosinophil counts observed at 3, 4, 5 and 72 hours was not statistically significant.
Additionally, peripheral blood lymphocytes were significantly increased from baseline at 4 ($p<0.01$, mean difference –0.54, 95% CI -0.97 - -0.11) and 5 hours ($p<0.001$, -0.59, -1.02 - -0.16) following intravenous but not inhaled IL-5 or placebo.

Effect of IL-5 on sputum eosinophils

The sputum eosinophil counts were 1.0% (0-6.3) (median (range)) at baseline. Following inhaled IL-5, sputum eosinophils increased to 5.0% (median) at 24 hours ($p=0.02$), and persisted at 72 hrs (4.6%, $p=0.02$). However, similar increases were observed after intravenous IL-5 and after placebo treatment (median at 24 hours: 3.6% and 3.7%, respectively, $p<0.01$). There were no significant differences in sputum eosinophils between the treatment groups (Figure 5).

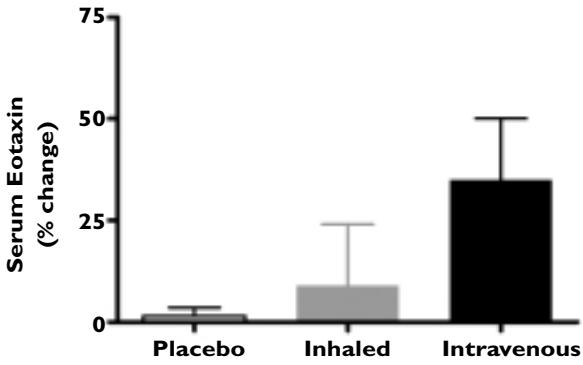
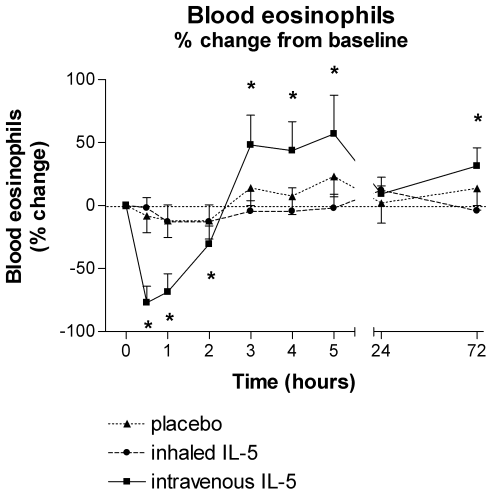


Figure 3. Serum eotaxin. There was a non-significant trend to increase in serum eotaxin levels 24 hours following intravenous IL-5 but not after placebo.



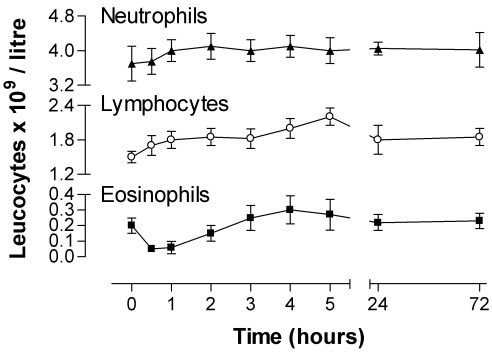


Figure 4A **Figure 4B**

Figure 4

Left panel: Time course of blood eosinophil counts after placebo or inhaled IL-5 or intravenous IL-5. Intravenous IL-5 induced a rapid fall in circulating eosinophil numbers ($p<0.05$), not seen following inhaled IL-5 or placebo. There was a prolonged but non-significant elevation of eosinophils at 3, 4, 5 and 72 hours after intravenous IL-5. Data shown as mean ± SEM.

Right panel: Time course of changes in blood neutrophil, lymphocyte and eosinophil counts after intravenous IL-5. Data shown as mean ± SEM.

Effect of IL-5 on lung function and bronchial responsiveness

Baseline FEV_1 was 95.1 ± 3.8% predicted, and PC_{20} was 1.15 ± 2.21 mg/ml. No significant changes were observed in FEV_1 or PC_{20} during the 3 different study periods (Figure 6). There were no significant correlations between sputum IL-5 levels and change in PC_{20}, nor between eosinophils in blood or sputum with levels of IL-5 measured in sputum or in serum.

Discussion

In this study, we report the first observations on the comparative effects of inhaled and intravenous IL-5 in mild asthma. We observed marked elevations of IL-5 levels in the serum but not in the sputum when administered intravenously, and the other way round when given by inhalation. Thus, inhaled IL-5 appeared confined to the lung and intravenous IL-5 to the blood compartment. Intravenous IL-5 induced a rapid decline and subsequent non-significant rise in blood eosinophils which was not associated with airway eosinophilia or bronchial hyperresponsiveness. Sputum eosinophils were indeed slightly increased after intravenous and inhaled IL-5 but were also observed following placebo, indicating that these increases were non-specific. Inhaled IL-5 had no systemic

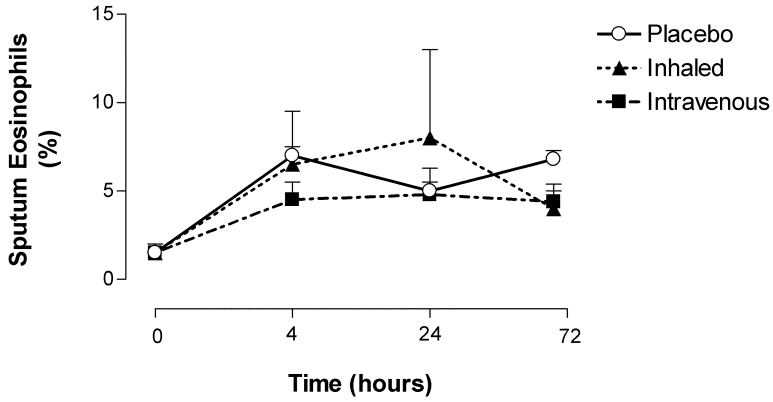


Figure 5. Mean sputtum eosinophil counts. Sputum eosinophils were elevated four hours from baseline in each of the treatment groups, but no between-group differences occurred at any of the time points measured.

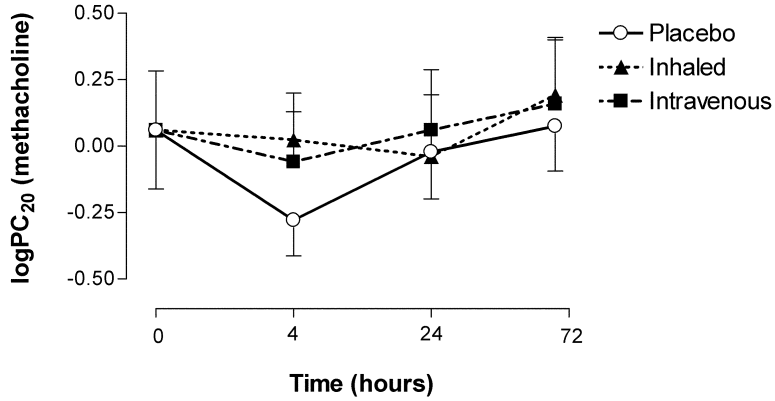


Figure 6. Mean log PC_{20} to methacholine. Bronchial responsiveness did not change significantly from baseline after inhaled IL-5, intravenous IL-5 or placebo.

effect as reflected by blood eosinophils or serum eotaxin levels and did not affect bronchial responsiveness. A trend to elevated serum eotaxin levels was observed 24 hours following intravenous IL-5. The cellular source of this increase in eotaxin levels could have been the vascular endothelial cells, or circulating eosinophil and T-cells. Systemic IL-5 is a potent stimulus to eosinophil mobilisation to the circulation, albeit not in itself sufficient to specifically induce lung eosinophilia (as measured in induced sputum) or airway hyperresponsiveness.

We observed significant, sequential and converse effects on peripheral blood eosinophils following intravenous IL-5. Between 0.5 and 2 hours, eosinophils were markedly reduced, and this effect has not been previously reported in animals or humans. At later time-points (4-72 hours) there was significant elevation of blood eosinophil counts. We speculate that these effects result from rapid vascular margination followed by, either

demargination or replenishment of the circulating eosinophil pool from bone marrow, and other extra-medullary leukocyte reservoirs. A rapidly-induced but short-lived peripheral eosinophilia has been observed previously following intravenous IL-5 in animals (20;21), while a prolonged peripheral blood eosinophilia occurred after inhaled IL-5 in human asthmatics (13).

That IL-5 did not specifically affect sputum eosinophils levels contradicts a previous study of IL-5 inhalation in mild asthmatics (14). We observed minor but significant increases in sputum eosinophil numbers in all groups. These changes are unlikely to be explained by methacholine challenge (22) or repeated sputum induction (23). Our findings are, however, consistent with previous studies in rodents. Wang et al (1998) (24) demonstrated that circulating IL-5 is essential for the development of airway eosinophilia, whereas local lung IL-5 production did not lead to eosinophil recruitment to the lungs. Similarly, Mould et al (2000) (11) observed a doubling of eosinophil counts in bronchoalveolar lavage fluid following the administration of intrapulmonary and intravenous IL-5 compared to intrapulmonary IL-5 administration alone in mice. Lee and coworkers (1997) created an IL-5 transgenic mouse expressing IL-5 in airway epithelium which developed baseline bronchial hyperresponsiveness and BAL eosinophilia; however, in this model IL-5 was expressed at unusually high levels in serum with concomitant prominent peripheral eosinophilia (12).

We saw no effect of IL-5 on bronchial responsiveness. Consistent findings are provided in human studies in which monoclonal anti-IL-5 blocking antibodies substantially reduced allergen-induced blood and sputum eosinophilia but had no effect on the late phase response and bronchial hyperresponsiveness to allergen challenge (25). These findings have been consistently confirmed in animal models (26;27). The contribution of IL-5 to bronchial hyperresponsiveness is contentious and in asthma models, difficult to dissociate from effects induced by mediators other than IL-5 (28-31). Consistently however, IL-5 transgenic mice have prominent airway eosinophilia but have acetylcholine responses similar to wild type mice (32). These studies collectively suggest a close association between IL-5 and both circulating and bronchial eosinophil numbers but a dissociation between pulmonary IL-5 and bronchial responsiveness in both animals and humans (25; 31-33).

Our observations are substantially different from those of Shi et al (14) in terms of sputum eosinophilia and bronchial responsiveness induced by IL-5, despite using similar amounts of inhaled IL-5 obtained from a similar source, and comparable cohorts of asthmatics in terms of severity and baseline sputum eosinophilia. These studies were performed in ethnically different populations raising the possibility that there may be racial susceptibility to the effects of IL-5. Another possibility is that the high level of endemic parasitic colonization in China (34-36) may induce an expanded population of activated tissue eosinophils(35-37), that are primed to respond more to IL-5. Sputum ECP levels in the mild asthmatics studied by Shi et al were, however, 2-log fold higher than levels previously described in severe asthma (38).

Collins et al 1995 (9) reported that in contrast with intravenous administration, local IL-5 did not induce tissue accumulation of eosinophils (9). This indicates that local IL-5 has little chemoattractant activity, whereas intravenous IL-5 has a rapid enhancing effect on eosinophil accumulation, by stimulating the release of a rapidly mobilizable pool of bone marrow eosinophils. Furthermore, although IL-5 is necessary, other signals from activated T lymphocytes may also be required to induce accumulation of eosinophils in the airways (20;39;40). Eotaxin, an eosinophil selective chemokine, augments the accumulation of eosinophils by IL-5 in a synergistic fashion. This implies that IL-5 and eotaxin act co-operatively to promote the recruitment of eosinophils into the tissue (9;20) Our data indicates that IL-5 when administered on its own to the airways by aerosol is not capable of inducing eosinophilic inflammation and bronchial hyperresponsiveness in mild asthmatic subjects. However, changes in circulating eosinophil numbers were observed with intravenous administration of IL-5, indicating potential systemic effects on the bone marrow. Thus, IL-5 may have systemic rather than pulmonary effects in asthma.

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Chapter 3

Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma

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Abstract

Background Airway hyperresponsiveness, induced sputum eosinophils and exhaled nitric oxide (NO) have all been proposed as non-invasive markers to monitor airway inflammation in patients with asthma. The aim of the present study was to compare the changes in each of these markers as obtained by inhaled glucocorticoids in one single study.

Methods In a randomized, double-blind, placebo-controlled, parallel study, 25 patients with mild asthma (19-34 yr, FEV_1>75% predicted, PC_{20}<4 mg/ml) inhaled fluticasone propionate (500 μg, bid) for 4 weeks. PC_{20} to histamine, sputum eosinophils and exhaled NO were determined at weeks 0, 2, 4, and after 2 weeks wash-out (week 6). Sputum was induced by inhalation of hypertonic (4.5%) saline, and eosinophils counts were expressed as % non-squamous cells. Exhaled NO (ppb) was measured by chemiluminescense.

Results Within the steroid group, there was a significant increase in PC_{20}, decrease in sputum eosinophils and decrease in exhaled NO as compared with baseline at weeks 2 and 4 of treatment ($p<0.01$). Subsequently, each of these variables showed significant worsening during two weeks run-out as compared with week 4 ($p<0.01$). These changes were significantly different from those in the placebo group ($p<0.05$), except for the changes in sputum eosinophils from baseline to week 2 and from week 4 to 2 weeks wash-out. There were no significant correlations between the changes in the three markers in either group at any time.

Conclusion We conclude that 4 weeks of treatment with inhaled steroids leads to improvements in airway hyperresponsiveness to histamine, eosinophil counts in induced sputum, and exhaled nitric oxide levels. Our results suggest that these markers may provide complementary information when monitoring anti-inflammatory treatment in asthma.

Introduction

Asthma is an inflammatory disease of the airways, associated with airway hyperresponsiveness to various bronchoconstrictor stimuli, such as histamine (1). The accompanying inflammation is characterized by the presence of inflammatory cells, such as T-lymphocytes, neutrophils and eosinophils, and their cytokines in the airway mucosa as demonstrated in bronchial biopsy specimens (2,3). The current treatment of asthmatic patients is based on reducing or preventing airway inflammation as guided by lung function and symptoms (1,4). To monitor airway inflammation more closely, measurement of non-invasive and sensitive markers of inflammation, such as airway hyperresponsiveness (5), sputum eosinophils (6) or exhaled NO (7) during treatment follow-up in patients with asthma has recently been suggested.

To date, inhaled glucocorticoids are the most effective treatment of asthma not only reducing symptoms and airway hyperresponsiveness (8), but also leading to an improvement of airway inflammation (9). However, there is recent evidence that therapy according to the present international guidelines provides only partial suppression of airway inflammation, as shown by persisting eosinophilic inflammation in the bronchial (sub)mucosa after long-term inhaled steroid treatment (5).

Among the non-invasive techniques, hypertonic saline-induced sputum has been shown to be a reliable method to measure eosinophilic airways inflammation (6,10,11). The number of eosinophils in sputum is associated with asthma severity (10) and decreases following inhaled steroid treatment (12). In addition, nitric oxide levels in exhaled air have also been proposed as marker for disease severity in asthma (7,13). Indeed, inhaled glucocorticoids decrease the levels of exhaled NO in patients with asthma (14), in a dose-dependent way (15).

Although the effects of inhaled steroids on sputum eosinophils and exhaled NO have been well established, comparative analysis is required before any of these markers can be recommended in the monitoring of asthma therapy. In the present study we investigated treatment-induced changes in airway hyperresponsiveness, sputum eosinophils and exhaled NO in asthma. To that end, we performed histamine challenge, induced sputum and exhaled NO measurements before, during and after 4 weeks treatment with fluticasone propionate or placebo in steroid-naive patients with asthma.

Methods

Subjects

Twenty-five non-smoking, atopic patients (9 female and 16 male, 19-34 years) with mild persistent asthma (1) voluntered to participate in this study (Table 1). Symptoms of episodic chest tightness and wheezing were treated by on-demand usage of inhaled salbutamol alone, which was discontinued at least 8 h before the measurements. Two

Table 1. Characteristics of the subjects

Subject No.	Sex	Age (yr)	FEV_1 (% pred)	PC_{20} (mg/ml)	EO (%)	NO (ppb)
Steroid						
1	M	24	77	0.07	1.4	5.85
2	M	24	104	0.37	3.8	10.75
3	M	21	104	0.39	5.8	11.81
4	F	23	88	0.55	7.6	5.28
5	F	20	83	0.71	4.0	3.42
6	M	24	103	0.72	0.0	8.24
7	M	23	94	1.29	3.6	10.21
8	F	24	101	1.81	0.2	7.25
9	F	21	99	2.05	NA	2.17
10	M	28	104	2.54	3.4	2.15
11	F	21	98	3.14	1.4	3.92
12	M	27	99	3.14	0.2	4.62
		23.3 (2.4)¶	96.2 (9.0)¶	0.91 (1.62)*	3.40 (0.0,7.6)§	5.57 (2.15,11.81)§
Placebo						
13	M	24	82	0.11	21.2	13.41
14	F	21	108	0.11	0.0	6.57
15	M	29	111	0.14	24.6	12.05
16	M	34	83	0.30	0.0	4.17
17	M	21	98	0.46	1.6	13.40
18	M	24	80	0.54	NA	14.08
19	M	25	86	0.73	0.0	3.26
20	M	24	98	0.77	1.8	4.22
21	F	24	106	0.89	3.2	2.48
22	M	28	90	1.00	1.2	3.36
23	F	28	106	1.20	NA	5.05
24	F	25	98	1.51	0.0	5.82
25	M	19	97	1.70	0.4	9.26
		25.1 (3.9)¶	95.6 (10.6)¶	0.52 (1.38)*	1.20 (0.0,24.6)§	5.82 (2.48,14.08)§

¶ = mean (SD), * = geometric mean (SD) in DD, § = median (range), NA = not applicable

weeks before the study all subjects were free of symptoms of respiratory tract infection. Atopy was indicated by a positive skin prick test (> 3 mm wheal) to one or more of 10 common airborne allergen extracts (Vivodi–agnost, ALK, The Netherlands). The forced expiratory volume in one second (FEV_1) was greater than 75 % of the predicted value (16), and all subjects were hyperresponsive to inhaled histamine (PC_{20} < 4 mg/ml) (17). The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and a signed informed consent was obtained from all volunteers.

Design

The study had a randomized, double-blind, placebo-controlled parallel design. During screening, the selection criteria were checked for all subjects. Before entering the treatment period baseline values of PC_{20} histamine and percentage eosinophils in

induced sputum were determined. These two measurements were carried out on two separate days, with a 2-4 days interval. Prior to histamine challenge and sputum induction, baseline values of FEV_1 and exhaled NO were recorded. This sequence of measurements was used at all time points of the study. Directly following the second baseline visit, the subjects were treated with inhaled fluticasone propionate (500 µg bid) or placebo for a period of four weeks. The measurement of PC_{20} histamine, sputum eosinophils, FEV_1 and exhaled NO were repeated during the treatment period (at weeks 2 and 4) and during wash-out at two weeks after the treatment period.

Histamine challenge

Histamine challenges were performed according to a standardized methodology (17). Histamine-di-phosphate (Sigma Chemicals, St.Louis, MO, USA) in PBS was stored at 4°C and administered at room temperature. Doubling concentrations between 0.06 and 16 mg/ml were used. The aerosols were generated by a DeVilbiss 646 nebulizer (output: 0.13 ml/min), connected to an in- and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). Each dose was inhaled through the mouth by tidal breathing for 2 minutes at 5-minute intervals, with the nose clipped (17). The airway responses to the inhaled aerosols were measured using FEV_1, recorded by a dry rolling-seal spirometer (Morgan Spiroflow, Morgan UK) and monitored on-line by a personal computer with a special soft-ware program. Before each test FEV_1 was measured in triplicate, for calculation of mean baseline levels (17). The airway response was recorded at 30 and 90 seconds after each dose. After each inhalation, the lowest, technically satisfactory FEV_1 value was applied in the analysis to calculate the percentage fall in FEV_1 from baseline. The test was discontinued if FEV_1 was decreased by 20% or more. The provocative concentration causing 20 % fall in FEV_1 (PC_{20}) was calculated by log-linear interpolation of the final two data points.

Sputum induction

Sputum was induced and processed by the so called full-sample method (18) according to a protocol that has been validated in our laboratory (6). Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer with a calibrated particle size (MMAD 4.5 mm) at maximal output (2.5 ml/min). The aerosols were administered to the subjects through a 100 cm long tube with an internal diameter of 22 cm, and inhaled via the mouth through a two-way valve (No. 2700; Hans-Rudolph, Kansas City, MO, USA), with the nose clipped. Before inhalation of the aerosols, baseline FEV_1 was recorded and, for safety reasons, 400 µg salbutamol was administered through a metered dose inhaler (Volumatic). Subsequently, the subjects inhaled hypertonic saline aerosols during 2 x 5 min and 1 x 10 min intervals. After each inhalation, or as soon as the subjects experienced cough, they were asked to blow their nose, to rinse their mouth and throat with water, and to expectorate sputum into a clean plastic container by coughing. After testing, FEV_1 was measured, and salbutamol was administered if needed.

Sputum processing and cell differential counts

The volume of the induced sputum samples was determined and mixed with an equal volume of 0.1% sputolysin (dithiotreitol, Calbiochem, USA) (6). To ensure complete homogenization, the samples were placed in a shaking water bath at 37 °C for 15 minutes, once interrupted by gently mixing the sample. The homogenized sputum was centrifuged (350 x g) for 10 minutes at room temperature. The cell pellet was resuspended in PBS to a final volume of 2-5 ml, followed by filtration through a gauze (pore-size approximately 1 mm) to remove clumbs. Total cell counts were performed in a haemacytometer (Tamson, Zoetermeer, The Netherlands). Subsequently the sample was diluted with PBS to a final concentration of $\pm 0.3 \times 10^6$ cells/ml which was used for preparation of the cytocentrifuge slides (1500 rpm, 3 minutes, 50 ml/slide) (Shandon 3, Life Sciences International, Veldhoven, The Netherlands). Differential cells counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells were performed on Diff-Quik stained, cytospins by a qualified cytopathologist. To correct for the variable salivary contamination, differential leukocyte and cylindric epithelial cell counts were expressed as a percentage of 250 nucleated cells excluding squamous cells. For each sample, differential cell counts were performed twice by the same observer, and the mean data were used in the analysis. A sputum sample was considered adequate when the percentage squamous cells was less than 80%. The reproducibility of the sputum cell counts as obtained by this method has been shown to be satisfactory (6). To ensure a blind analysis of the sputum samples, all cytocentrifuge slides were coded before analysis by an investigator who was not involved in the counting.

Exhaled NO

Exhaled NO levels were measured by a chemiluminescense analyzer (Sievers NOA 270B) according to a standardized procedure (7), which has previously been applied by our lab (19). The subjects were connected to a closed system to avoid contamination of the measurements with ambient NO. Pressured air with low NO concentration (< 1ppb) was administered through a 150 L reservoir connected to the inspiratory side of a Hans-Rudolph three-way valve. The subjects performed a slow vital capacity manoeuvre with a constant expiratory flow of 10L/min against an expiratory resistance of 3-4cm H_2O. Expiratory NO concentration was sampled continuously from the centre of the mouthpiece at a sample flow of 440 ml/min, and the average concentration (in parts per billion; ppb) was determined for a period of 10 seconds (7). Baseline values of exhaled NO were obtained from the mean values of the two NO measurements recorded before histamine challenge and sputum induction, because the reproducibility was good (intraclass correlation coefficient, $R_i > 0.92$).

Analysis of data

PC_{20} was log-transformed before statistical analysis, and expressed as geometric mean (SD) in doubling dose. To test for differences between and within the treatment groups in general, multivariate analysis of variance (MANOVA) was applied for FEV_1 and log PC_{20}, whilst Kruskal-Wallis test was used for sputum eosinophils and exhaled NO. The changes in PC_{20} (expressed in doubling doses: DD) within each treatment group were

analysed using Student's paired t-test, whilst the changes in PC_{20} between both groups were tested using Student's unpaired t-test. Since exhaled NO levels and sputum eosinophils were not normally distributed, these markers were analysed non-parametrically. The Wilcoxon signed-rank test was used to test for the differences within each treatment group. Furthermore, the Mann-Whitney signed-rank test was applied to test for the differences between the groups in changes in sputum eosinophils and exhaled NO at all time-points as compared with baseline. Finally, Spearman rank correlation analysis was used to examine the relationship between the changes in PC_{20}, sputum eosinophils and exhaled NO. Results were considered significant if p value < 0.05. All statistical analyses were performed using SPSS.

Results

Three of the subjects dropped out during the run-out period due to a history of respiratory tract infection (#5 and #6), or because of taking an anti-histamine (#11). Three subjects (#9, #18 and #23) did not produce adequate sputum at baseline, whilst subject 21 and 7 were not able to produce sputum at week 2 and week 4, respectively These time points were handled as missing data.

Lung function and histamine challenge

At baseline there were no significant differences in FEV_1 and PC_{20} between the groups (p>0.19; table 1). During the study there were no significant changes in FEV_1 in the two groups (p>0.96, MANOVA). In the placebo group there were no significant changes in PC_{20} (p = 0.92, MANOVA) while in the steroid treated group PC_{20} increased significantly at week 4 compared with baseline values (mean change 2.01 (95% CI 0.683 to 2.090); p = 0.001; fig 1). After a two week washout period PC_{20} decreased again compared with week 4 by –1.75 (–1.831 to –0.582) doubling doses (p = 0.002; table 2, fig 1). These changes were significantly different from the changes in the placebo group (p<0.003; table 3).

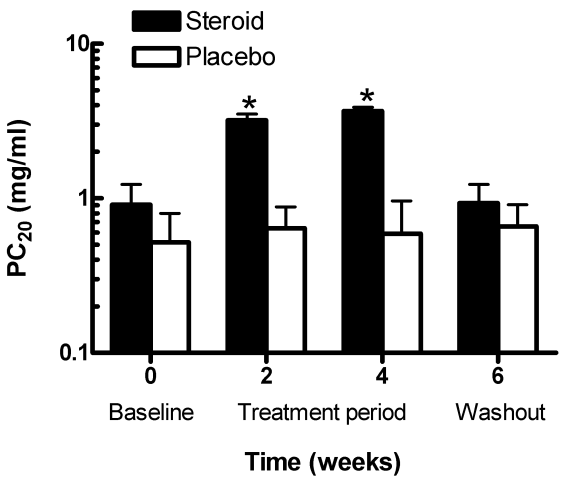

Figure 1. Airway hyperresponsiveness to histamine (PC_{20}) at baseline, at 2 and 4 weeks of treatment, and after 2 weeks wash-out in steroid (closed bars) and placebo group (open bars). shown as geometric mean doubling dose. * = significant difference between the two groups.

Table 2. Airway hyperresponsiveness, sputum eosinophils and exhaled NO during and after steroid and placebo treatment

	Baseline	Week 2	Week 4	Run-out
Steroid group				
PC_{20} (mg/ml)	0.91 (1.62)	3.19 (1.54)¶	3.67 (1.05)¶	0.93 (1.50)§
Eosinophils (%)	3.40 (0.00,7.60)	0.30 (0.00,3.00)¶	0.20 (0.00,1.60)¶	4.41 (1.40,20.00)§
NO (ppb)	5.57 (2.15,11.81)	1.54 (0.11,4,86)¶	1.48 (0.59,3.68)¶	3.50 (0.90,12.89)§
Placebo group				
PC_{20} (mg/ml)	0.52 (1.38)	0.64 (1.21)	0.59 (1.86)	0.66 (1.26)
Eosinophils (%)	1.20 (0.00,24.60)	1.20 (0.00,18.56)	3.47 (0.00,16.60)	3.75 (0.60,30.00)
NO (ppb)	5.82 (2.48,14.08)	5.03 (0.59,18.73)	5.26 (0.17,11.38)	5.36 (1.94,21.28)

Values of PC_{20} expressed as geometric mean (SD) in DD, values of sputum eosinophils and exhaled NO expressed as median (range), ¶p<0.01 as compared to baseline, §p<0.01 as compared to week 4

Sputum eosinophils

The mean (SD) percentage of squamous cells in this study was 33.4 (17.6)%. Baseline sputum eosinophils were not significantly different in the two groups (p = 0.31; table 1). There were no significant changes in sputum eosinophils within the placebo group (p = 0.85, MANOVA), but in the steroid treated group a significant decrease in sputum eosinophils was observed compared with baseline values (mean change at week 4 –2.46 (95% CI –4.260 to –0.660)%; p = 0.01) with a subsequent worsening in the washout period compared with week 4 (mean change 6.13 (95% CI 0.804 to 11.459)%; p = 0.03; table 2, fig 2). The changes in sputum eosinophils were not significantly different between the two groups when baseline values were compared with week 4, or week 4 values were compared with those in the washout period (table 3).

Exhaled NO

At baseline exhaled NO levels were not significantly different in the two groups (p = 0.55; table 1). During the study there were no significant changes in exhaled NO levels

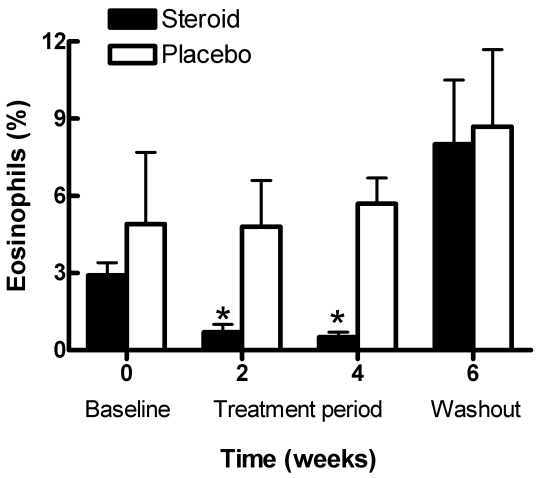

Figure 2. Mean eosinophil counts in induced sputum at baseline, at weeks 2 and 4 of treatment, and after 2 weeks of wash-out in steroid treated (closed bars) and placebo groups (open bars). * = significant difference between the two groups.

Table 3. Comparison of change in airway hyperresponsiveness, sputum eosinophils and exhaled NO between steroid and placebo treatment

	Baseline – Week 2	Baseline – Week 4	Week 4 – Run-out
Δ PC_{20} (mg/ml)			
Steroid	1.80 (1.38)	2.01 (1.60)	-1.75 (1.17)
Placebo	0.32 (0.59)	0.19 (0.97)	0.17 (1.15)
p-value	0.004	0.003	0.001
Δ Sputum eosinophils (%)			
Steroid	–1.40 (-7.60,0.40)	-1.90 (-7.60,0.00)	2.81 (1.40,18.80)
Placebo	0.20 (-18.80,9.80)	1.00 (-11.40,11.20)	0.82 (-7.45,20.80)
p-value	0.13	0.03	0.15
Δ Exhaled NO (ppb)			
Steroid	-3.81 (-10.10,-1.09)	-3.89 (-9.90,-0,75)	2.12 (0.16,9.21)
Placebo	-0.62 (-2.67,4.65)	-1.71 (-5.15,1.09)	0.40 (-1.21,10.76)
p-value	0.0001	0.007	0.049

Values of changes PC_{20} expressed as geometric mean (SD) in DD, values of changes in sputtum eosinophils and exhaled NO expressed as median (range)

in the placebo group (p = 0.54, MANOVA; table 2) but in the steroid treated group the levels of exhaled NO decreased significantly at week 4 compared with baseline values with a mean change of –4.88 (95% CI –6.862 to –2.892) ppb (p < 0.001), with a subsequent increase during the washout period compared withweek 4 of 3.65 (95% CI 0.882 to 6.423) ppb (p = 0.016; table 2, figure 3). These changes in exhaled NO levels were significantly different from the changes in the placebo group between baseline and week 4 (p = 0.005; table 3).

Relationship between observed changes

Within the steroid group there were no significant correlations between the changes in PC_{20}, sputum eosinophils and exhaled NO at any time point (Pearson's r< 0.56, p>0.15; figures 4-6).

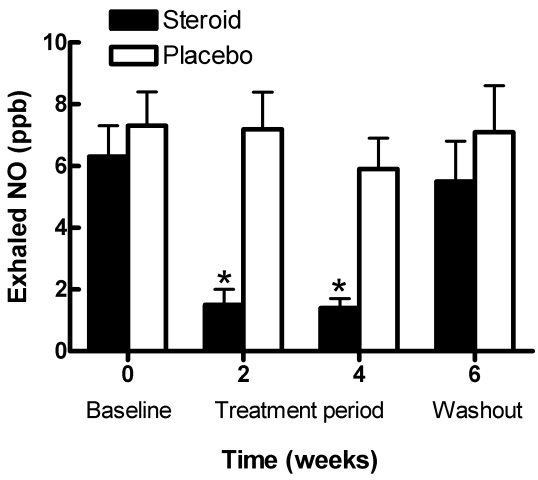

Figure 3. Mean levels of exhaled nitric oxide (NO) at baseline, at weeks 2 and 4 of treatment, and after 2 weeks of wash-out in the steroid treated (closed bars) and placebo groups (open bars). * = significant difference between the two groups.

Discussion

The results of this study indicate 4 weeks of therapy by inhaled steroids lead to improvements in airway hyperresponsiveness, sputum eosinophils, and levels of exhaled NO in patients with mild atopic asthma. In addition, it appears that the improvement in these markers are lost 2 weeks after cessation of treatment. This suggests that each of these markers is useful for monitoring patients with asthma, even though there might be small differences between the markers in the earliest response to anti-inflammatory treatment.

To our knowledge this is the first study comparing the treatment-induced changes in airway hyperresponsiveness to histamine, eosinophils counts in induced sputum, and exhaled NO in a group of asthmatic patients. Our study confirms and extends the results of others who have demonstrated the beneficial effect of glucocorticoids on each of these markers separately. In accordance with Kraan *et al.*, we showed an improvement of 2 doubling doses in airway hyperresponsiveness after 4 weeks of treatment with inhaled

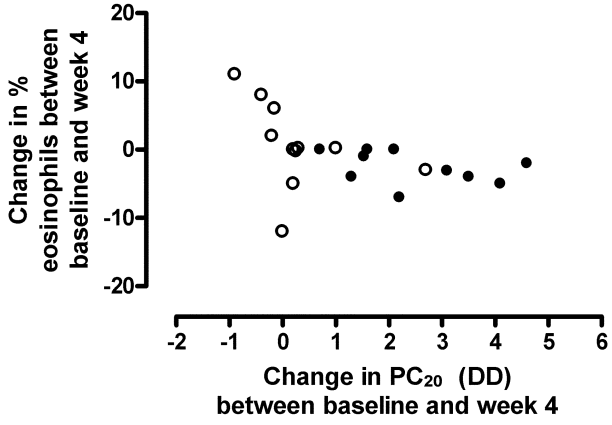

Figure 4. Relationship between the change in sputum eosinophils and the change in PC_{20} histamine at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

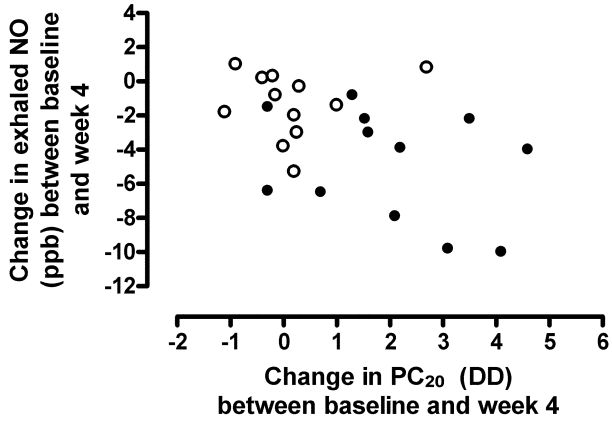

Figure 5. Relationship between the change in exhaled NO levels and the change in PC_{20} histamine at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

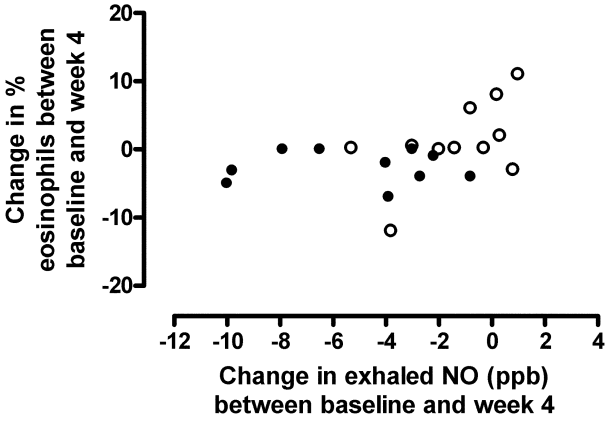

Figure 6. Relationship between the change in sputum eosinophils numbers and the change in exhaled NO levels at week 4 compared with baseline (closed circles = steroid group; open circles = placebo group).

steroids (20). Furthermore, our findings are in agreement with those of Keatings *et al.* (12) and Kharitonov *et al.* (14), who demonstrated a decrease in sputum eosinophils and exhaled NO, respectively, after inhaled steroid treatment.

Although cross-sectional relationships between airway hyperresponsiveness, sputum eosinophils and exhaled NO in asthma have been previously reported (10,21), there are only limited data on the comparison of within-subject changes in these markers during treatment follow-up. Our results are in agreement with those of Baraldi *et al.* who also failed to demonstrate a correlation between steroid-induced changes in PD_{20} and sputum eosinophils (22). The absence of such relationships may reflect the partially distinct pathophysiological backgrounds of these markers, and might be indicative of possible independent, complementary clinical information during anti-inflammatory therapy.

We do not believe that our data were influenced by measurement errors, since we used validated and reproducible methods (6,7,17,19). All subjects in this study were carefully selected to be non-smokers with stable, atopic asthma, who had not used inhaled steroids for at least 1 month prior to the study. We had chosen a relatively high dose of inhaled steroids as the present intervention in order to ensure an optimal anti-inflammatory effect. To avoid carry-over effects, the histamine challenge for determination of PC_{20} and the sputum induction were separated by 2-4 days. Furthermore, exhaled NO levels at these two days appeared to be highly reproducible. Our inability to demonstrate significant improvement in lung function following steroid treatment may be due to the normal baseline levels of FEV_1 in our study (77-111% of the predicted value).

How can the present findings be interpreted? First, glucocorticoids are likely to decrease the percentage sputum eosinophils by reducing the release and effects of cytokines like interleukin-5 (IL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on eosinophil infiltration and survival (23-25). Second, the steroid-induced reduction in exhaled NO can be explained by the inhibition of inducible NO synthase (iNOS) expression directly and/or indirectly by reduction in the levels of stimulatory cytokines,

for instance in epithelial cells (26). Finally, the improvement in the physiological marker, PC_{20}, is likely to be due to effects of steroids on the presence and activity of multiple (infiltrative and resident) cells (5,8,9,27). Hence, it may not be surprising that the steroid-induced changes in the three markers were not significantly correlated to each other. Apparently, the earliest improvements of eosinophils in response to steroid treatment is somewhat out of phase as compared to the other two markers. However we believe that this has little implications, given the consistency in the changes between the markers after 4 weeks of treatment.

What are the clinical implications of the present findings? Treatment according to the current international guidelines is based on minimising symptoms and optimising lung function (1). However, frequently, this fails to provide complete suppression of airway inflammation (5). It has been postulated that persistent airway inflammation in asthma leads to airway remodelling and an irreversible loss of lung function (28,29). This may require the use of more direct markers for monitoring airway inflammation (10,30). Indeed, a recent study by Sont *et al.* demonstrated that the adjustment of long-term inhaled steroid treatment, additionally guided by the level of airway hyperresponsiveness, leads to a significantly better clinical, as well as histological, outcome as compared to treatment based on symptoms and lung function alone (31). Based on the present data, it needs now to be addressed in long-term prospective trials as to whether monitoring sputum eosinophils and/or exhaled NO can provide similar benefits in asthma management.

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Chapter 3

Chapter 4

Assessment of microvascular leakage via sputum induction: the role of substance P and neurokinin A in patients with asthma

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Abstract

Background Microvascular leakage is an important feature of inflammation. However, the assessment of vascular leakage has seldom been used to monitor airway inflammation in asthma. The aim of this study was to determine the effect of inhaled substance P, a potent NK1-agonist and mediator of plasma extravasation, on markers of microvascular leakage in induced sputum in patients with asthma.
Methods In a cross-over study, sputum was induced before and 30 min. after inhalation of substance P or neurokinin A (as control) in 12 atopic and mild, steroid-naive asthmatic subjects. The levels of alpha-2-macroglobulin, ceruloplasmin, albumin, and fibrinogen were determined in induced sputum as markers of leakage.
Results Substance P induced a significant increase in the levels of alpha-2-macroglobulin, ceruloplasmin, and albumin in induced sputum (median fold change 3.1, 2.2 and 2.9, respectively) ($p < 0.013$), whereas inhaled neurokinin A was not able to induce significant changes ($p>0.31$). The increase in sputum leakage markers was not associated with the cumulative dose of substance P ($p>0.12$).
Conclusion These results indicate that NK1-receptor stimulation causes a rapid increase in microvascular leakage as shown in induced sputum in patients with asthma. This investigational model of "dual induction" (first leakage, then sputum) may therefore be useful to test the anti-exudative effect of newly develop drugs, such as NK1-antagonists.

Introduction

Asthma is a chronic disease of the airways, characterized by variable airway obstruction and airway hyperresponsiveness to various stimuli (1). Mucosal inflammation of the airways can be considered as one of the major components of asthma (2). The role of cell activation and the subsequent release of mediators in this inflammatory process has been extensively studied (2). On the other hand, microvascular leakage and edema are also prominent features of airways inflammation in asthma (3). Remarkably, leakage has not often been measured to monitor inflammation in asthma, which is probably due to the difficulties of the measurement in bronchial tissue specimens and luminal fluids. Thus far, there are only few studies demonstrating the effect of treatment intervention on leakage parameters in asthma (4).

In animal models, several inducers of airway microvascular leakage have been identified (5). Amongst these, the tachykinin substance P (SP) appears to be one of the most potent mediators causing leakage in guinea pig airways (6). It is likely that tachykinins play a role in the pathogenesis of asthma. Tachykinins have been detected in airway sensory nerves and in secretions recovered from human airways (7). More importantly, elevated levels of SP have been demonstrated in BAL fluid and sputum, and a further increase occurred following allergen challenge in patients with asthma (8;9). In human tissue, the distinct NK1- and NK2-tachykinin receptors have recently been identified using immunohistochemistry (10). The NK2-receptor mediates smooth muscle contraction in human airways (11), whereas the NK1–receptor primarily induces pro-inflammatory effects: plasma extravasation, mucus secretion, inflammatory cell chemotaxis and activation (7). Interestingly, NK1- and NK2-receptor mRNA expression appears to be increased in asthmatics as compared to normals (12;13).

It is still unknown whether NK1-stimulation leads to microvascular leakage in the airways of asthmatics *in vivo*. So far, SP-induced effects on microvascular leakage have only been demonstrated in the airways of guinea pigs (14). In humans *in vivo*, both neurokinin A (NKA) and SP cause airway narrowing, particularly in patients with asthma (15). This may predominantly be due to NK2-mediated smooth muscle contraction (11). In addition, inhaled SP enhances airway hyperresponsiveness, as demonstrated by an increase in the maximal response to inhaled methacholine in asthmatic patients *in vivo* (16). We postulate that this is associated with an NK1-mediated increase in microvascular permeability in the airway wall.

Therefore, in the current study, we investigated whether inhaled SP induces microvascular leakage in asthmatic patients *in vivo*. To that end, we determined the levels of albumin, fibrinogen, ceruloplasmin and alpha-2-macroglobulin as markers of leakage in induced sputum before and after SP challenge in patients with asthma. Since challenges with methacholine or histamine are known to induce leakage (17), inhaled NKA was used as a control challenge, in order to compare the leakage markers in sputum between a NK1- and NK2-agonist at a given degree of bronchoconstriction.

Methods

Subjects

Twelve non-smoking atopic asthmatic volunteers (8 female, 20-26 years) participated in the study (Table 1). All subjects had a history of episodic chest tightness and wheezing and none was using medication except for short-acting ß$_2$-agonists as needed. Atopy was determined by a positive response to a standardized skin prick test with 10 common allergens (Vivodiagnost, ALK, Benelux). The baseline forced expiratory volume (FEV_1) was > 80% predicted (18) and all subjects were hyperresponsive to inhaled histamine (provocative concentration causing a 20% fall in FEV_1 (PC_{20}) <4 mg/ml) (19). All patients were clinically stable and had no history of recent allergen exposure or respiratory chest infection. The study was approved by the medical ethics committee of the Leiden University Medical Center and all volunteers gave a written informed consent.

Design

The study had a single blind randomized, cross-over design. At least one week before entering the study, inclusion and exclusion criteria were examined and PC_{20} histamine was determined. Inhaled SP or NKA challenge was performed on two randomized study days with an interval of at least one week. Sputum was induced 2 days before and 30 minutes following the SP or NKA challenge. In addition, a venous blood sample was obtained before each sputum induction.

Inhalation challenges

Histamine challenge was performed according to a standardized procedure (19) using the tidal breathing method. SP and NKA challenges were performed according to a previously validated protocol (16;20;21). SP (Sigma, St. Louis, USA) was inhaled in serial doubling concentrations (0.25 - 8 mg/ml) and NKA (Bachem, Budendorf, Switzerland)

Table 1. Characteristics of the subjects

Subject no	Sex (F/M)	Age (years)	FEV_1 (%pred)	PC_{20} his (mg/ml)
1	F	23	86.0	0.20
2	F	23	87.7	0.65
3	F	22	96.0	0.74
4	F	23	100.8	0.98
5	M	22	100.0	1.37
6	M	21	91.4	1.76
7	F	24	102.5	1.82
8	F	18	102.6	1.89
9	F	20	80.6	2.03
10	M	26	82.8	2.19
11	M	24	82.1	2.73
12	F	24	105.9	3.90
		22.5 (2.11)*	93.2 (9.13)*	1.35 (1.15)†

FEV_1 = forced expiratory volume in one second; PC_{20}his = provocative concentration of histamine causing a 20% fall in FEV_1; * mean (SD), † geometric mean (SD in doubling doses).

in concentrations between 8 and 1000 mg/ml. SP and NKA were aerosolized by a jet-nebulizer into a collapsible bag (Mallinckrodt, Petten, The Netherlands) (22). The aerosols were subsequently inhaled by tidal breathing for 3-4 min. at 7 min. intervals. The airway responses to SP and NKA were measured using FEV_1 (18), at 90 and 180 seconds after each dose (16). Additionally, systolic and diastolic blood pressure were monitored after each dose. Both challenges were discontinued when PC_{20} was reached (FEV_1 dropped by 20% from baseline).

Sputum induction and processing

Prior to induction, each subject inhaled 200 µg salbutamol. Sputum was induced by inhalation of NaCl 4.5% during 3x5 minutes intervals, according to a recommended protocol validated in our lab (23;24). Sputum samples were processed according to a validated method (24). An equal volume of 0.1% w/v dithiotreitol was added to the whole sample. Supernatant was aspirated following centrifugation and the total cell counts were determined. Differential cell counts were expressed as a percentage of 250 non-squamous cells (24).

Measurement of microvascular leakage

Fibrinogen was measured in coded sputum samples using a commercially available ELISA (Kordia, Leiden, The Netherlands) and albumin was determined by rate nephelometry (24). The concentrations of alpha-2-macroglobulin and ceruloplasmin in sputum and serum were measured by ELISA. Commercially available antibodies for alpha-2-macroglobulin (code A033) and ceruloplasmin (code A031) were obtained from DAKO (DAKO, Glostrup, Denmark) (25;26). To correct for protein concentrations in blood, sputum-to-serum ratios were determined as follows: sputum-protein (mg/l)/serum-protein (g/l) (26).

Analysis

The PC_{20} for histamine, SP and NKA were log transformed before statistical analysis and expressed as geometric mean (SD in doubling doses) The markers of microvascular leakage were expressed as median (range). Wilcoxon Signed Rank test was applied to test for differences before and after, and between challenges. Correlations were analysed using Spearman rank test. A p-value of < 0.05 was used as statistical significance and all analysis were performed using SPSS 10.0.

Results

Two subjects (nos. 7 and 12) did not produce sputum after the NKA challenge, whilst subject 8 was not able to produce sputum following SP. These time points were handled as missing data. Neither SP nor NKA had a significant effect on systolic or diastolic blood pressure (data not shown), though most subjects experienced transient flushing and warmth after 4 and 8 mg/ml SP. The geometric mean value (SD in doubling doses) for PC_{20} SP was 1.61 (1.62) mg/ml, whereas for PC_{20} NKA was it 130.83 (1.55) mg/ml. As a

consequence, the molar concentration to reach PC_{20} was 7.5 fold higher for SP than for NKA. There was a moderate correlation between the PC_{20} values for NKA and histamine (Rs = 0.59, p = 0.04), whereas neither of these correlated with PC_{20} SP (Rs < 0.19, p > 0.56). The maximal drop in FEV_1 following SP- and NKA challenge was similar (median (range) 25.4 (20.0-30.6) and 24.0 (20.0-28.4) %fall from baseline, respectively) (p = 0.31). There were no significant changes in the differential cell counts 30 minutes after the SP or NKA challenge (p > 0.09). Furthermore, total cell counts per gram sputum were not altered by the challenges (p > 0.11) (Table 2).

Markers of microvascular leakage

There were no significant differences in the baseline levels of all sputum markers between the SP- and the NKA challenge days (Table 3) (p > 0.44). The concentrations of alpha-2-macroglobulin, ceruloplasmin and albumin in sputum increased markedly 30 minutes following SP as compared to before the challenge (median fold change 3.1, 2.2 and 2.9, respectively) (p < 0.013) (Figure 1; Table 3). In contrast, there were no significant changes in these markers after NKA challenge (median) fold change 1.0, 1.0 and 1.1 (p > 0.31) (Figure 1). Neither SP nor NKA had a significant effect on the levels of fibrinogen in sputum (median fold change 1.4 and 1.5, respectively) (p > 0.29) (Figure 1d). At 30 min following challenge, the levels of alpha-2-macroglobulin were significantly higher after SP as compared to NKA (p = 0.028) (Figure 1a) (Table 3). The sputum-to-serum ratios for alpha-2-macroglobulin, ceruloplasmin and albumin increased significantly after the SP challenge (median prior to post challenge 1.22 to 2.94, 3.83 to 7.26 and 2.94 to 10.72, respectively) (p < 0.021), but not after the NKA (1.14 to 1.56, 2.77 to 5.21 and 2.94 to 5.34) (p > 0.33). The alpha-2-macroglobulin sputum-to-serum ratio following SP challenge was higher than after the NKA challenge (p = 0.05). The cumulative dose of inhaled SP was (median (range)) 1.88 (0.13-6.38) mg and for NKA 121.10 (27.35-996.10) mg. These cumulative doses were not correlated with the percentage increase in sputum leakage markers (p>0.12) or sputum-to-serum ratios (p>0.08). Furthermore, there was no relationship between the maximal % fall in FEV_1 following SP or NKA and the rise in leakage markers (p>0.10) or sputum-to-serum ratios (p>0.17).

Table 2. Differential cell counts in induced sputum

	Neurokinin A		Substance P	
	pre	post	pre	post
macrophages (%)	48.6 (9-82)	45.2 (24-77)	52.2 (34-77)	35.0 (14-82)
neutrophils (%)	36.1 (6-72)	38.9 (16-59)	27.8 (15-59)	41 (15-78)
eosinophils (%)	1.3 (0-18)	2.8 (0-30)	2.2 (0-21)	1.8 (0-32)
lymphocytes (%)	1.6 (0-6)	2.0 (0-8)	1.6 (0-10)	2.4 (0-13)
epithelial cells (%)	6.8 (1-45)	5.3 (1-18)	5.6 (3-35)	5.2 (2-25)

Differential cell counts are expressed as percentage of non-squamous cells. Values are presented as median (range)

Table 3. Markers of microvascular leakage in induced sputum

	Neurokinin A		Substance P	
	pre	post	pre	post
α2-macroglobulin	4.8	6.2	3.2	14.9*†
(µg/ml)	(0.4-25.5)	(0.4-41.1)	(0.4-22.1)	(0.4-139.9)
Ceruloplasmin	0.56	0.65	0.62	1.70*
(µg/ml)	(0.2-3.3)	(0.2-5.2)	(0.2-3.5)	(0.2-4.9)
Albumin	132.7	238.8	141.2	471.6*
(µg/ml)	(19.6-1889.6)	(25.8-1795.6)	(21.6-629.4)	(83.4-1834.0)
Fibrinogen	13.1	12.9	12.2	16.4
(µg/ml)	(1.0-28.7)	(1.6-44.1)	(1.9-41.4)	(3.6-44.2)

* p-value < 0.013 compared with pre-challenge; † p-value = 0.028 compared with post NKA-challenge. Data are expressed in median (range)

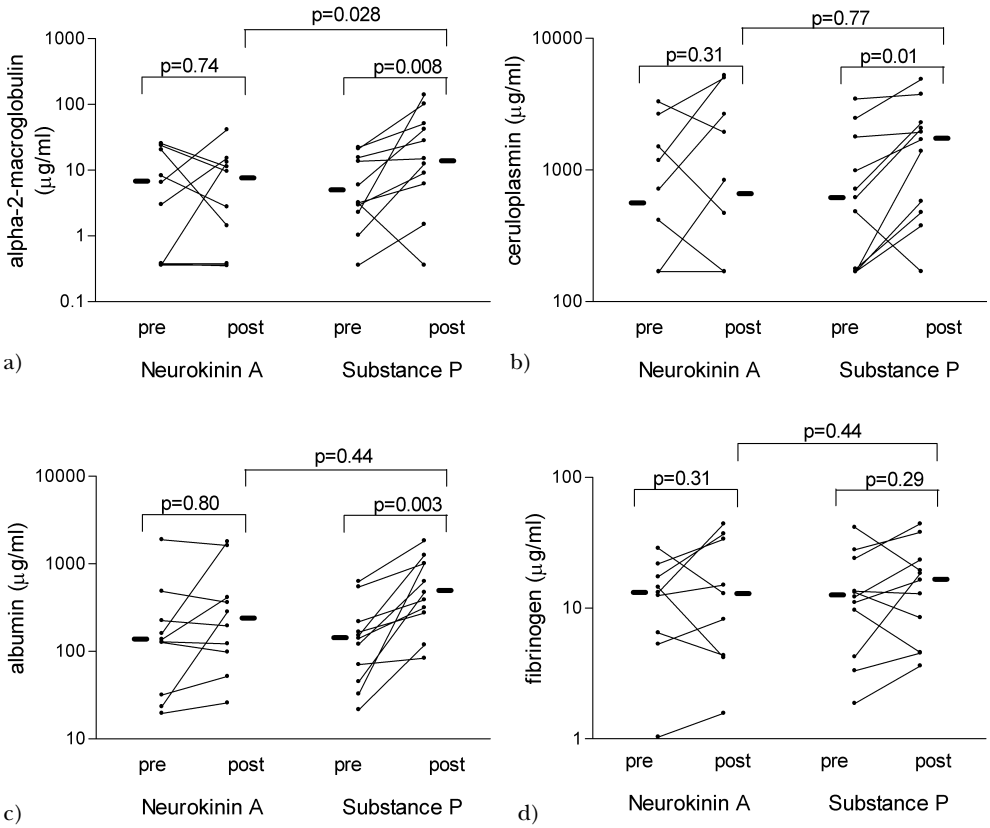

Figure 1. Markers of microvascular leakage in induced sputum pre and 30 min. post inhalation of neurokinn A or substance P.

a) alpha-2-macroglobulin in µg/ml; b) ceruloplasmin in µg/ml; c) albumin in µg/ml;

d) fibrinogen in µg/ml)

Discussion

This study has shown that inhaled substance P induces a rapid increase in the levels of alpha-2-macroglobulin, albumin and ceruloplasmin in induced sputum in patients with asthma. In contrast, inhaled neurokinin A was not able to induce such changes. These results indicate that NK1-receptor stimulation enhances microvascular leakage in the airways of patients with asthma *in vivo*, whereas NK2 stimulation does not. Furthermore, it becomes apparent that induced sputum can be a suitable method of monitoring microvascular leakage as a component of airways inflammation in asthma.
This is the first study examining the effects of SP on microvascular leakage in humans *in vivo*. Similar results have been found in previous animal studies. Lötvall *et al.* have demonstrated in guinea pigs, that microvascular leakage in the airways occurred after aerosolized SP (14), which appears to be inhibited by a selective NK1 receptor antagonist (27). In addition, the presently observed absence of leakage following NKA inhalation is also in agreement with results reported in guinea pig airways (28). Interestingly, our data demonstrate that induced exudation can be measured using induced sputum. This confirms and extends the findings by Halldorsdottir *et al.*, who have shown that inhaled histamine induces a comparable increase in the levels of alpha-2-macroglobulin in induced sputum (17).

It seems unlikely that the present findings can be explained by measurement errors, since the data were obtained by using validated methods of performing SP and NKA challenge (16;21), sputum induction and processing (24) and determination of markers of microvascular leakage (25). All subjects were non-smokers, and were having clinically stable asthma without using steroids. Furthermore, we have applied a cross-over, randomized design, using NKA as a control challenge. This allowed comparison of the leakage markers between distinct stimuli at a given degree of bronchoconstriction.

We have chosen NKA instead of histamine or methacholine challenge, because these mediators have previously been shown to induce an increase in alpha-2-macroglobulin (17). Moreover, NKA is a tachykinin and therefore member of the same family of neuropeptides as SP. For each of these agonists the receptors have been demonstrated within human airways (10), and by using these two tachykinins we aimed to distinguish preferential NK1- and NK2-stimulation within the airways (7;29). Though, only when using selective receptor antagonists, it would be possible proof the unique involvement of the NK1 receptor in our model (30). With selective antagonists, it has indeed been demonstrated that stimulation of the NK1-receptor induces microvascular leakage, whereas the NK2-receptor exerts its effects on bronchoconstriction in man and animals (31;32). We can not exclude that the differences between SP and NKA in inducing microvascular leakage are related to the 7.5-fold difference in molar concentrations between these compounds required to reach the PC_{20}. Therefore, we may have missed a potential possitive effect of NKA due to the lower concentration nebulized. However, the absence of relationships between the doses of SP or NKA and the increase of leakage

markers in sputum does not favour such explanation. In addition, similar differences between SP and NKA in potency have been found in animal models (33).

We had chosen to start sputum induction at 30 minutes after the challenges, based on the study of Halldorsdottir *et al.* (17), who demonstrated an increase in alpha-2-macroglobulin in sputum 45 minutes following a histamine challenge. It can be argued that the extravasation of plasma occurs earlier, based on the rapid effect of mediators on microvascular permeability (5). This would imply that the maximal increase in these markers, is even more pronounced than we observed. The time-course of leakage may also explain why we did not observe a significant SP-induced increase in the levels of sputum fibrinogen. It can not be excluded that the leakage of fibrinogen occurred at an earlier time point and had dissipated by the time of the sputum induction. Furthermore, after escaping the vasculature, fibrinogen could polymerize into fibrin and thereby cannot gain access to the airway. A recent study of Peebles *et al.*, also failed to show plasma extravasation, as demonstrated by fibrinogen in BAL, 24 hours following an allergen challenge in patients with asthma (34).

The SP-induced increase in permeability of the airways appears to be associated with the formation of small interendothelial pores. Subsequently, the extravasating bulk plasma is moving through these pores into the airway lumen, which is likely to be driven by an increase in hydrostatic pressure (35). Hirata *et al.* have demonstrated that injection of SP causes the formation of intercellular gaps between endothelial cells in postcapillary and collecting venules of rat trachea (36). Microvascular leakage *in vitro* can be reduced using cAMP elevating drugs e.g. PDE inhibitors and formoterol by inhibiting endothelial gap formation (37;38). In healthy subjects, such anti-exudative effect of formoterol could indeed be confirmed in sputum, indicating that this mechanism plays a role in humans *in vivo* (4).

What are the clinical implications of our findings? The SP-induced increase in microvascular leakage in our study suggests that the phenomenon of neurogenic inflammation can be relevant in asthmatics *in vivo*. Indeed, elevated levels of SP have been found in BAL and sputum of patients with asthma (8;9). In particular, during unstable episodes of asthma, NK1-activity seems to have functional significance, for instance, during cold air-, virus- (39) or allergen- (40) induced plasma extravasation. Taken together, this implicates that NK1-receptor antagonists may reduce microvascular leakage in asthma and thereby could have beneficial effects in the treatment of asthma. Using the current investigational model of “dual induction”, that is induced exudation and induced sputum, it will be possible to demonstrate microvascular leakage in humans *in vivo*. Therefore, this method can be applied when testing the anti-exudative effect of newly developed drugs, such as NK1-antagonists.

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Chapter 4

Chapter 5

Bronchial CD8 cell infiltrate and lung function decline in asthma

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Abstract

Background Patients with asthma have an accelerated decline in lung function, which can lead to irreversible airway obstruction. It is generally assumed that this is related to specific aspects of airway inflammation and/or remodelling. We investigated the prognostic significance of bronchial eosinophil and CD8+ cell counts and subepithelial reticular layer thickness for the subsequent decline in lung function in patients with asthma after 7½ years of follow-up.

Methods In a prospective study, pre- and post-bronchodilator lung function (FEV_1) was measured at baseline, after 2 years and 7½ years in 32 patients with asthma. Annual decline in lung function after 7½ years of follow-up was related to type and severity of airway inflammation and remodelling in bronchial biopsies, which were taken at baseline and at year 2.

Results Annual decline in post-bronchodilator FEV_1 (mean (SD) 46.6 (53.4) ml/yr) was significantly larger than the decline in pre-bronchodilator FEV_1 (mean (SD) 27.5 (62.5) ml/yr), indicating loss in reversibility. Whereas, annual fall in post-bronchodilator FEV_1 was not related to thickness of the reticular layer or to eosinophil counts in bronchial biopsies, there was a significant correlation with CD8 positive T-cells ($r=-0.39;p=0.032$). Analyzing the biopsies taken at year 2, the significant association between annual fall in post-bronchodilator FEV_1 and CD8 cells could independently be confirmed ($r=-0.39;p=0.036$).

Conclusion The outcome of asthma, as determined by the annual decline in FEV_1, can be predicted by the bronchial CD8+ cell infiltrate. This suggests that the inflammatory phenotype in asthma has prognostic relevance, which may require phenotype-specific therapeutic strategies.

Introduction

Asthma is a chronic inflammatory disease that is characterized by variable airway obstruction to various inhaled stimuli (1). Although this is largely reversible in most patients, some asthmatics develop persistent non-reversible airway obstruction despite adequate treatment (2). Longitudinal studies have shown that adult patients with asthma have an accelerated decline in lung function (FEV_1) as compared to controls (3-5). However, the rate of decline demonstrates large variability between patients, which seems to be associated with disease duration, baseline lung function and airway responsiveness (6;7). Eventually, the lung function decline may progress to irreversible airway obstruction in a subgroup of patients with asthma (8).

The current working hypothesis is that chronic inflammation promotes restructuring of the airways, which in turn results in accelerated decline in lung function in some but not all asthmatics. Airway inflammation in asthma is characterized by infiltration of lymphocytes and eosinophils in the bronchial epithelium and lamina propria (9) and of mast cells in the smooth muscle layer (10). Due to the release of growth factors and other mediators, the infiltrate is thought to induce structural changes in the bronchial wall often referred to as tissue remodelling (11). Since this process begins early in the development of asthma, remodelling may occur in parallel or could even be required for the development of persistent inflammation (12). The features of airway remodelling in asthma include thickening of the sub-epithelial reticular layer, changes of the interstitial matrix composition, increases in blood vessel area, airway smooth muscle, goblet cells in the surface epithelium and number of mucous glands (11).

The prognostic significance of airway inflammation and remodelling for the decline in lung function is still unclear. In patients with chronic obstructive pulmonary disease (COPD) fixed airway obstruction is often found to be associated with bronchial CD8+ T cell infiltration (13;14). Cross-sectional studies in severe asthma have demonstrated that sputum and tissue eosinophil counts are associated with a lower lung function (8;15). Furthermore, in some (16;17), but not all studies (15), the thickness of the sub-epithelial reticular layer was inversely associated with the level of lung function in asthma. However, it remains questionable whether these cross-sectional associations hold after longitudinal follow-up.

We postulated that the type and severity of inflammation or remodelling in bronchial biopsies are predictive of the subsequent annual decline in lung function in patients with asthma. For this reason, we performed a prospective follow-up study in a previously reported group of patients with asthma (AMPUL-cohort) (18) who underwent repeated bronchoscopies and extensive clinical measurements at baseline. We aimed to investigate the relationship of bronchial eosinophil and CD8+ cell counts and the thickness of the sub-epithelial layer as measured at baseline with the subsequent annual decline in lung function after 7½ years of follow-up.

Methods

Subjects

75 Atopic patients with mild-moderate persistent asthma participated in the study (18). 45 Patients underwent a successful bronchoscopy at entry and 37 patients at t=2 years. At inclusion, all patients (18-50yr) were non- or ex-smokers (< 5 pack-years), all had symptoms of episodic chest tightness and wheezing, whilst 77% of patients was using regular inhaled steroids. Pre-bronchodilator forced expiratory volume in one second (FEV_1) was >50% of predicted and >1.5L, whilst post-bronchodilator was within the normal range (>80% predicted) (20). All patients were hyperresponsive to methacholine (provocative concentration causing 20% fall in FEV_1 (PC_{20}) <8mg/ml). The medical ethics committee of the Leiden University Medical Center approved the study and all participants gave written informed consent.

Design

In a prospective study design, pre- and post-bronchodilator FEV_1 and PC_{20} were measured at baseline, at years 2 and 7½. Bronchoscopies were performed at baseline and year 2. During the first two years patients were treated according to standardized guidelines, and treatment was adjusted by a chest physician every 3 months (18). In order to make this study representative for daily practice, the own physician of each patient was instructed to adjust treatment according to Dutch GINA-derived guidelines between 2 and 7½ years of follow-up.

Spirometry and airway responsiveness

Spirometry was performed according to the same procedures throughout the study (18). Patients withheld short-acting $ß_2$-agonists for 8 hours and long-acting $ß_2$-agonists for at least 24 hours prior to the measurements. Post-bronchodilator FEV_1 was measured 15 minutes following inhalation of 400 µg salbutamol (20). Airway hyperresponsiveness was determined using a methacholine challenge and was expressed as PC_{20}.

Bronchoscopy and immunohistochemistry

At baseline and after 2 years five bronchial biopsies were taken for electron and light microscopy from right lower lobe subsegments, the middle lobe and the main carina using a pair of cup forceps (Olympus FB-21C, Japan). Two biopsies were fixed immediately in Trump's fixative and ultra thin sections were processed for electron microscopy. The thickness of the sub-epithelial reticular basement membrane was determined by measuring area divided by length on electron microscopy pictures in 2-5 well oriented electron micrographs (X5700, 35x42mm), using computerized analysis(18). Three biopsies were immediately embedded in ornithyl carbamyltransferase medium and snap-frozen in isopentane. Immunohistochemistry was performed on 6mm cryostat sections. Sections were stained with monoclonal antibodies against EG2 (eosinophils) (Pharmacia, Sweden), and CD8+ cells (Becton Dickinson, USA).A validated method using computerized analysis was applied to examine the coded biopsy specimens(21). Two areas were selected and the number of positively stained cells was

determined in the lamina propria. Values were expressed as cells/0.1 mm^2. Detailed biopsy methods and cell number data, including AA1 (mast cells), CD3 and CD4+ cells, have been previously published(18).

Analysis

Post-bronchodilator FEV_1 was applied in the analysis in order to minimize the contribution of varying degrees of smooth muscle contraction to the level of airway obstruction. The decline in post-bronchodilator FEV_1 was determined between baseline and t=7½ years (FEV_1 at 7½ years – FEV_1 at baseline / 7½) and between t=2 and t=7½ years (FEV_1 at 7½ years – FEV_1 at 2 years / 5½) and was expressed as annual decline in ml/years. The declines in pre- and post-bronchodilator FEV_1 were compared using a paired t-test. Linear regression analysis was used to investigate the association between inflammation (EG2 and CD8 positive cells and reticular layer thickness) in bronchial biopsies and annual decline in post-bronchodilator FEV_1 during follow-up.

Results

Patient characteristics

Thirty-two of the 45 patients who underwent the bronchoscopy at baseline, participated at follow-up after 7½ years (71% response rate) [table 1]. The participating patients were not different from the non-participants with respect to disease severity, spirometry, reticular layer thickness, EG2 and CD8+ cells ($p>0.2$). In 30 of these 32 patients biopsies were also taken at year 2. The total follow-up period was 7.6 (0.6) (mean (SD)) years. At all three time points, about 70% of the patients were using inhaled steroids [table 2]. None of the patients were using long-acting $ß_2$-agonists at t=0 and t=2, compared to six patients at t=7½ years. Seven patients stayed under regular control of a chest physician, whereas 23 patients were treated by a general practitioner. Only 2 patients stopped using any asthma medication and were free of symptoms. During the follow-up period, 1 in 5 received treatment with one or more courses of oral corticosteroids. Two patients had become current smokers after 7½ years, whereas none smoked during the first two years. PC_{20} methacholine was <8 mg/ml in all patients at baseline, and in 28 of the 32 patients at t=7½ (range 0.02 to 16.3 mg/ml) [table 2].

Lung function decline

The mean pre- and post-bronchodilator FEV_1 in % predicted stayed within the normal range at all visits with considerable scatter [table 2]. The annual decline in pre-bronchodilator FEV_1 during follow-up was (mean (SD)) 27.5 (62.5) ml/yr, whereas the annual drop in post-bronchodilator FEV_1 was 46.6 (53.4) ml/yr [figure 1]. The variability in decline in post-bronchodilator FEV_1 between individual patients was large, ranging from an annual increase by 39 ml/yr to an annual fall by 149 ml/yr. The decline in post-bronchodilator FEV_1 was significantly larger than in pre-bronchodilator FEV_1 ($p=0.022$), indicating loss in reversibility [figure 1].

Table 1. Patient recruitment: flow chart

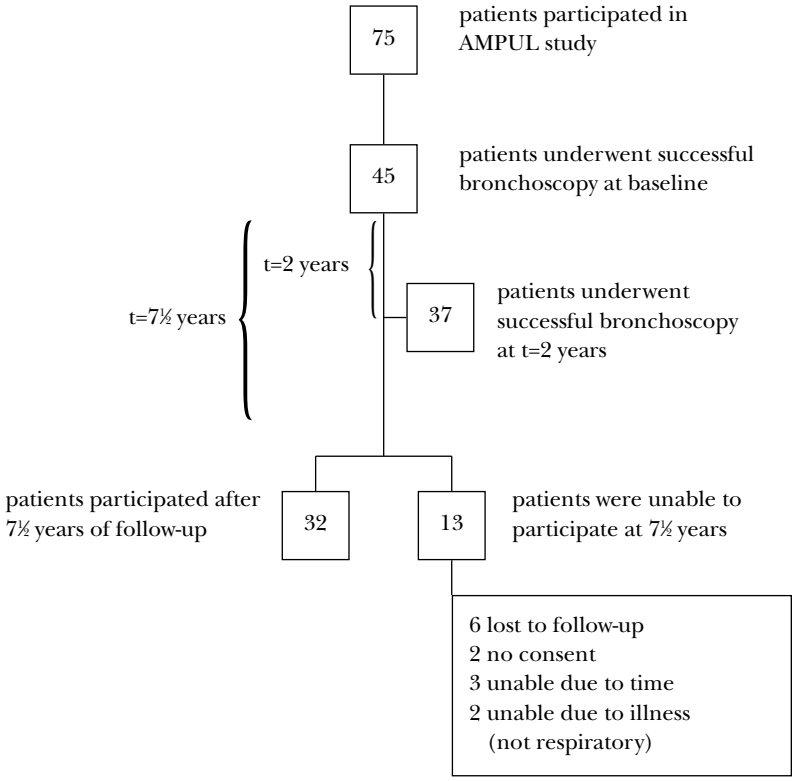

Table 2. Patient characteristics

	baseline	t=2 years	t=7½ years
age (years)	30.8 (8.9)		
follow-up (years)		2.0 (0.0)	7.6 (0.6)
inhaled steroids (% patients)	72%	75%	69%
pre-bronchodilator FEV_1 (% pred.)	87.2 (13.4)	86.3 (14.0)	84.7 (16.9)
post-bronchodilator FEV_1 (% pred.)	99.3 (11.0)	96.7 (12.6)	93.2 (15.8)
PC_{20} methacholine (mg/ml)*	0.67 (2.2)	0.88 (1.73)	0.91 (2.8)

Data in mean (SD); *geometric mean (SD in DD)

Prognostic significance of airway inflammation

The annual decline in post-bronchodilator FEV_1 during the follow-up period was not related to thickness of the bronchial subepithelial reticular layer at t=0 (r=-0.02; p=0.92) [figure 2]. In addition, the fall in post-bronchodilator FEV_1 during follow-up showed no correlation with eosinophils at baseline (r=0.02;p=0.90). On the other hand, the annual change in post-bronchodilator FEV_1 during the follow-up period of 7½ years was significantly and inversely correlated with the bronchial CD8+ cells at t=0 (r=-0.39;p=0.032). The slope of the linear regression analysis showed that for each doubling in CD8+ cells, post-bronchodilator FEV_1 declined with an additional 13.8 ml/yr. When

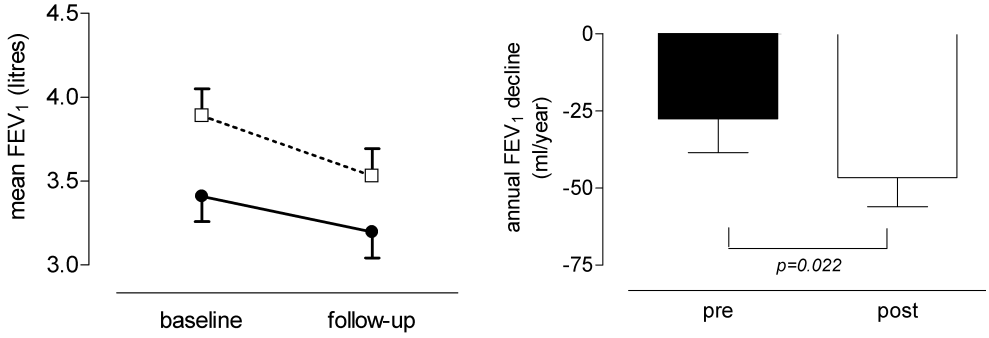

Figure 1. Left panel: mean pre-bronchodilator (closed circles) and post-bronchodilator (open squares) FEV_1 at baseline (t=0) and at follow-up (t=7½ years). Right panel: annual decline in FEV_1 from baseline to follow-up for pre-bronchodilator (black bar) and post-bronchodilator (white bar).

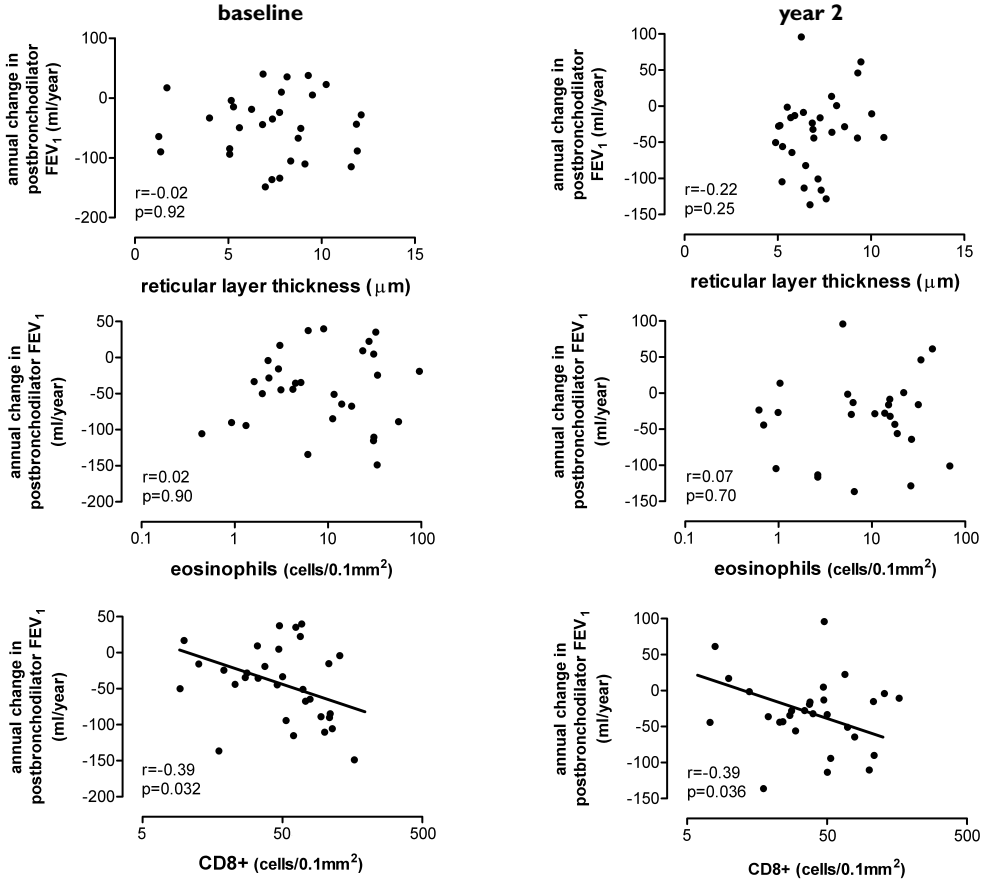

Figure 2. Left panel: associations of annual change (year 0 to 7½) in post-bronchodilator FEV_1 with reticular layer thickness, eosinophils and CD8+ at baseline. Right panel: associations of annual change (year 2 to 7½) in post-bronchodilator FEV_1 with reticular layer thickness, eosinophils and CD8+ at t=2 years.

repeating the analysis using the bronchial biopsies taken at 2 years these findings were entirely confirmed. There was a consistent, significant correlation of annual fall in post-bronchodilator FEV_1 with the number of CD8+ cells at t=2 years (r=-0.39;p=0.036), but not with bronchial eosinophils or reticular layer thickness [figure 2]. All other cell types (AA1, CD3 and CD4) demonstrated no significant associations with the annual change in FEV_1 (r<-0.20;p>0.28).

Discussion

The results of this study show that the number of CD8+ cells in bronchial biopsies in patients with asthma is associated with disease outcome, as determined by loss of lung function. Other markers of inflammation or remodeling were not related to the decline in lung function during follow-up. Furthermore, the loss in post-bronchodilator FEV_1 was significantly larger than the decline in pre-bronchodilator FEV_1. These findings indicate that CD8 cells can be predictive of disease outcome in asthma and therefore suggest that targeting specific elements of inflammation may be required when aiming to prevent the accelerated decline in lung function in patients with asthma.To our knowledge, this is the first longitudinal study showing the prognostic significance of type and severity of inflammation on the outcome of asthma. A cross-sectional relationship between CD8+ cells and the outcome of asthma has been observed in patients with fatal asthma (22). Recently, increased cytokine production of sputum CD8+ cells has been shown in patients with asthma that was related with disease severity (23). Interestingly, the association between lung function and CD8 cells has also been demonstrated in other diseases: not only by cross-sectional analysis in COPD patients (13), but also regarding decline in lung function in patients with systemic sclerosis (24). This shows that our longitudinal findings in asthma are in line with those in other inflammatory lung disorders.

The magnitude of the annual decline in lung function is in keeping with other longitudinal studies in patients with asthma and is higher as compared to the figures previously published for normals (normals: 22 ml/year and asthma: 38 ml/year) (5). Our results extent previous findings by demonstrating that the decline in post-bronchodilator FEV_1 is larger than the decline in pre-bronchodilator FEV_1. This puts emphasis on measuring post-bronchodilator FEV_1, as a ceiling of lung function, in prospective studies in asthma.

The present study design may have potential limitations. During the follow-up period of 7½ years, the patients were treated by their own physician as opposed to controlled standardized therapy. This may have introduced variability in asthma control, since some patients were seeing a chest physician regularly (22% of patients), whereas others had not been visiting their doctor for asthma symptoms at all (6% of patients). We consider this strategy to be representative for the daily practice. Prior to the baseline bronchoscopy the patients were also treated by their own physician, or were newly

diagnosed (23% of patients). Moreover, any variability in therapy may have led to a broader disease outcome, which is likely to be represented by the large range in annual decline in FEV_1. For that reason, we chose common asthma management as opposed to protocolized therapy during follow-up of this cohort.

It is unlikely that the present association between decline in FEV_1 and CD8+ cells, is due to chance. It was a consistent finding when using bronchial biopsies of two separate bronchoscopies two years apart. The first and second bronchoscopy differed in such way that the second biopsy was taken following 2 years optimal treatment according to the GINA guideline or management additionally based on airway hyperresponsiveness (18). This suggests that treatment level is not affecting the association between airway inflammation and lung function decline in asthma.

How can CD8 cells contribute to the accelerated, irreversible airway obstruction in asthma? In vitro studies have characterized CD8+ cells in regard to their cytokine production (Tc1 vs Tc2) and populations (effector vs memory) (25). Interestingly, a subset of antigen specific "non-lymphoid" memory CD8 T cell population, which can be isolated from several organs including the lungs, demonstrate a high lyctic activity and proliferate rapidly (26). Various antigens, like allergens and viruses can rapidly activate specific effector/memory T cells (27). In mice models, CD8 cells are required for the development of airway hyperresponsiveness following allergic sensitization (28) leading to increased inflammation (29). During respiratory virus infections, CD8 cells appear to be essential for the influx of eosinophils into the lung and the development of airway hyperresponsiveness in mouse models (30). Indeed, we have recently demonstrated that rhinovirus infection in asthmatic subjects is associated with accumulation of CD8 cells (31). Interestingly, antigen specific CD8 cells can persist in the lung for several months (32) and may also activate resident cells such as epithelial cells (33). Therefore, CD8 cells can induce potential conditions that are required for changes in airway structure, which eventually may lead to changes in airway structure. However, we cannot exclude the possibility that the association of CD8 cells with lung function decline is just an epiphenomenon and a marker of a complex immunopathologic pathway.

Eosinophils were not predictive for the decline in lung function in our study. Increased numbers of sputum and tissue eosinophils have been associated with persistent airway obstruction in patients with severe asthma (8;15). However, these conclusions were derived from cross-sectional data. Interestingly, it has been shown that elevated sputum eosinophil numbers may predict the short-term worsening of asthma as reflected by exacerbations (34). This suggests that the inflammatory profile may have distinct effects on short- and long-term disease outcome.

Remarkably, the thickness of the sub-epithelial reticular layer was not related to lung function decline either. This probably illustrates that restructuring of the airways as measured in large airway biopsies is not sufficient to represent other aspects of (small) airways remodeling (35). When sampling the latter in patients with COPD, Hogg et al.

recently did show an association between airway structure and lung function level (36). However, comparable data in asthma will not be readily available.

Our findings can have implications for clinical management and drug development. First, the consistent association between FEV_1 decline and CD8 cells even after 2 years of optimal standardized therapy in our study suggests that the current treatment strategies for asthma may not be effective in preventing or reversing the accelerated fall in lung function in patients with asthma. Second, even though the CD8 cell may just be a marker of another causative mechanism, the possibility to manipulate the presence and/or phenotype of CD8 cells should be considered. Glucocorticoids are able to induce a CD8 cell phenotype that is producing high levels of IL-10 and reduced levels of IL-4 and IL-5 (37). However, the effect of glucocorticoids modulation of CD8 cell cytokine production is much smaller as compared to CD4 cells (37). Therefore, the development of new interventions, specifically targeting CD8+ T cells, may need to be explored when aiming to prevent the persistent airway obstruction in asthma.

In conclusion, we have shown that outcome of asthma, as determined by the annual decline in FEV_1, can be predicted by bronchial CD8 cell infiltrate. CD8+ cells may have, as previously suggested in patients with COPD, a significant role in the clinical course of asthma. We could speculate that this requires phenotype-specific therapeutic strategies in order to prevent the accelerated decline of lung function in asthma.

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Chapter 5

Chapter 6

Asthma guidelines: towards evidence-based application of peak flow

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Abstract

Background: In the current GINA guidelines, the assessment of asthma control is based on symptoms and lung function. We investigated the value of including PEF-variability in addition to symptoms and $ß_2$-agonist use, in predicting the development of poor asthma control in a prospective study.
Methods: 75 patients with asthma had GINA step determined and treatment adjusted at baseline and after 3 months of follow-up. Data were analysed to ascertain the extent to which each clinical feature was involved as decisive factors in determining the GINA grade. Logistic regression analysis was applied to determine the value of PEF-variability in predicting asthma control at the second visit in the whole group and in a sub-group of patients with GINA grade 1 and 2 at entry. The optimal cut-off value for loss of control was determined by the receiver operating characteristic (ROC) curve.
Results: A PEF-variability of ≥20% determined in 0% of cases the GINA step at baseline. However, as a continuous variable, PEF-variability provided additional information on top of symptoms and $ß_2$-agonist use in predicting loss of control (total group: OR=1.14; p=0.003 and sub-group: OR=1.37; p=0.012). Patients in GINA step 1-2 with PEF-variability >10% at visit one had a RR of 7.7 (p=0.034) for an increase towards GINA step 3-4 at follow-up as compared to patients with PEF-variability ≤10%.
Conclusions: PEF-variability provides useful information in addition to symptoms and $ß_2$-agonist use and may therefore be valuable to adjust therapy in order to prevent loss of asthma control.

Introduction

The current Global Initiative for Asthma (GINA) guidelines for the treatment of asthma recommend the assessment of symptoms and lung function for successful management of asthma (1). Since their first publication in 1993, the GINA guidelines have taken a stepwise standardized approach to asthma treatment, with the treatment level based on four grades or steps which are determined by clinical features such as daytime and night-time symptoms and PEF-variability (Figure 1A). The presence of one of the features of a GINA step is sufficient to place a patient in that category; thus, the GINA step is determined by the worst among the patient's clinical features. The cut-off values between the GINA steps are not evidence-based, but are derived from presumed clinical significance as assessed by an expert panel through consensus (1). In the 2002 GINA guidelines, a separate table was added for ongoing assessment of control *during* treatment. This table (Figure 1B) utilised a similar categorisation of symptoms and lung function, in combination with the "Treatment Step" of daily medication. The clinical

Figure 1A

Figure 5-6. Classification of Asthma Severity by Clinical Features Before Treatment

STEP 1: Intermittent
Symptoms less than once a week Brief exacerbations Nocturnal symptoms not more than twice a month ● FEV1 or PEF ≥80% predicted ● PEF or FEV1 variability <20%
STEP 2: Mild Persistent
Symptoms more than once a week but less than once a day Exacerbations may affect activity and sleep Nocturnal symptoms more than twice a month ● FEV1 of PEF ≥80% predicted ● PEF or FEV1 variability 20-30%
STEP 3: Moderate Persistent
Symptoms daily Exacerbations may affect activity and sleep Nocturnal symptoms more than once a week Daily use of inhaled short-acting ß2-agonist ● FEV1 or PEF 60-80% predicted ● PEF or FEV1 variability >30%
STEP 4: Severe Persistent
Symptoms daily Frequent exacerbations Frequent nocturnal asthma symptoms Limitation of physical activities ● FEV1 or PEF ≤60% predicted ● PEF or FEV1 variability >30%

Figure 1B

Figure 5-7. Classification of Asthma Severity by Daily Medication Regimen and Response to Treatment

	Current Treatment Step		
	Step 1: Intermittent	Step 2: Mild Persistent	Step 3: Moderate Persistent
Patients Symptoms and Lung Function on Current Therapy	Level of Severity		
Step 1: Intermittent			
Symptoms less than once a week		Mild	Moderate
Brief exacerbations	Intermittent		
Nocturnal symptoms not more than twice a month		Persistent	Persistent
Normal lung function between episodes			
Step 2: Mild Persistent			
Symptoms more than once a week but less than once a day	Mild	Moderate	Severe
Nocturnal symptoms more than twice a month but less than once a week	Persistent	Persistent	Persistent
Normal lug function between episodes			
Step 3: Moderate Persistent			
Exacerbations may affect activity and sleep			
Nocturnal symptoms at least once a week	Moderate	Several	Severe
60% < FEV1 < 80% predicted OR	Persistent	Persistent	Persistent
60% < PEF < 80% personal best			
Symptoms daily			
Step 4: Several Persistent			
Symptoms daily			
Frequent exacerbations	Severe	Severe	Severe
Frequent nocturnal asthma symptoms	Persistent	Persistent	Persistent
FEV1 ≤60% predicted OR			
PEF ≤60% of personal best			

Figure 1. Classification of asthma severity in GINA guidelines (A) before treatment and (B) during treatment (1).

features were now considered to reflect the level of control which had been achieved with treatment (1;2). It can be seen from this approach that the underlying asthma severity is perceived as driving the appropriate maintenance treatment in order to achieve control of asthma. Indeed, a prospective evaluation showed that acute health care utilisation was predicted by an index of asthma control (3).

There is some controversy over whether the assessment of asthma control should include PEF measurements, at least partly because of difficulty in achieving good adherence with monitoring. In clinical practice guidelines, the rationale for including both symptoms and lung function in the classification of asthma control in individual patients has been

that both symptoms and airway obstruction are integral to the definition of asthma, and there is a wide variation in the way individual patients manifest inadequacy of asthma control (4). Monitoring of peak expiratory flow (PEF) has been recommended to overcome poor perception or under-reporting of symptoms by patients. Furthermore, it has been found that successful control of both symptoms and peak expiratory flow (PEF) leads to improvements in quality of life which therefore benefits the patient (5).
Peak flow measurements may be summarised in several different ways, including the PEF-level (clinic PEF measured by the clinician, or, alternatively, mean daily PEF, expressed as a percentage of predicted or of personal best) and PEF-variability (amplitude as percentage of mean value). In the 2002 GINA guidelines, there is an unexplained distinction between the initial assessment, when both PEF-variability and PEF-level are used (Figure 1A), and the ongoing assessment during treatment, when PEF-level alone is used (Figure 1B) (1).
To date, there are few data on the relative contribution of symptoms and PEF to the classification of asthma control and the resulting maintenance treatment according to GINA guidelines in clinical practice. We assessed the extent to which different levels of PEF-variability provide information on asthma control in addition to that provided by symptoms and $ß_2$-agonist use. Furthermore, we evaluated the value of including PEF-variability in addition to symptoms and $ß_2$-agonist use in predicting the development of poor asthma control in a prospective study.

Methods

Subjects

Seventy-five atopic patients with mild to moderate persistent asthma, who participated in the 2-years prospective AMPUL (Asthma Management Project University Leiden) study, were included in the analysis (6). All patients had a history of episodic chest tightness or wheezing and prior to entry were treated with or without inhaled steroids. They were 18-50 years and non- or ex-smokers (>1 yr; <5 packyrs). The pre-bronchodilator forced expiratory volume in one second (FEV_1) was more than 50% of predicted, whilst post-bronchodilator FEV_1 was within the normal range (>80% predicted). All were hyperresponsive to methacholine (PC_{20} < 8 mg/ml) (6).
The medical ethics committee of the Leiden University Medical Center approved the study and all participants gave written informed consent.

Design

In the prospective study, treatment was adjusted every 3 months for 2 years in a referral hospital according to the GINA guidelines. Full details of the methodology have previously been published (6). For the present analysis, the first 2 visits (i.e. baseline visit, at which the first treatment adjustment was made, and the first follow-up visit, 3 months later) were used.
Before each visit, patients kept a diary card for 2 weeks on which day- and night-time symptoms, use of ß2-agonists and AM and PM PEF were recorded. Symptoms were

recorded twice daily on a 0-4 scale (6), and the scores were converted to a categorical scale equivalent to that in the GINA guidelines. AM and PM peak flows were used to calculate PEF-variability (amplitude%mean) (1), using the PEF cutpoints specified in the GINA guidelines (7). As recommended in the guidelines, the worst feature was used to determine the GINA step and hence the level of treatment for the first 3 months. Twenty-three patients had newly detected asthma and were classified according to Figure 1A of the guidelines, whilst the other 52 patients were on regular inhaled steroids and were therefore classified according to Figure 1B.

Analysis

We analysed data from the first visit to ascertain the extent to which each clinical feature was involved as (one of) the decisive factors in determining the GINA grade. The clinical features which are accessible to a general practitioner (day and night-time symptoms, bronchodilator use and PEF variability) were included in the analysis. Second, logistic regression analysis was applied to determine the value of PEF-variability as a continuous variable, in addition to symptoms, in predicting asthma control at the second visit (dependent variable: GINA grade 1 and 2 *versus* GINA grade 3 and 4 at visit two; independent variables: GINA grade and PEF variability at visit one). A sub-group analysis was performed for the patients in GINA grade 1 and 2 at visit one. Only these patients might be classified in a higher GINA grade based on their PEF-variability, whereas for the patients in GINA grade 3 and 4, adding PEF variability could not change their classification, since symptoms and or bronchodilator use had already placed them in a higher GINA grade. Next, based on a receiver operating characteristic (ROC) curve, an optimal cut-off value for PEF variability was selected for prediction of loss of control (GINA step 1 and 2 versus GINA step 3 and 4) at the second visit. Finally, logistic regression analysis was carried out to determine the risk of patients in GINA grade 1 and 2 with a PEF-variability higher than the selected cut-off value to be classified in GINA grade 3 or 4 at the second visit.
P-values of less than 0.05 were considered as significant and STATA was used to analyse the data.

Results

Contribution of clinical features to GINA grade

At Visit 1, the distribution of patients among the GINA steps was Step 1 17%, Step 2 28%, Step 3 35% and Step 4 20%. The GINA grade was determined by one single factor in 82.1% of the patients. Figure 2 shows the percentage of patients in whom a particular clinical feature was involved as (one of) the determining feature(s) to allocate an individual patient to GINA step 2 or higher. Symptom was by far the most dominant feature in determining the GINA level and thereby the level of asthma treatment (figure 2, top panel). For no patient (0%) was the level of control determined by PEF-variability, using the cut-off value of 20% which is specified in the GINA guidelines.

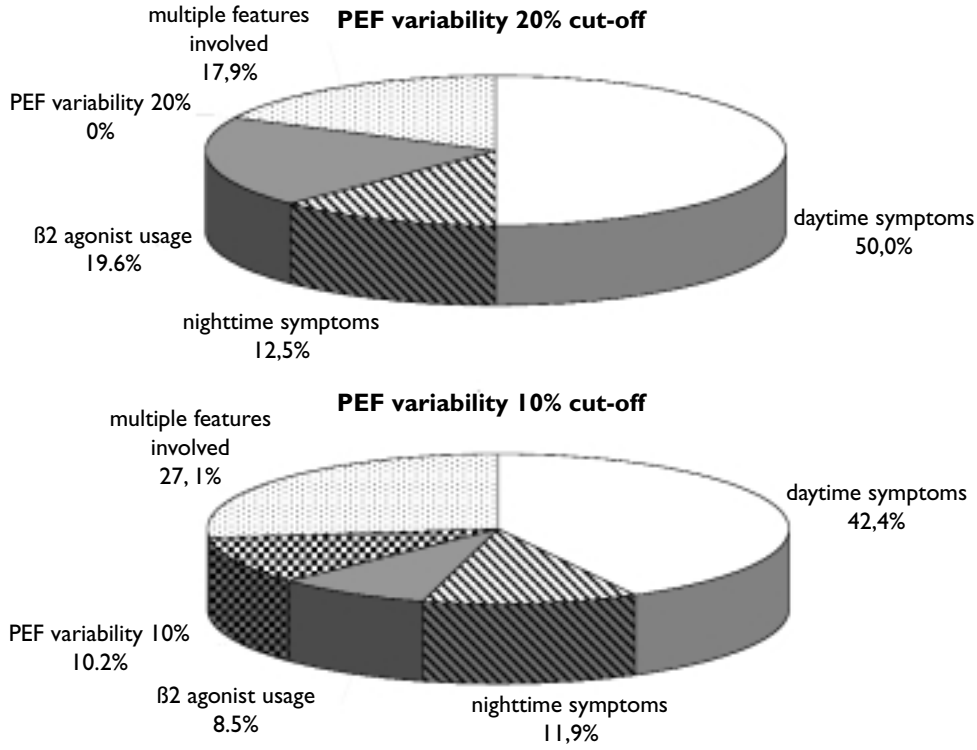

Figure 2. Percentage of visits on which a particular clinical feature was involved as (one of) the driving features to allocate a patient in GINA severity grade 2 or higher. Two different cut-off value for PEF-variability were used in the analysis: when using PEF-variability < 20% (top panel) and when using PEF-variability < 10% (bottom panel).

Prognostic value of PEF-variability

Logistic regression analysis showed that at the first visit, PEF-variability significantly provided additive information on top of symptoms in predicting the level of asthma control (GINA grade 1 and 2 *versus* GINA grade 3 and 4) at the second visit (RR=1.14; p=0.003).

Sub-group analysis

A logistic regression, analysing the additional value of PEF-variability in determining asthma control, was repeated in the sub-group of 40 patients categorised in grade 1 and 2 at the first visit. In this sub-group, again PEF-variability on top of symptoms significantly predicted asthma control at the second visit (RR=1.37; p=0.012).
The area under the curve (AUC) of the ROC curve for using PEF-variability on top of the current guidelines for predicting asthma control at the second visit was 0.75. From this ROC curve (Figure 3) a cut-off value of PEF-variability >10% was selected based on the high specificity and likelihood ratio.

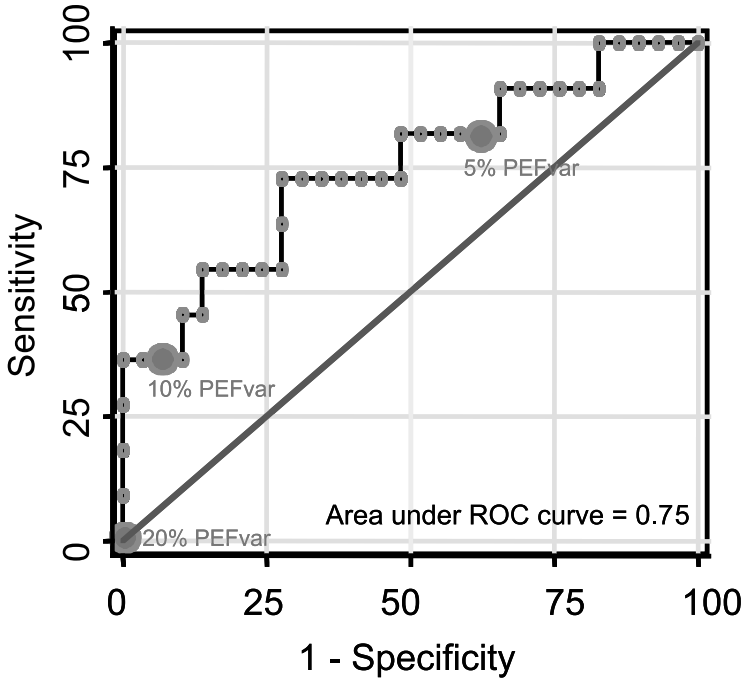

Figure 3. ROC curve for using PEF-variability on top of the current guidelines for predicting asthma severity (GINA grade 1 and 2 versus GINA grade 3 and 4) at the second visit. The area under the curve was 0.75.

Applying cut-off value of PEF-variability >10%

The cut-off value of PEF variability 10% gave a sensitivity of 36%, a specificity of 93% and a likelihood ratio (LR) for a positive result of 5.27.

Logistic regression analysis showed that patients in GINA control step 1 or 2 with PEF variability >10% at baseline had an OR of 7.71 (p=0.034) for an increase in GINA level at the second visit as compared to patients with PEF variability ≤10%.

A retrospective re-classification of GINA categorisation at baseline was performed using PEF-variability with the proposed cut-off value of >10%, instead of the existing cutpoint of ≥20%. With this cutpoint, PEF-variability would have determined the GINA step at baseline in 10.2% of the patients [figure 2, bottom panel].

Discussion

Our results have shown that PEF variability provides information about asthma control in addition to symptoms and $ß_2$-agonist use. Furthermore, this study demonstrated that patients who were classified as being in GINA step 1 and 2 using the existing GINA criteria, but who had PEF-variability >10% had an almost 8 times higher risk for loss of asthma control 3 months later compared to patients whose PEF-variability was ≤10% at baseline. This suggests that including PEF-variability in the assessment of asthma control during treatment can improve the current guidelines for the treatment of asthma, but that the existing cut-points for PEF-variability are too high.

This is the first study showing the additional value of measuring PEF-variability in the management of asthma. These results extend previous findings which showed that PEF-variability in selected populations is valuable for epidemiological and diagnostic purposes (8-10). We have demonstrated that when using a cut-off value of 10%, PEF-variability is able to identify patients with an increased risk of loss of control of asthma. Previous studies have shown that patients with poor asthma control demonstrate higher PEF-variability compared to stable asthmatics (11). Furthermore, our findings are consistent with results from Nathan *et al.* who showed that a 10% cut-off value for PEF variability identifies patients with greater benefit from treatment (12).

We believe that our results have not been affected by patient selection or the methods of asthma monitoring. The current group of asthmatics seems to be representative of a broad range of asthma severity, since our patients were equally distributed over the 4 GINA grades. We cannot exclude that a patient group with a different balance of asthma severity would lead to different results. However, all our measurements were strictly based on asthma guidelines, allowing extrapolation of our findings to asthma care elsewhere.

The 10% cut-off value of PEF-variability was selected based on the high specificity and likelihood ratio observed. Even though we have shown that this cutpoint identifies patients at greater risk of loss of control, the most optimal cut-off value has to be investigated in large follow-up studies.
The cut-off value of PEF variability of 10% is in contrast with the 20% cut-off used in the current GINA guidelines. The number of PEF readings per day could readily explain this lower value. Indeed, reducing the frequency of measurements has been shown to underestimate the diurnal variation in PEF (13,14). The cut-off value of 20% stated in the present guidelines was based on studies where PEF was determined more frequently (18), whereas when PEF was measured twice daily, as is current practice, the upper 95% confidence limit for normal PEF-variability was 8-9% (15;16). Indeed in our study, in which PEF was measured twice daily, the PEF-variability was below the conventional cut-off value of 20% in almost all patients and therefore never determined the level of treatment, although these patients by other criteria had sub-optimally controlled asthma.

How can these results be interpreted? Airway hyperresponsiveness is an important characteristic of asthma (1). Previously, we have shown that asthma treatment aimed at reducing airway hyperresponsiveness is more effective in gaining control than therapy adjustment based on symptoms and level of lung function alone (6). Diurnal changes in PEF have been suggested as an indicator of the responsiveness observed in asthma (17). In this respect, the additional value of PEF-variability in asthma management observed in our study is not surprising. In addition, PEF-variability appears to be associated with eosinophilic inflammation in sputum in patients with asthma (18). Again, a strategy that minimises eosinophilic inflammation leads to improved asthma management as compared with a standard strategy (19). This implies that a feature reflecting airway hyperresponsiveness and/or airway inflammation should be included in the guidelines

of asthma management to optimise control of the disease. PEF-variability might be an appropriate indirect marker for this, which is accessible even in primary care settings where bronchial provocation testing or sputum induction are not readily available.

In conclusion, PEF-variability with a cut-off level of 10% provides additional information to monitor asthma control in addition to symptoms and lung function. Our data suggest that determining PEF-variability is not only useful for the diagnosis of asthma, but is also valuable for adjusting therapy of patients during treatment to thereby prevent loss of asthma control. These findings imply that the current cut-off values of PEF-variability should be revisited.

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Chapter 7

Anti-IgE-induced reduction in airway responses to inhaled allergen is paralleled by decreased eosinophilia in bronchial biopsies and sputum in patients with asthma

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Abstract

Background Anti-IgE, omalizumab, has been shown to inhibit the allergen response in patients with asthma. This has not been directly related to changes in inflammation.
Objective We hypothesised that anti-IgE exerts its effects by reducing airway inflammation. To that end, the effect of anti-IgE on allergen-induced inflammation in bronchial biopsies in 25 patients with asthma was investigated in a randomised, double blind, placebo-controlled study.
Methods Allergen challenge followed by a bronchoscopy at 24h was performed at baseline and after 12 weeks of treatment with subcutaneous anti-IgE or placebo. PC_{20} methacholine and induced sputum was performed at baseline, 8 and 12 weeks of treatment. Changes in the early and late responses to allergen, PC_{20}, inflammatory cells in biopsies and sputum were compared between anti-IgE and placebo.
Results Both the early and late asthmatic response were suppressed to 15.3% and 4.7% following anti-IgE as compared with placebo ($p<0.002$). This was paralleled by a decrease in eosinophil counts in sputum (from 4% to 0.5%) and post-allergen biopsies (from 15 to 2 cells/0.1mm^2) ($p<0.03$). Furthermore, biopsy IgE+ cells were significantly reduced between both groups, whereas high-affinity IgE receptor and CD4+ cells were decreased within the anti-IgE group. There were no significant differences for PC_{20} methacholine
Conclusion The response to inhaled allergen in asthma is diminished by anti-IgE, which is paralleled by a reduction in eosinophilic inflammation in bronchial mucosa and sputum and a decline in bronchial IgE positive cell counts post-allergen without changing PC_{20} methacholine. This suggests that the benefits of anti-IgE in asthma may be explained by a decrease in eosinophilic inflammation and IgE bearing cells.

Introduction

Thirty years after it was first identified, there is substantial evidence that immunoglobulin E (IgE) plays a key role in allergic asthma (1). In a population-based study, Burrows *et al.* were the first to show a strong association between serum IgE levels and self-reported asthma (2). Furthermore, high levels of circulating IgE have been shown to correlate with the risk of emergency room admissions in patients with asthma (3). IgE induces mediator release of mast cells and basophils via binding to high-affinity receptors, and thereby leads to activation of immune responses (4). Taken together, this makes immunomodulation of IgE an interesting strategy for new therapeutic interventions in asthma (5).

A recombinant, humanized, monoclonal antibody directed against IgE has been developed. This antibody decreases the levels of circulating IgE by binding to the constant region of the IgE molecule, which prevents free IgE from interacting with high- and low-affinity IgE receptors (FcεRI and FcεRII) (6). Anti-IgE has been found to decrease IgE levels and downregulate FcεRI expression on basophils and dendritic cells in blood (7;8).

The first clinical studies with intravenous anti-IgE have shown that both the early (EAR) and late (LAR) asthmatic response to inhaled allergen are attenuated in patients with asthma (9;10). Subsequent large phase 3 trials, involving both pediatric and adult patients with moderate to severe asthma, have demonstrated the clinical beneficial effect of subcutaneous anti-IgE treatment by improving asthma control (11-13). A recent double-blind, placebo-controlled study confirmed the effectiveness of anti-IgE treatment in inadequately controlled severe persistent asthma by showing a reduction in exacerbation rate and emergency room visits and an improvement in quality of life and morning peak flow (14).

The anti-inflammatory activity of anti-IgE was established in a biopsy study of mild asthmatic patients (15). Following 16 weeks treatment with anti-IgE significant reductions in IgE positive cells and eosinophils in the bronchial mucosa were found (15). It remains unclear whether the inhibition of the allergen response by anti-IgE treatment can be explained by a reduced airway inflammation.

The aim of the present study was to determine whether treatment with anti-IgE decreases the early and late responses to inhaled allergen and whether this is associated with a reduced allergen-induced airway inflammation in bronchial biopsies. Furthermore, the effect of anti-IgE on peak flow, airway hyperresponsiveness and inflammatory cells in sputum was investigated in patients with asthma in a randomized, double blind, placebo-controlled study.

Table 1. Patient characteristics

characteristic	anti-IgE (n=12)	placebo (n=13)
age (years)	20.5 (18-24)	21 (19-29)
female sex, n (%)	12 (100)	10 (77)
smoking history, n (%)		
never	11 (92)	12 (92)
ex-smoker	1 (8)	1 (8)
duration of asthma (years)	10.5 (1-19)	9.0 (4-22)
total IgE (IU/ml)	154 (51-674)	321 (35-593)
FEV_1 (%pred)	96.0 (82-115)	88.8 (72-114)
PC_{20} methacholine (mg/ml)*	0.48 (1.61)	1.02 (1.93)

Data are presented as median (range); * g-mean (SD in dd);
There were no significant differences between the groups.

Methods

Subjects

Twenty-five non-smoking asthmatic volunteers (18-29 years) participated in the study [Table 1]. All patients had a history of episodic chest tightness and wheezing and were only using short-acting $ß_2$-agonists on demand. All were atopic to house dust mite (HDM) and were having a total serum IgE between 30 and 700 IU/ml. The baseline forced expiratory volume (FEV_1) was > 70% predicted (16) and all subjects were hyperresponsive to inhaled methacholine (provocative concentration causing a 20% fall in FEV_1 (PC_{20}) <4 mg/ml) (17). The fall in FEV_1 during the late asthmatic response (LAR) following inhaled allergen was at least 15%. All patients were clinically stable and had no respiratory chest infection 2 weeks prior to the study. The study was approved by the medical ethics committee of the Leiden University Medical Center and all volunteers gave a written informed consent.

Design

This study had a randomized, placebo-controlled, parallel, double-blind design. Anti-IgE or placebo was administered for 12 weeks every 2 or 4 weeks. At baseline, after 8 and 12 weeks of treatment, PC_{20} methacholine was determined and sputum induced. Allergen challenge followed by a bronchoscopy at 24 hours was performed at baseline and 12 weeks.

Treatment

The dose (150 mg to 375 mg) and frequency (every 2 or 4 weeks) of treatment was determined by weight and baseline total serum IgE level of each patient and had to be al least 0.016 mg/kg per IgE (IU/ml) (18). A research nurse who was not involved in any other measurement of the study administered the subcutaneous anti-IgE or placebo.

Diary cards

Patients kept diary cards from 2 weeks prior to and during the 12 weeks of the study. Morning and evening pre-bronchodilator peak flow measurements were recorded. Mean PEF values of the 2 weeks prior to baseline measurements of the study and the mean PEF values of the 2 weeks prior to the 12 week-measurements of the study were used in the analysis.

Spirometry and Airway hyperresponsiveness

Patients were not allowed to take any short-acting ß2-agonists for at least 8 hours prior to spirometry. A standardized methacholine challenge was applied to determine airway hyperresponsiveness (17). To determine the PC_{20} methacholine, patients inhaled increasing doses of methacholine for 2 minutes until a fall of at least 20% in FEV_1 had been reached.

Sputum induction and processing

Prior to induction, each subject inhaled 200 µg salbutamol. Sputum was induced by inhalation of NaCl 4.5% during 3x5 minutes intervals, according to a recommended protocol validated in our laboratory (19;20).
Sputum samples were processed according to the whole sample method. Differential cell counts were expressed as a percentage of 250 non-squamous cells (20).

Allergen challenge

Allergen challenges were performed according to a standardized protocol (17;21). Purified aqueous allergen extract of *Dermatophagoides pteronyssinus* (SQ 503; Vivodiagnost, ALK, Benelux), with 0.5% phenol as a preservative, was diluted ranging from 2,000-15.63 BU/ml. PC_{20} allergen was predicted from PC_{20} methacholine and skin-test sensitivity, derived from a multi-dose skin prick test, according to Cockcroft's method (21). Starting three concentrations below the predicted PC_{20} allergen, 3 ml of consecutive doubling concentrations of allergen were aerosolized for 2 minutes using a DeVilbiss 646 nebulizer (output 0.13 ml/min). The response to allergen was determined by measuring FEV_1 in duplicate 10 min after each inhalation of allergen. After reaching a fall of at least 20%, FEV_1 measurements were repeated 10, 20, 30, 40, 50, 60, 90, and 120 minutes and then hourly until 7 hours after the last inhalation. In the analysis, EAR (from 0-3 hours post-allergen) and LAR (from 3 to 7 hours post-allergen) were defined as the maximum % fall in FEV_1 from baseline and as the area under the time-response curve (AUC) (17). Patients received exactly the same allergen dose at the end of the study as they inhaled during the baseline allergen challenge.

Bronchoscopy and immunohistochemistry

Fiberoptic bronchoscopy was performed according to a standardized and validated protocol (22). Six biopsy specimens were taken at (sub)segmental level from either the right lung (first bronchoscopy; right lower lobe and/or the middle lobe) or the left lung (second bronchoscopy; lingula and left lower lobe). Two biopsies were immediately frozen and stored at –80°C.

The remaining 4 biopsies were fixed for 24 hrs in buffered formalin and paraffin embedded. Three µm thick, HE stained slides were used for checking biopsies quality (size, crushing, epithelial and mucosal representation) and the two technically best biopsies were selected for immonuhistochemistry. Slides were immunostained for IgE, high and low affinity IgE receptor (FcεRI and FcεRII), eosinophils (EG2), mast cells (AA1), neutrophil elastase (NE), macrophages (CD68), and T lymphocytes CD3, CD4, and CD8. In short, the sections were incubated with an optimal dilution of the primary antibodies in 1% BSA/PBS at room temperature for 60 min. As a secondary antibody, the horseradish peroxidase conjugated anti-mouse or anti-rabbit EnVision system (DAKO, Glostrup, Denmark) was used, with NovaRED (Vector, Burlingame, CA) as the chromagen. The sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, the Netherlands). For negative controls, the first antibody was omitted from this procedure. All biopsies were coded and sections analysed in a blinded fashion, using a fully automated image analysis system (23). Images were digitized using a three-chip colour camera (433.10^3 pixels, 660x496µm^2, 3x256 grey values) (KS-400 System, Kotron/Zeiss). The whole available area of lamina propria was determined by manually delineating the basement membrane. Lamina propria was defined by the widest possible 125 µm deep zone beneath the basement membrane of at least 86,000 µm^2. The automated counting of the number of positively staining cells consisted of the following steps: level off background noise, normalize staining intensity, delete noise, fuse stained fragment, delineate stained clusters, determine cell counting by an algorithm. This method has been shown to be fully reproducible and to have good agreement with interactive cell counting (23). Data were expressed as cells/0.1mm^2.

Analysis

The PC_{20} methacholine, cell counts in sputum and biopsies were log transformed before statistical analysis. All data are presented as median (range), except for PC_{20} methacholine which is presented as geometric mean (SD expressed as doubling dose). Paired t-test was applied to test for changes within groups, whereas unpaired t-test was used for changes from baseline to end between anti-IgE and placebo treatment groups. A p-value of < 0.05 was considered as statistical significance and all analyses were performed using SPSS 12.0.

Results

From one patient in the placebo group, no biopsies were obtained during the second bronchoscopy. Consequently, a total of 24 patients completed the study (anti-IgE: n=12; placebo: n=12). Three patients in the anti-IgE group and 4 patients in the placebo group did not produce sputum at one of the time points. Matched sputum samples were therefore obtained from 18 patients (anti-IgE: n=9; placebo: n=9).
Baseline FEV_1 was 96.3% predicted in anti-IgE group and 90.8% predicted in the placebo group. There were no significant changes for FEV_1 within or between groups during the study ($p>0.07$).

Diary cards

Morning PEF was significantly increased from 383.6 L/min (314.6-469.2) at baseline to 430.0 L/min (325.8-503.3) after 12 weeks of anti-IgE (p=0.038). This increase was significantly different from the change in the placebo group (398.5 L/min (342.1-613.8) to 392.1 L/min (349.3-608.5) (p=0.53 for the change within the placebo group) (p=0.041 for the change from baseline between anti-IgE and placebo groups). Similar improvements were observed for evening PEF [Figure 1].

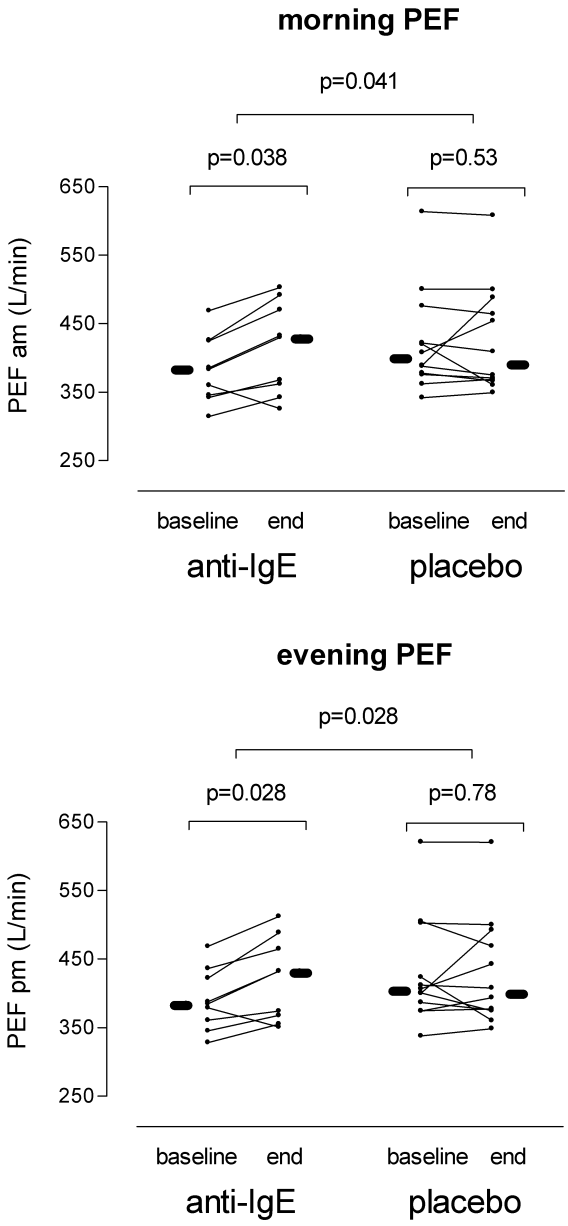

Figure 1. PEF
Individual values of morning (top panel) and evening (bottom panel) PEF at baseline and end of the study in anti-IgE and placebo treated patients.

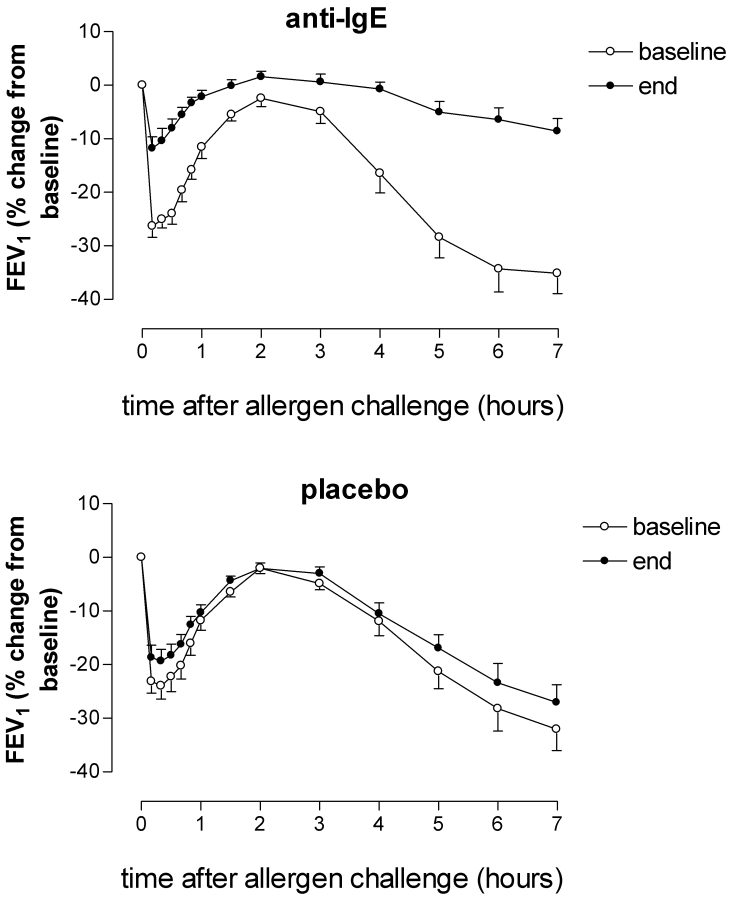

Figure 2. Allergen challenge
Airway responses to inhaled allergen between 0 to 7 hours following allergen challenge for the anti-IgE group (top panel) and for the placebo group (bottom panel). In the anti-IgE group, EAR and LAR were significantly reduced from baseline (open circles) to end (closed circles), which was also significant between the groups.

Allergen Challenge

At baseline, the maximum % fall in FEV_1 during the EAR was (median (range)) 28.9% (19.4-38.6) for the anti-IgE group and 27.0% (20.3-35.9) for placebo. Anti-IgE treatment significantly inhibited the EAR to 15.3% (0.0-23.8) (p=0.000). This change was significantly larger as the change in the placebo group (p=0.002). The AUC of the EAR was also significantly reduced following anti-IgE treatment (28.0 percentage fall per hour (%fall*h) (2.7-49.3) to 15.3 %fall*h (0.0-23.8)) as compared to placebo (33.5 %fall*h (14.2-41.9) to 23.2 %fall*h (6.0-37.1) (p=0.002) [Figure 2].

The maximum % fall during the LAR was suppressed following anti-IgE treatment from 36.1% (18.9-52.7) to 4.7% (0.0-20.6). This reduction was significantly larger as compared with placebo (31.1% (12.8-54.0) to 25.4% (14.0-48.2)) (p=0.000 for the change from baseline between anti-IgE and placebo). In addition, the reduction in AUC of the LAR was significantly larger in the anti-IgE group (95.6 %fall*h (13.9-182.9) to 9.3 %fall*h (-23.0-52.8) as compared with the placebo group (89.9 %fall*h (12.4-152.2) to 56.0 %fall*h (25.2-122.9) (p=0.000) [Figure2].

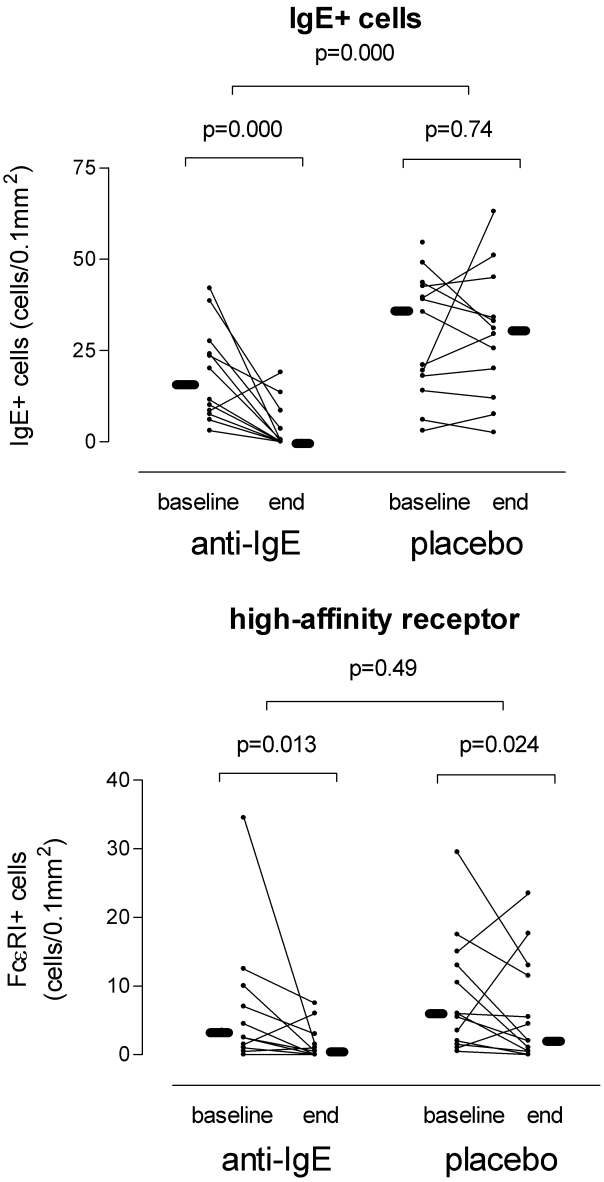

Figure 3. Biopsy IgE+ and FcεRI+ cells

Individual values of IgE+ (top panel) and FcεRI+ cells (bottom panel) at baseline and end of the study in anti-IgE and placebo treated patients.

Bronchial biopsies

Anti-IgE treatment markedly reduced the submucosal IgE+ cells from 15.8 cell/0.1mm^2 (3.0-42.0) to 0.0 (0.0-19.0) (p=0.000). This reduction was significantly larger as compared with the placebo group (35.5 cell/0.1mm^2 (3.0-54.5) to 30.3 (2.5-63.0) (p=0.000 for changes from baseline between anti-IgE and placebo) [Figure 3]. FcεRI+ cells were also significantly reduced within the anti-IgE group (p=0.013). However this change was not significantly different from the change in the placebo group (p=0.49 for the change from baseline between anti-IgE and placebo) [Figure 3]. Most biopsy samples did not show any FcεRII+ cells and no changes following treatment were observed (p>0.18).

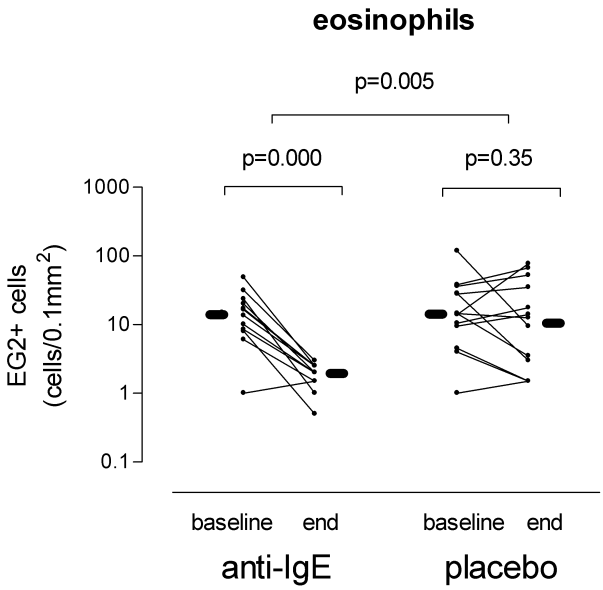

Figure 4. Biopsy eosinophils
Individual values of eosinophils at baseline and end of the study in anti-IgE and placebo treated patients.

There was a clear reduction in eosinophil counts in the anti-IgE group (15.0 cells/0.1mm^2 (1.0-48.5) to 2.0 cells/0.1mm^2 (0.5-3.0)) (p=0.000), which was significantly different from the placebo group (14.5 cells/0.1mm^2 (1.0-118.5) to 11.0 cells/0.1mm^2 (1.5-77.0) (p=0.005 for the change from baseline between anti-IgE and placebo) [Figure 4].
There were no significantly differences between anti-IgE and placebo observed for mast cells, macrophages, neutrophil elastase and B-lymphocytes (p>0.09). CD4+ T-lymphocytes were significantly reduced after anti-IgE treatment (p=0.021), however, there were no significant between-group differences for the T-lymphocytes (p>0.14).

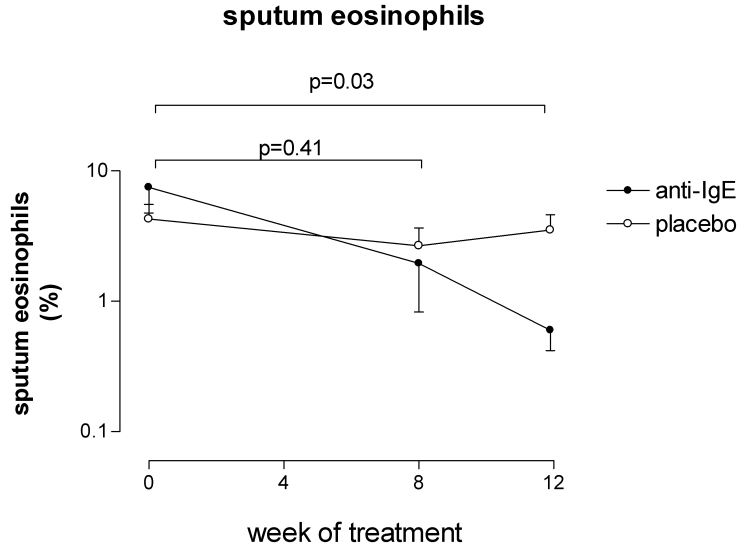

Figure 5. Sputum eosinophils
Change in sputum eosinophils from baseline, to 8 weeks and at the end of the study for anti-IgE (closed circles) and placebo (open circles).

Sputum

The median (range) % sputum eosinophil decreased in the anti-IgE treated group from 4.0% (0.2–28.0) at baseline, to 0.8% (0.2-10.8) after 8 weeks and to 0.5% (0.0-1.6) at the end of the study (after 12 weeks of treatment). This change was significantly different with placebo (baseline: 2.2% (0.4-10.2); 8 weeks: 1.0% (0.2-10.8); 12 weeks: 2.6% (0.4-13.8) after 12 weeks of treatment (p=0.03 for the change from baseline between anti-IgE and placebo) [Figure 5].
None of the other cell types in induced sputum showed any significant changes.

Airway hyperresponsiveness

PC_{20} methacholine was measured (geometric mean (SD expressed as doubling dose) 0.48 (1.61) at baseline, 0.94 (1.89) after 8 weeks and 1.04 (2.06) after 12 weeks in the anti-IgE group and 1.02 (1.93), 1.30 (2.16), 1.90 (2.26) in the placebo. There were no significant differences for the changes between the groups (p>0.18) [Figure 6].

Discussion

The results of this study show that anti-IgE treatment leads to a marked reduction of eosinophil counts in sputum and biopsies and IgE+ cells in biopsies. This suppression of inflammation is paralleled by a clear inhibition of both early and late response to inhaled allergen and a significant improvement in morning and evening PEF rates. However, PC_{20} methacholine was not changed by anti-IgE treatment. These findings indicate that the blunting of the allergen response by anti-IgE may be explained by a decrease in eosinophilic inflammation and IgE bearing cells. Interestingly, our results suggest that airway hyperresponsiveness to methacholine in atopic asthma is independent from IgE.

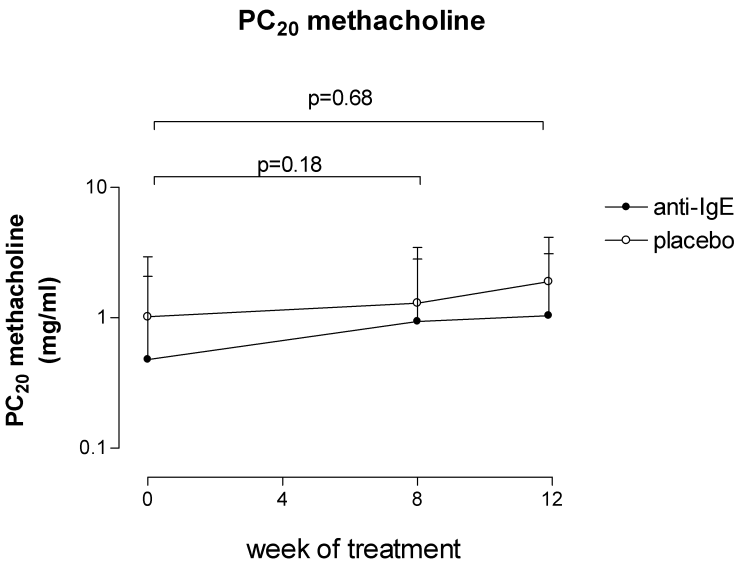

Figure 6. Airway hyperresponsiveness Change in PC_{20} methacholine from baseline, to 8 weeks and at the end of the study for anti-IgE (closed circles) and placebo (open circles).

To our knowledge, this is the first clinical study with anti-IgE demonstrating that the effects on allergen-induced airway responses are paralleled by a reduction in airway inflammation in patients with asthma. Our functional outcomes are in line with others who showed the inhibitory effect of anti-IgE treatment on early and late asthmatic response (24;25). Our results indicate that the major anti-inflammatory effects of anti-IgE are based on a marked fall in eosinophils and local IgE production in the airways. This extends the effects shown on nasal inflammation in patients with rhinitis and on airway inflammation in patients with asthma (26;27). Like Djukanovic *et al.*, we have demonstrated the anti-inflammatory effect of anti-IgE treatment in bronchial biopsies (26). In contrast with their study, we showed also a clear clinical beneficial effect of anti-IgE on morning and evening PEF and on response to inhaled allergen (26).
In the present study, airway hyperresponsiveness was not improved following anti-IgE treatment as compared with placebo. Our results are in keeping with previous studies also showing no or marginal effects on PC_{20} methacholine (24-26;28).

We do not believe that our data were influenced by measurement errors, since we used validated and reproducible methods (16;17;20;22;23). All subjects in this study were carefully selected non-smokers with stable, atopic, mild-intermittent asthma, who had not used inhaled steroids for at least 1 month prior to the study. In order to compare the allergen-induced inflammation in the bronchial biopsies, the allergen challenge at the end of study was performed in a similar manner to the baseline allergen challenge.

What is the mechanism by which anti-IgE treatment gives such a marked reduction in eosinophilic inflammation? Via cross-linking with high-affinity receptors on mast cells, IgE induces the release of preformed mediators, such as histamine and thereafter the release of newly formed mediators and cytokines such as TNF-α, IL-4 and IL-5, leading to the accumulation of eosinophils (4). Treatment with anti-IgE is likely to intervene in this mechanism. Indeed, a reduction in cell surface IL-4 following anti-IgE treatment has been demonstrated (15). A second mechanism, by which anti-IgE affects eosinophils, might be related to the inhibitory effects of anti-IgE on dendritic cell FcεRI expression (8). It has been proposed that anti-IgE can alter allergen presentation by dendritic cells (1). Anti-IgE treatment may decrease the uptake and presentation of allergens by dendritic cells, leading to a reduced T cell response, which may diminish eosinophilic airway inflammation (29).
Interestingly, we have shown that the effect of anti-IgE is much larger for the late asthmatic response than for the early response. The EAR is predominantly mediated by IgE-triggered mast cell mediator release of histamine and tryptase (30). Free IgE is markedly reduced following anti-IgE treatment, the number of mast cells was not changed in our study. Apparently, it is not the number of mast cells that is important, but it is whether they are loaded with IgE. Furthermore, resulting from the minimal amount of IgE that is probably available after treatment, cross-linking with high-affinity receptors still might occur and thereby leading to an early asthmatic response.

Airway hyperresponsiveness is an important feature of patients with asthma (31), but anti-IgE treatment did not appear to affect it. We cannot exclude that airway hyperresponsiveness might improve after longer treatment with anti-IgE, since our patients were only treated for 12 weeks. Results of therapy with anti-IL5, which also results in an abolishment of eosinophils, have shown no effect on airway hyperresponsiveness either (32). These findings confirm the dissociation of eosinophils and airway hyperresponsiveness in asthma as observed in some animal models (33). Treatment with inhaled steroids, in contrast, reduces both eosinophils and airway hyperresponsiveness (34). Possibly, inhaled steroids not only affect eosinophilic inflammation, but also affects the functional properties of airway smooth muscle, whereas the effects of anti-IgE treatment are mediated through anti-inflammatory properties alone.

Our findings may have several clinical implications. First, we have shown that PEF, allergen-induced airway responses and airway inflammation are strongly reduced after anti-IgE treatment within one study. This implies that indeed anti-IgE exerts its clinical beneficial effects by reducing airway inflammation and thereby might be an effective therapy for asthma. On the other hand, anti-IgE treatment had no effect on airway hyperresponsiveness in our study. We have previously shown that treatment aimed at reducing airway hyperresponsiveness leads to more effective control and thereby a reduction of exacerbations in patients with asthma (35). Therefore, the inability of anti-IgE treatment to normalize airway hyperresponsiveness requires further investigation.

In conclusion, treatment with anti-IgE inhibits both allergen-induced airway response and airway inflammation in patients with asthma. We have shown that the most important anti-inflammatory effect of anti-IgE is on reducing eosinophilic inflammation and IgE-bearing cells. The suggestion that airway hyperresponsiveness appears to be independent of IgE is of interest with respect to the role of anti-IgE as a new therapy for asthma.

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Chapter 8

Summary and general discussion

Summary

Airway inflammation is the key characteristic of asthma. In this thesis, we have investigated different aspects of this inflammatory process in patients with asthma. First, the pathogenesis of the disease was explored in a proof of concept study. Second, monitoring of airway inflammation was studied by examining markers of airway inflammation. Third, treatment of airway inflammation was investigated by studying improvements for asthma management. The main conclusions from these studies are summarized below.

Proof of concept

In **chapter 2**, we have shown that exogenous IL-5 is mainly effective in the circulation by enhancing the number of eosinophils to the circulation. There was no effect on the number of eosinophils in the lungs or on airway hyperresponsiveness. This suggests that IL-5 is able to promote the recruitment of eosinophils to the circulation.

Monitoring

The steroid-induced changes in airway hyperresponsiveness, sputum eosinophils and exhaled NO were compared in **chapter 3**. Treatment with inhaled steroids leads to significant improvements in airway hyperresponsiveness, reduced numbers of eosinophils in sputum, and decreased levels of exhaled nitric oxide in patients with asthma. The steroid-induced changes for each of the three different markers were not related. Therefore, the data suggest that these markers may provide different information when monitoring anti-inflammatory treatment in asthma.

Microvascular leakage is an important feature of inflammation. In **chapter 4**, an investigational model of "dual induction" was introduced to determine the level of microvascular leakage in patients with asthma. Using this model, it was possible to detect microvascular leakage by first inducing leakage with inhaled substance P and then measuring leakage in induced sputum. Alpha-2-macroglobulin appeared to be the most appropriate marker. This implies that this "dual induction" model can be applied when testing the antiexudative effect of newly developed drugs.

Chapter 5 demonstrated that the outcome of asthma, as determined by the annual decline in FEV_1, can be predicted by the bronchial CD8+ cell infiltrate. On the other hand, eosinophils in bronchial biopsies and the thickness of the sub-epithelial reticular layer were not associated with the decline in lung function. This suggests that inflammatory phenotypes in asthma may have prognostic relevance.

Management

In **chapter 6**, we have shown that PEF variability provides information about asthma severity in addition to symptoms and $ß_2$-agonist use. Patients who were classified as being in severity step 1 and 2 using the existing GINA criteria, but who had PEF-variability >10% had an almost 8 times higher risk for an increase in asthma severity 3 months later

compared to patients whose PEF-variability was ≤10% at baseline. Therefore, the current guidelines for the treatment of asthma can be improved by including PEF-variability in the assessment of asthma severity during treatment.

Treatment with anti-IgE, omalizumab, has recently been FDA approved for patients with moderate to severe persistent, IgE-mediated asthma that is sub-optimally controlled with inhaled steroids. We have demonstrated in **chapter 7** that PEF values were improved and that the response to inhaled allergen in asthma was diminished by anti-IgE. This was paralleled by a reduction in eosinophilic inflammation in bronchial mucosa and in induced sputum and a decline in bronchial IgE positive cell counts post-allergen. On the other hand, anti-IgE treatment did not improve airway hyperresponsiveness in these patients. This suggests that the clinical benefits of anti-IgE in asthma may be explained by a decrease in eosinophilic inflammation and IgE bearing cells. Furthermore, airway hyperresponsiveness appears to be independent of IgE.

General discussion

Proof of concept: is inflammation the right target?

Airway inflammation in asthma is complex in origin, regulation and outcome. There is still a lack of understanding of the mechanisms involved in asthma (1). Proof of concept studies may be useful to investigate the underlying pathogenesis of asthma. In this thesis we have performed a proof of concept study by questioning whether exogenous IL-5 leads to airway inflammation in asthma and whether the route of IL-5 production is crucial to its effects on the airways. IL-5 plays an important role in the mobilisation, differentiation and maturation of eosinophils (2). A causal relationship between the key pathological feature of asthma: eosinophils, and the key physiological characteristic: airway hyperresponsiveness, has been a long question of debate. The effects of inhaled steroids are suggestive for a causal relationship, since they both improve eosinophilia and airway hyperresponiveness in patients with asthma. On the other hand, the steroid-induced changes between sputum eosinophils and airway hyperresponsiveness were not related (Chapter 3). Some animal models, investigating the effect of IL-5 on eosinophilia, have shown the subsequent development of airway hyperresponsiveness, whereas others have not (3-6). In Chapter 2, we have demonstrated that intravenous administration of IL-5 to patients with asthma leads to an increased number of eosinophils in blood, but not in sputum. However, airway hyperresponsiveness was not affected. On the other hand, Shi *et al* did show an increase in airway hyperresponsiveness following IL-5 administration (7). This discrepancy may be related to differences in racial susceptibility to the effects of IL-5, since these studies were performed in ethnically different populations. The development of a monoclonal antibody against IL-5 further challenged the hypothesis that the eosinophil is the central effector cell in asthma (8). Anti-IL-5 treatment in patients with asthma abolishes eosinophils in blood and sputum, but this fall in eosinophils was not accompanied by changes in airway hyperresponsiveness or response to inhaled allergen (9). On the other hand, Flood-Page

and co-authors have demonstrated that anti-IL-5 treatment only partially depletes the numbers of eosinophils in bronchial biopsies of patients with asthma (10). Interestingly, anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane (11). The finding that the response to inhaled allergen is unaffected by anti-IL5 makes its unlikely that anti-IL5 treatment will be beneficial to control asthma. The drugs that are effective in asthma control (inhaled steroids, cromolyn, theophylline, leukotriene antagonists, and anti-IgE) all inhibit the late response to allergen. Long-acting $ß_2$-agonists also appeared to inhibit the late response by reducing airway inflammation (12). However, a study showed that long-acting $ß_2$-agonists modify allergen-induced airway responses through functional antagonism rather than the inhibition of inflammatory cell infiltration (13).

But, is it also true for airway hyperresponsiveness, that all effective asthma drugs improve airway hyperresponsiveness? Indeed, treatment with inhaled steroids, cromolyn, theophylline and leukotriene antagonists reduce airway hyperresponsiveness in asthma. Anti-IgE treatment, however, apparently has no effect on airway hyperresponsiveness after short-term treatment (Chapter 7) (14). Therefore, it seems that eosinophils and airway hyperresponsiveness are not causally related. Nevertheless, the clinical beneficial effect of anti-IgE treatment has been demonstrated in large phase 3 trials, involving both pediatric and adult patients with moderate to severe asthma (15-17). Based on these data, anti-IgE has recently been approved by the Food and Drug Administration in the US for patients with moderate to severe persistent, IgE-mediated asthma that is sub-optimally controlled with inhaled steroids. Furthermore, we have shown that in patients with mild persistent asthma treatment with anti-IgE for 12 weeks significantly improves morning and evening PEF and reduces early and late allergen response (Chapter 7). Thus, the question remains whether drugs have to reduce airway hyperresponsiveness in order to be beneficial for asthma treatment. Therefore, the issue arises which outcome parameters should prevail in proof of concept studies: the cellular and pathological outcome, or the functional endpoints?

What is the implication of the persistent airway hyperresponsiveness under anti-IgE treatment? IgE+ cells in the bronchial mucosa are significantly reduced following anti-IgE treatment (Chapter 7). Apparently, airway hyperresponsiveness in patients with asthma is independent of IgE. The clinical consequences of airway hyperresponsiveness are reflected in an increased variation in airway caliber both within and between days (PEF variability) (18). Indeed, PEF variability in our study did not change either. However, this may be explained by the low level of PEF variability in our patient group (Chapter 7). In patients with more severe asthma an effect of anti-IgE on PEF variability has not been published.

The pathological mechanisms responsible for airway hyperreponsiveness may be related to the altered behaviour of airway smooth muscle (10). Changes in the organization of contractile filaments or in the plasticity of smooth muscle may underlie the persistence of airway hyperresponsiveness (20). It could be argued that inhaled steroids are not only

anti-inflammatory, but also change the functional properties of airway smooth muscle, whereas anti-IgE treatment does not (21). Remarkably, it has recently been suggested in a pilot study, that anti-TNFα treatment reduces airway hyperresponsiveness and increases FEV_1, but does not affect inflammation (22). This may suggest that inflammation per se, is not the right target for asthma therapy. Nevertheless, inflammation in the airway wall may enhance airway narrowing during smooth muscle contraction and thereby lead to airway hyperresponsiveness (23). Interestingly, the number of mast cells in the smooth muscle of patients with asthma is inversely correlated with the PC_{20} methacholine in the subjects with asthma (24). Following anti-IgE, the number of mast cells in the lamina propria was not decreased (Chapter 7). Although, we have not analyzed the number of mast cells in the airway smooth muscle, this might provide an explanation for the unchanged airway hyperresponsiveness following anti-IgE treatment.

Monitoring inflammation: there is more than eosinophils

The current GINA guidelines recommend that lung function and symptoms are measured in order to adjust (anti-inflammatory) treatment and thereby maintain asthma control (1). It is an interesting hypothesis that more direct monitoring airway inflammation will lead to improved asthma control. Markers for monitoring airway inflammation were investigated in this thesis in three different ways. First, inhaled steroids improved airway hyperresponsiveness, sputum eosinophils and exhaled nitric oxide; however these changes were not interrelated. Therefore, these markers may provide complementary information when monitoring anti-inflammatory treatment in asthma (Chapter 3). Second, anti-exudative effect of treatments can be determined via the assessment of microvascular leakage in induced sputum following inhalation of substance P (Chapter 4). Third, CD8 cells in bronchial biopsies predicted lung function decline and thus demonstrated the prognostic value of inflammation in asthma (Chapter 5). Therefore, inflammation in asthma does not only consist of eosinophilic inflammation.

Which criteria can be identified to determine the usefulness of markers for monitoring inflammation in asthma? First, it is important to distinguish between markers for short- and long-term outcome of asthma. At this moment most research is focused on the short-term outcome of the disease. However, the inflammatory process within the airway may have different effects on short- and long-term outcome of the disease. Indeed, eosinophils have been shown to predict asthma exacerbations in a study with a follow-up period of 8 weeks (25). On the other hand, we could not observe the prognostic value of eosinophils in our follow-up study of 7½ years (Chapter 5). Therefore, the prognostic value of inflammatory markers may be different for short- and long-term follow-up. Markers for short-term outcome of asthma have to be safe, non-invasive, reproducible, accurate and easy to perform, since they have to be measured more often in the same patient. Furthermore, these markers should be responsive to the effects of (or to changes in) treatment, exposure or avoidance to allergens. This means that they should mirror changes in the degree of inflammation. Next, markers may be selected on their ability to discriminate between different diseases and thereby be of use for the diagnosis

of asthma. For example, the negative predictive value of airway hyperresponsiveness for asthma is very high (26). The diagnostic accuracy appears to increase if sputum eosinophils and levels of exhaled NO are used in comparison with conventional approaches as recommended in the guidelines (27). Alternatively, a marker should be able to reliably distinguish between different disease severities. Whether a marker meets this criterion is often tested by investigating the correlations with other measures of asthma severity (symptoms and lung function). However, the lack of such a correlation could also imply that this marker is reflecting a different component of the disease. We also failed to demonstrate a correlation between steroid-induced changes in airway hyperresponsiveness, sputum eosinophils and levels of exhaled NO (Chapter 3). This could imply that the first improvements induced by inhaled steroid for the different markers are "out of phase". On the other hand, it could mean that these three markers represent different features of asthma.

Measuring airway hyperresponsiveness has demonstrated its usefulness in asthma management (28). Although it is safe and non-invasive, it may not be easy to perform in a non-specialized setting. Nevertheless, measures of airway hyperresponsiveness may provide additional and useful information, which is probably not always picked-up by other markers of inflammation (29). Sputum eosinophils have also been effectively used to guide anti-inflammatory treatment (30). Again, specialized personnel time is needed for the induction and processing of sputum. Therefore, cost-effectiveness analyses are needed to investigate the repetitive use of these markers in regular patient care, outside a research setting. In Chapter 4, we have demonstrated that induced sputum can be used to assess microvascular leakage. Neurogenic inflammation, which may result in microvascular leakage, is an important component of the pathology in asthma (31). Monitoring this feature of the inflammatory process might also lead to better asthma control and should, therefore, be further explored. Improved asthma management based on monitoring the levels of exhaled NO has been used in adults and children (32;33). The measurement of exhaled NO is safe and non-invasive, but in contrast with AHR and sputum eosinophils, it is easy to perform. However, the equipment needed to measure exhaled NO is still very expensive. In the future, the measurement of exhaled NO might be used in regular patient care. On the other hand, it may be questioned whether these asthma management studies are sufficient proof that exhaled NO is a appropriate marker for adjusting treatment. Indeed, the levels of exhaled NO appeared not to predict loss of asthma control (25). Interestingly, the same study showed that changes in sputum eosinophils were prognostic for loss of control (25). The study by Leuppi and co-authors confirmed that both AHR and sputum eosinophils, but not exhaled NO, are predictive for asthma exacerbations (34).

Assessing airway inflammation for markers of long-term outcome is important for investigating the underlying mechanisms of the disease and for following the progression and resolution of the disease. Consequently, the criteria "non-invasive" and "easy to perform" may be less important, since these measures will not be performed frequently. The prognostic significance of airway inflammation for the long-term outcome of asthma

is still unclear. The lack of scientific evidence is probably due to the long duration needed to investigate markers for longitudinal follow-up. To overcome this problem, several cross-sectional studies have been performed. These cross-sectional studies have shown associations between eosinophils and persistent airflow limitation (35;36). Furthermore, others have demonstrated that the thickness of the sub-epithelial reticular layer was inversely associated with the level of lung function in asthma (37;38). Still, it is not known whether these cross-sectional associations hold after long-term follow-up. Indeed, we have found that the cross-sectional associations with eosinophils and sub-epithelial reticular layer thickness are not established in a longitudinal follow-up study (Chapter 5). It is not unexpected that cross-sectional and follow-up studies have different results, since cross-sectional studies do not include changes over time. Thus, longitudinal follow-up studies are required to examine the usefulness of markers for monitoring inflammation of long-term asthma outcome.

Management: how to distinguish asthma severity from asthma control?

Asthma is a heterogeneous disease. Patients who participate in clinical trials have to be classified according to the severity of their disease for enrolment. Traditionally, asthma severity is defined by the clinical features that are present in the absence of therapy. This approach has also been used in the current GINA guidelines (Table 5-6) (1). Under appropriate treatment these clinical features should be absent, otherwise there is lack of control. To be able to identify patients who are at increased risk for exacerbation, there is a growing need for a distinction between asthma severity and asthma control (39-41).

What is asthma severity? Asthma severity is meant to grade the underlying disease state. In the current asthma guidelines, classification of asthma severity is assessed by the clinical features that are present before treatment (1). These clinical features would include symptoms and lung function. However, these clinical features are modified by therapy. Therefore, treatment level should be taken into account relating asthma severity to clinical symptoms (39). Asthma severity may vary from time to time in a single patient, however, changes in asthma severity occur only relatively slowly over time.

What is asthma control? Asthma control, on the contrary, is meant to grade the current expression of the disease as a result of treatment intervention. It is based on the goals of optimal treatment as described in the asthma guidelines. These goals include the absence of symptoms. On the other hand, minimal symptoms are allowed if they do not (or only minimally) require rescue medication. Furthermore, lung function should be normal or at least near the patient's best. Asthma control will also mean control of exacerbations. However, when defining disease control, inflammation should also be taken in to account. Moreover, chronic control of asthma would indicate the prevention of loss of lung function.

Control can be achieved by patient education, environmental control and adequate treatment. There are several reasons why patients with asthma may have poorly-controlled disease. The most important reason is failure to adhere to treatment recommendations. Indeed, poor compliance to asthma medication has been repeatedly

reported (42). Second, untreated non-asthmatic conditions (gastroesophageal reflux, rhino sinusitis, psychopathology co-morbidity) associated with asthma may lead to poor asthma control. Finally, very severe asthma could also lead to uncontrolled asthma, but only if the two reasons mentioned above are excluded (39).

Although measures to assess control and severity of asthma overlap, there should be emphasis on the distinction between asthma control and asthma severity. The common perception that well-controlled asthma is synonymous with mild asthma and that poorly-controlled asthma is synonymous with severe asthma is wrong. Asthma severity should be defined by the minimum medication required to achieve asthma control (39). PEF variability may be a measure that can be used to assess and improve asthma control. Indeed, we have shown that PEF variability provides information in addition to symptoms and $ß_2$-agonist use and may therefore be valuable to adjust therapy in order to prevent loss of asthma control (Chapter 6). The current GINA guidelines use PEF variability only for the initial assessment before treatment. For the ongoing assessment of asthma control during treatment, PEF variability is not included in the guidelines (1). Asthma management studies using airway hyperresponsiveness (28), sputum eosinophils (30) or exhaled NO (33) as a marker to adjust treatment have demonstrated that the current guidelines are not optimal and can be improved. In addition, a treatment algorithm, which includes the reduction of PEF variability, might also improve the asthma management guidelines. Furthermore, the use of a composite measure to determine asthma control has been proposed (43;44). In the current GINA guidelines the presence of one of the features of a severity step is sufficient to place a patient in that category (1). Finally, it has been suggested that the patient perception should be taken into account (45). This would imply the inclusion of patient-centred outcomes in the asthma management guidelines.

Directions for future research

The studies described in this thesis have gained more insight into airway inflammation in patients with asthma. Despite the growing knowledge about the concept, monitoring and management of asthma, many issues remain to be explored. Interesting questions for futures studies may include:

- Which parameters should prevail in proof-of-concept studies?
- What is the clinical implication of the persistent airway hyperresponsiveness under anti-IgE treatment?
- Can measurement of microvascular leakage be used to monitor airway inflammation and thereby improve asthma control?
- Is it effective to adjust asthma treatment based on the levels of PEF- variability?
- What is the cost-effectiveness of non-invasive markers of airway inflammation to monitor asthma treatment?
- Which markers are useful for monitoring inflammation of long-term asthma outcome?
- Is it possible to improve the current GINA guidelines by including non-invasive markers of airway inflammation or by using a composite outcome?

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Chapter 9

Nederlandse samenvatting

Achtergrond

Wat is astma?

Patiënten met astma hebben het regelmatig benauwd. Kenmerkend voor de ziekte is dat de benauwdheid gepaard gaat met een piepende ademhaling, hoesten en het opgeven van slijm. Tussen de aanvallen van benauwdheid zijn patiënten doorgaans klachten vrij. Meestal ontstaan de klachten op jonge leeftijd en is astma een chronische ziekte waarvan patiënten gedurende hun hele leven last hebben. Het is echter ook mogelijk dat de eerste symptomen pas op latere leeftijd optreden. Allergieën en astma gaan vaak samen. Het merendeel van de patiënten is allergisch voor huisstofmijt, katten, honden, gras- en/of boompollen. Tot op heden is de ziekte nog niet te genezen; een goede behandeling is echter wel vaak mogelijk. Desondanks is het hebben van de ziekte astma voor veel patiënten een grote beperking in het dagelijks leven.

Het percentage van de bevolking dat astma heeft, verschilt van land tot land. Van een paar procent tot 1/3 van de mensen heeft symptomen. Bij 2 tot 12% van de personen is ook de diagnose astma gesteld. Voor Nederland betekent dit dat er ongeveer 430.000 mensen astma hebben. Dit maakt astma wereldwijd tot een van de meest voorkomende chronische ziekten. In de laatste decennia is het vóórkomen van astma flink toegenomen. In enkele landen zijn de percentages sinds de jaren 70 zelfs verdubbeld. Er is geen duidelijke verklaring voor deze stijging. Recente onderzoeken tonen echter aan dat het aantal mensen met astma momenteel gelijk blijft of zelfs afneemt. Het zal in de komende jaren duidelijk worden of deze trend doorzet.

Risicofactoren voor het krijgen van astma

Astma is deels een erfelijke ziekte. Dit betekent dat wanneer de moeder of de vader astma heeft, er een grote kans is dat hun kinderen de ziekte ook zullen krijgen. Het lijkt er echter niet op dat één duidelijke genetische afwijking hiervoor verantwoordelijk is. Zoals eerder aangegeven gaan allergieën en astma vaak samen. Allergisch zijn is dan ook de belangrijkste risicofactor voor het ontwikkelen van de ziekte. Ook is aangetoond dat een frequente blootstelling aan allergenen (huisstofmijt of dieren) in de omgeving, de kans op het ontstaan van astma vergroot. Daarnaast lijkt het erop dat het doormaken van bepaalde virusinfecties op jonge leeftijd het vervolgens krijgen van de ziekte waarschijnlijker maakt. Kinderen die opgroeien op een boerderij daarentegen blijken juist minder vaak allergieën en astma te hebben. Het bovenstaande suggereert dat het voorkomen van astma mogelijk zou moeten zijn. Onderzoeken die gedaan zijn op het gebied van preventie laten tot op heden teleurstellende resultaten zien. Zo bleek bijvoorbeeld het verminderen van de blootstelling aan huisstofmijt bij pasgeborenen niet te leiden tot minder astma. De meest overtuigende resultaten werden vooralsnog gevonden bij onderzoek naar het effect van probiotica. Het gebruik van deze probiotica (gezonde bacteriën) door zwangere vrouwen resulteerde namelijk in minder eczeem bij hun kinderen. Misschien biedt dit een mogelijkheid om in de toekomst de ontwikkeling van astma te voorkomen.

Luchtwegontsteking en astma

Het afweersysteem van patiënten met astma reageert uitzonderlijk op prikkels zoals bijvoorbeeld katten. Hierdoor ontstaat de chronische ontsteking van de luchtwegen die kenmerkend is voor astma. De slijmvliezen aan de binnenkant van de luchtwegen zwellen op en produceren meer slijm dan normaal. Ook trekken de spieren om de luchtwegen samen, waardoor de luchtwegen zich vernauwen. Dit geeft benauwdheid, hoesten en/of een piepende ademhaling. Diverse stoffen en cellen komen vanuit de bloedbaan de luchtwegen binnen en veroorzaken daar een ontstekingsreactie. Deze ontsteking begint direct na het inademen van allergenen en gaat ook nog langer door zodat de ontstekingsreactie chronisch wordt. Daarnaast leidt dit tot permanente veranderingen in de structuur en samenstelling van het longweefsel. Hierdoor is de longfunctie bij sommige patiënten blijvend verminderd.

Dit proefschrift

Dit proefschrift gaat over de luchtwegontsteking bij patiënten met astma. Hierbij is op drie verschillende manieren naar deze ontsteking gekeken.

- ontstekingsmediatoren bij astma (dit zijn stoffen die het lichaam zelf aanmaakt tijdens de ontstekingsreactie)
- het monitoren van luchtwegontsteking
- het behandelen van luchtwegontsteking

Rol van ontstekingsmediatoren

Achtergrond

IL-5 is een ontstekingsmediator die bij astma een belangrijke rol lijkt te spelen. Deze stof wordt door het lichaam zelf gemaakt. Bij gezonde personen is IL-5 niet of nauwelijks aanwezig, echter in patiënten met astma kan deze stof zowel in het bloed als in de longen aangetoond worden. Hoe ernstiger de ziekte, des te meer IL-5 er door het lichaam geproduceerd wordt. In het laboratorium en bij proefdieren is aangetoond dat IL-5 in actieve rol speelt tijdens de ontstekingsreactie. IL-5 blijkt namelijk de eosinofiel (een ontstekingscel) te laten rijpen en te activeren. Geactiveerde eosinofielen gaan dan vanuit de bloedbaan naar de longen. Hier veroorzaken zij de ontstekingsreactie, die vervolgens de voor astma zo bekende klachten van benauwdheid geeft. Het is echter nog onduidelijk of IL-5 ook de gevoeligheid van de luchtwegen voor prikkels verhoogt.
In **hoofdstuk 2** gaat het over de volgende vragen:

Onderzoeksvragen

- Verhoogt IL-5 de gevoeligheid van de luchtwegen voor prikkels?
- Is het effect dat IL-5 heeft op de ziekte afhankelijk van de plaats (bloedbaan of longen) in het lichaam waar deze stof zich bevindt?

Onderzoeksmethode
Patiënten met een lichte vorm van astma kregen IL-5 op 2 manieren toegediend: via inademing zodat de stof direct in de longen komt en via een prik zodat het direct in de bloedbaan komt. Vervolgens werden de hoeveelheid eosinofielen in het bloed en in de longen gemeten. Ook werd de gevoeligheid van de luchtwegen voor prikkels bepaald.

Resultaten
IL-5 in de bloedbaan bleek direct de hoeveelheid eosinofielen in het bloed te doen toenemen. IL-5 had echter geen effect op het aantal eosinofielen in de luchtwegen. Interessant was dat de gevoeligheid van de luchtwegen voor prikkels niet veranderde door de toediening van IL-5.

Conclusies
Het effect dat IL-5 heeft op de ontstekingsreactie bij patiënten met astma richt zich met name op de bloedbaan. Omdat IL-5 geen invloed blijkt te hebben op de gevoeligheid van de luchtwegen, speelt IL-5 waarschijnlijk geen cruciale rol bij astma. Een nieuw geneesmiddel dat de werking van IL-5 blokkeert, bleek inderdaad bij patiënten met astma niet effectief te zijn.

Monitoren van luchtwegontsteking

Achtergrond
De behandeling van astma is erop gericht om de luchtwegontsteking zoveel mogelijk te verminderen. Hierbij is het van belang dat een arts precies weet hoe ernstig deze ontsteking bij een patiënt is. Immers, ernstige luchtwegontsteking zou zware aanvallen van benauwdheid en ook blijvende schade aan de luchtwegen kunnen veroorzaken. Maar aan de andere kant leidt te veel medicatie ook tot onnodige bijwerkingen. De ernst van de ontsteking varieert van patiënt tot patiënt, maar ook bij een patiënt kan de luchtwegontsteking het ene moment ernstiger zijn dan op het andere. Het vragen naar de hoeveelheid klachten zou een betrekkelijk eenvoudige manier kunnen zijn om de ernst vast te stellen. Helaas blijken deze klachten geen goede weerspiegeling te zijn van de ernst van de ontsteking. Dit komt mogelijk omdat patiënten klachten niet altijd goed herkennen. Het kan ook veroorzaakt worden doordat klachten en ontsteking niet altijd hand in hand gaan.

Er zijn drie verschillen methoden ontwikkeld waarmee luchtwegontsteking op een voor de patiënt niet belastende manier gemeten kan worden.

Ten eerste het bepalen van de luchtwegovergevoeligheid voor de stof methacholine. Het inademen van methacholine geeft bij gezonde personen geen klachten. Patiënten met astma worden echter benauwd zodra zij methacholine inademen. Patiënten met ernstige luchtwegontsteking blijken al van een lage hoeveelheid methacholine benauwd te worden, terwijl patiënten met nauwelijks ontsteking pas na een hoge concentratie

methacholine klachten krijgen. In het laboratorium kan op een veilige en gecontroleerde manier de gevoeligheid van de luchtwegen voor methacholine bepaald worden.

Het bepalen van de hoeveelheid eosinofielen (ontstekingscellen) in het slijm is de tweede methode om luchtwegontsteking te bepalen. Een probleem hierbij is dat niet alle patiënten dit slijm spontaan kunnen ophoesten. Echter na het inademen van een zoute nevel lukt dit vaak wel. Vervolgens kan in dit opgehoeste slijm het aantal eosinofielen bepaald worden. Hiermee wordt de mate van luchtwegontsteking vastgesteld.

Tot slot komt er in de uitgeademde lucht een stof voor die geproduceerd wordt tijdens de ontstekingsreactie. Deze stof heet stikstof mono-oxide, afgekort NO. Door te blazen in een apparaat (als bij een alcohol blaastest) wordt de hoeveelheid NO in de uitgeademde lucht gemeten.

In **hoofdstuk 3** van dit proefschrift zijn deze drie methoden met elkaar vergeleken. Hierbij is gebruik gemaakt van een standaard onderhoudsmedicatie voor astma: inhalatie cortico-steroïden. Van deze medicatie is bekend dat het de ontstekingsreactie remt.

Onderzoeksvraag

- Welk effect heeft een handeling met inhalatie cortico-steroïden op gevoeligheid van de luchtwegen voor methacholine, op het aantal eosinofielen in het slijm en op de hoeveelheid NO in de uitgeademde lucht bij patiënten met astma?

Onderzoeksmethoden

Vijfentwintig patiënten met astma zijn gedurende 4 weken behandeld met inhalatie cortico-steroïden of placebo (een nepmiddel). Voor, tijdens en na deze behandeling zijn gevoeligheid van de luchtwegen voor methacholine, het aantal eosinofielen in het slijm en de hoeveelheid NO in de uitgeademde lucht bepaald.

Resultaten

De behandeling met inhalatie cortico-steroïden verminderde significant de gevoeligheid van de luchtwegen voor methacholine, het aantal eosinofielen in het slijm en de hoeveelheid NO in de uitgeademde lucht. Bij de patiënten die de placebo behandeling hadden gekregen, werden geen veranderingen gemeten. Echter de door inhalatie cortico-steroïden veroorzaakte afnames waren niet vergelijkbaar tussen de drie methoden. De ene patiënt kon bijvoorbeeld een grote daling in het aantal eosinofielen hebben en een kleine afname in NO, terwijl dit bij een andere patiënt juist weer het omgekeerde was.

Conclusies

Alle drie de methoden zijn goed bruikbaar om de ernst van de luchtwegontsteking vast te stellen. Ze geven echter wel verschillende informatie over deze ontsteking en moeten daarom misschien naast elkaar gebruikt worden.

Achtergrond
Luchtwegontsteking bij astma bestaat niet alleen uit ontstekingscellen en mediatoren. Het ontstaan van oedeem (zwelling door vocht) vormt ook een belangrijk onderdeel van het ontstekingsproces. Het is echter niet goed mogelijk om de ernst van het oedeem bij patiënten te meten. Het is bekend dat bij oedeemvorming, als gevolg van lekkage van de bloedvaten, eiwitten in de luchtwegen komen. Deze eiwitten kunnen gemeten worden in het opgehoeste slijm.
In **hoofdstuk 4** is onderzocht of opgehoest slijm geschikt is voor het monitoren van de ernst van oedeem. Hierbij is gebruikt gemaakt van substance P. Substance P wordt geproduceerd tijdens de ontstekingsreactie door de zenuwen in de luchtwegen. Het is een van de belangrijkste veroorzakers van oedeem en lekkage. Het inademen van substance P veroorzaakt benauwdheid bij patiënten. Als controle is gebruikt gemaakt van neurokinine A. Deze stof is erg vergelijkbaar met substance P. Het wordt ook door de zenuwen geproduceerd en geeft tevens benauwdheid. Neurokinine A veroorzaakt aan de andere kant geen oedeem en lekkage.

Onderzoeksvraag
- Is het mogelijk om in opgehoest slijm de ernst van oedeem te bepalen?

Onderzoeksmethoden
Zowel voor als na het inademen van substance P werden patiënten met astma gevraagd slijm op te hoesten. In dit slijm werden verschillende eiwitten die vrijkomen bij oedeemvorming gemeten. Tevens werden dezelfde eiwitten bepaald voor en na het inademen van neurokinine A.

Resultaten
Na inademing van substance P werden significant meer eiwitten gemeten dan na neurokinine A. Dit verschil was het duidelijkst meetbaar bij het eiwit alpha-2-macroglobuline.

Conclusies
Door middel van het inademen van substance P en het vervolgens ophoesten van slijm is het goed mogelijk om de ernst van oedeem te bepalen. Hiermee kan in de toekomst bijvoorbeeld ook het effect van nieuwe medicatie op oedeemvorming bepaald worden.

Achtergrond
Bij een bepaalde groep astmapatiënten blijkt de longfunctie na verloop van tijd blijvend af te nemen. Op dit moment is het nog niet mogelijk om te voorspellen bij welke patiënten dit het geval zal zijn. Het lijkt erop dat luchtwegontsteking hierin een belangrijke rol speelt.
In **hoofdstuk 5** is onderzocht of aan de hand van de ontsteking in de luchtwegen voorspeld kan worden welke patiënten een blijvende vermindering in longfunctie zullen krijgen.

Onderzoeksvragen

- Voorspelt luchtwegontsteking de blijvende vermindering in longfunctie?

Onderzoeksmethoden

Bij 32 patiënten met astma was, in een ander onderzoek 7½ jaar geleden, de ontsteking in de luchtwegen bepaald. Deze bepaling was gedaan in een biopt dat in de luchtwegen was genomen door middel van een bronchoscopie. Hierbij gaat de longarts met een slangetje via de keel naar de longen en neemt daar minuscule hapjes (biopten). In deze biopten kan bepaald worden welke ontstekingscellen er in de luchtwegen aanwezig zijn. In het huidige onderzoek is bij dezelfde mensen opnieuw de longfunctie bepaald. Vervolgens is berekend of en zo ja hoeveel, deze longfunctie was verminderd ten opzichte van 7½ jaar geleden. Tot slot is de relatie tussen de vermindering in longfunctie en de ernst van de ontsteking bepaald.

Resultaten

De verschillen tussen de patiënten waren erg groot. In de afgelopen 7½ jaar was bij sommige patiënten de longfunctie verbeterd, terwijl bij anderen de longfunctie met ruim 1 liter was afgenomen. Patiënten met een grote vermindering in longfunctie bleken meer CD8 cellen te hebben, dan patiënten bij wie de longfunctie hetzelfde was gebleven. Deze CD8 cel is een bekende ontstekingscel in de luchtwegen. De cel herkent virussen en allergenen die ingeademd worden. Nadat de cel geactiveerd is, worden andere ontstekingscellen aangetrokken en ook actief. Hierdoor ontstaat een ontstekingsproces waarbij ook structurele veranderingen in de luchtwegen zouden kunnen optreden.

Conclusies

Luchtwegontsteking, en specifiek de hoeveelheid CD8 cellen, voorspelt de vermindering in longfunctie bij patiënten met astma. Dit suggereert dat medicatie die gericht is tegen CD8 cellen mogelijk in de toekomst de blijvende vermindering in longfunctie kan voorkomen.

Behandelen van luchtwegontsteking

Achtergrond

Een arts zal bij de behandeling van astma er naar streven dat een patiënt zo min mogelijk symptomen heeft. De belangrijkste medicijnen hiervoor zijn ontstekingsremmers en luchtwegverwijders. Ontstekingsremmers bestrijden de ontsteking in de luchtwegen. Luchtwegverwijders zorgen ervoor dat de spiertjes rondom de luchtwegen verslappen, waardoor de luchtwegen bijna direct weer wijder worden en de benauwdheid verdwijnt. Ontstekingsremmers moet een patiënt dagelijks innemen, terwijl luchtwegverwijders alleen ingenomen hoeven te worden bij klachten van benauwdheid om deze snel te verminderen. Daarnaast is het van belang dat de longfunctie goed blijft. Deze longfunctie kan in het ziekenhuis gemeten worden, maar

ook thuis op een klein apparaat. Dit apparaat meet de maximale hoeveelheid uitgeademde lucht, de zogenaamde PEF. Door de PEF dagelijks 's ochtends en 's avonds te meten krijgt de arts een goed beeld van de longfunctie van een patiënt. Dus de hoeveelheid ontstekingsremmers die een arts voorschrijft, is afhankelijk van de symptomen, hoe vaak een patiënt luchtwegverwijders gebruikt en de longfunctie.
Als er een groot verschil is tussen de PEF die 's ochtends en 's avonds gemeten wordt, kan dit wijzen op een instabiele fase van de ziekte. Een maat om dit aan te geven, is de PEF variabiliteit. Deze PEF variabiliteit wordt op dit moment niet meegenomen in de behandeling van astma.
In **hoofdstuk 6** is onderzocht of behandeling op basis van de PEF variabiliteit zinvol is.

Onderzoeksvraag

- Voorspelt de mate van PEF variabiliteit of de behandeling van astma onder controle is?

Onderzoeksmethoden

Bij 75 patiënten met astma, die volgens de huidige richtlijnen behandeld werden, werd de PEF variabiliteit bepaald. Drie maanden later werd gekeken of bij deze patiënten het astma onder controle was.

Resultaten

De patiënten bij wie de PEF variabiliteit in het begin van het onderzoek hoog was, hadden 8 keer zoveel kans dat hun astma drie maanden later niet meer onder controle was.

Conclusies

De mate van PEF variabiliteit voorspelt of astma onder controle is. Deze maat dient daarom meegenomen te worden in de behandeling van astma.

Achtergrond

Recent is er een nieuw medicijn voor astma ontwikkeld: omalizumab. Dit medicijn is een antilichaam tegen IgE. Het stofje IgE wordt in het lichaam van allergische patiënten veel geproduceerd en staat aan de basis van het ontstekingsproces. Omalizumab bindt aan het IgE en zou op deze manier ontsteking verminderen, waardoor de allergie en daarmee astmaklachten zouden afnemen.
In **hoofdstuk 7** is onderzocht welk effect omalizumab heeft op de ernst van astma.

Onderzoeksvragen

- Remt omalizumab de benauwdheid na het inademen van allergenen?
- Voorkomt omalizumab ontsteking na het inademen van allergenen?
- Verbetert omalizumab de PEF?
- Verlaagt omalizumab de gevoeligheid van de luchtwegen voor methacholine?
- Vermindert omalizumab het aantal eosinofielen in het slijm?

Onderzoeksmethoden
Gedurende 12 weken zijn 25 astmapatiënten behandeld met omalizumab of placebo. Voor en na deze behandeling is de benauwdheid na het inademen van allergenen gemeten en is vervolgens met behulp van een bronchoscopie in een biopt de ontsteking bepaald. Daarnaast hebben de patiënten dagelijks hun longfunctie door middel van de PEF gemeten. Ook werd de gevoeligheid van de luchtwegen voor methacholine vastgesteld en het aantal eosinofielen in het slijm bepaald.

Resultaten
Na 12 weken was de PEF verbeterd in de patiënten die met omalizumab behandeld waren. Daarnaast bleek het inademen van allergenen geen benauwdheid of luchtwegontsteking meer te geven. Ook was het aantal eosinofielen in het slijm sterk verminderd. De gevoeligheid voor methacholine echter was onveranderd gebleven.

Conclusies
Omalizumab blijkt zowel luchtwegontsteking als klachten te doen afnemen bij patiënten met astma. Het feit dat omalizumab de gevoeligheid voor methacholine niet vermindert, dient verder onderzocht te worden.

Conclusies

De belangrijkste conclusies die getrokken kunnen worden, staan hieronder vermeld:

- Het effect dat IL-5 heeft op de ontstekingsreactie bij patiënten met astma richt zich met name op de bloedbaan (hoofdstuk 2).
- De gevoeligheid van de luchtwegen voor methacholine, het aantal eosinofielen in slijm en de hoeveelheid NO in de uitgeademde lucht zijn 3 methoden die goed bruikbaar zijn om de ernst van de luchtwegontsteking vast te stellen (hoofdstuk3).
- Door middel van het inademen van substance P en het vervolgens ophoesten van slijm is het goed mogelijk om de ernst van oedeem te bepalen (hoofdstuk 4).
- Luchtwegontsteking, en specifiek de hoeveelheid CD8 cellen, voorspelt de vermindering in longfunctie bij patiënten met astma (hoofdstuk 5).
- De mate van PEF variabiliteit voorspelt of astma onder controle is (hoofdstuk 6).
- Omalizumab blijkt zowel luchtwegontsteking als klachten te doen afnemen bij patiënten met astma (hoofdstuk 7).

Vragen

Dit proefschrift levert naast resultaten en conclusies, ook een aantal vragen op. Hieronder staan de belangrijkste:

- Welke methode om luchtwegontsteking te monitoren is het meest kosten-effectief?
- Is het mogelijk om de behandeling van astma te verbeteren als de ernst van luchtwegoedeem wordt meegenomen?
- Welke meting voorspelt de ernst van astma op de lange termijn?
- Is het mogelijk om astma beter onder controle te krijgen als de mate van PEF variabiliteit bepaald wordt?
- Is het op den duur ongunstig voor de patiënt dat de verhoogde gevoeligheid van de luchtwegen voor methacholine na behandeling met omalizumab onveranderd aanwezig blijft?

Chapter 9

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Curriculum Vitae

Liesbeth van Rensen was born on 5 October 1973, in Venlo, The Netherlands. From 1986 she received secondary education at the Thomas College in Venlo. After graduation (VWO) in 1992, she started to study Biomedical Sciences at the University of Leiden. Practical training periods were performed at the departments of Rheumatology (Prof. dr. T.W.J. Huizinga) and Pulmonology (Prof. dr. P.J. Sterk) of the Leiden University Medical Centre (LUMC). Additional courses were followed on health promotion at the Faculty of Social and Behavioural Sciences and on health law at the Faculty of Law at the University of Leiden. A Master of Science degree was obtained in 1998.

From June 1998 until June 1999 she worked as a research fellow at the department of Thoracic Medicine, National Heart and Lung Institute, Imperial College, London, United Kingdom (Prof. dr. K.F. Chung and Prof. dr. P.J. Barnes). From June 1999 until August 2005 she worked as a research fellow at the department of Pulmonology of the LUMC (Prof. dr. P.J. Sterk and Prof. dr. K.F. Rabe). Most of the studies that were performed during this period are included in the thesis.

Between August 2000 and March 2005 courses were followed at the Netherlands School of Public and Occupational Health, Amsterdam, and the Netherlands Institute for Health Sciences, Rotterdam, and an international Master of Public Health degree was obtained.

Since May 2005 she is working as a programme officer for the Health Care Efficiency Research Programme of ZonMw. Here she is a co-ordinator of the Early Evaluation of Medical Innovation programme.

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