Difficult-to-treat asthma : mechanisms and risk factors

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Chapter 8

Summary and general discussion

Summary

Around 5 to 10% of patients with asthma do not respond adequately to inhaled steroids and long-acting bronchodilators and become difficult-to-treat; they remain symptomatic, have recurrent exacerbations or persistent airflow limitation. This thesis focuses on the mechanisms that may explain why these patients become difficult-to-treat, describes risk factors that are associated with difficult-to-treat asthma and investigates biomarkers that can predict the development of specific asthma phenotypes. The main conclusions from the studies in this thesis are summarized below.

Mechanisms that may explain the development of persistent airflow limitation
In **chapter 3** we investigated the role of alpha-1-antitrypsin deficiency in the development of persistent airflow limitation in patients with difficult-to-treat asthma. The prevalence of a non-PiM alpha-1-antitrypsin phenotype in our cohort of patients with difficult-to-treat asthma was low (4.9%). We demonstrated that persistent airflow limitation was not associated with a non-PiM phenotype in these patients. Therefore alpha-1-antitrypsin heterozygosis does not seem to be an important contributing factor for a rapid decline in lung function in asthmatic patients.

The aim of the study in **chapter 4** was to investigate the degree of peripheral airway dysfunction and peripheral airway inflammation, and their relationship in patients with severe asthma compared to patients with mild-to-moderate asthma. To assess peripheral airway inflammation a rather new measure was used; alveolar nitric oxide (alveolar NO), derived from nitric oxide levels in exhaled breath. The results showed that alveolar NO is closely related to parameters of peripheral airway dysfunction in patients with severe asthma, but not in patients with mild-to-moderate asthma. The fall in forced vital capacity (FVC) at the provocative concentration of methacholine causing a 20% fall in FEV_1 (ΔFVC) was higher, and the slope of the nitrogen washout curve (dN_2) was steeper in patients with severe asthma. There was no difference in alveolar NO between patients with severe and mild-to-moderate asthma. However, those patients who were on continuous oral corticosteroid treatment had more peripheral airway inflammation and dysfunction than patients with difficult-to-treat asthma who were treated with inhaled corticosteroids only, and than patients with mild-to-moderate asthma. This suggests that patients on chronic oral steroid treatment have more extensive airway disease and require additional anti-inflammatory treatment to better target the peripheral airways. Alveolar nitric oxide may become an important tool to detect peripheral airway disease in patients with asthma and to evaluate new therapeutic strategies.

The obese phenotype

In **chapter 5** we investigated the clinical and inflammatory profile of obese patients with difficult-to-treat asthma as compared to non-obese patients who were difficult-to-treat. The results of this study showed that obese patients with difficult-to-treat asthma do not have more severe airway inflammation as compared to nonobese patients. However, differences were observed in lung function parameters and co-morbid factors: obese patients had a lower FRC/TLC (functional residual capacity/total lung capacity) and an increased number of co-morbid factors as compared to non-obese patients. We concluded that obese patients with asthma may exhibit more severe asthma symptoms or even become difficult-to-treat because of an unfavourable effect of overweight on lung function, or because of aggravating co-morbid factors such as gastroesophageal reflux and obstructive sleep apnoea.

Predictors of an accelerated decline in lung function

It has been shown that a subgroup of patients with asthma have an accelerated decline in lung function. In **chapter 6** we studied the decline in FEV_1 in our cohort of patients with difficult-to-treat asthma over a period of 5-6 years and determined predictors of an accelerated decline in FEV_1. A subgroup of patients (39%) had an accelerated decline in FEV_1 defined as a fall in FEV_1 ≥ 25ml/year. Patients with a high level of exhaled nitric oxide (FeNO ≥ 20ppb) had an increased risk for this accelerated decline (relative risk (RR):1.9) as compared to patients with low FeNO levels, especially when they had normal lung function at baseline (RR:3.1).

Persistence of the eosinophilic phenotype

Chapter 7 shows that the presence of sputum eosinophilia, defined as sputum eosinophils ≥ 2%, in patients with difficult-to-treat asthma is a consistent feature over a period of five years in the vast majority of patients and that the percentage of sputum eosinophils is highly reproducible. Adult onset asthma, extensive sinus disease, high peripheral blood eosinophil counts, and persistent airflow limitation are associated with permanent sputum eosinophilia, but extensive sinus disease is the only independent factor. FeNO levels are only weakly correlated with sputum eosinophils, but show similar consistency over time. This implies that patients with eosinophilic airway inflammation despite vigorous steroid treatment do represent a separate phenotype that persists over time, and that FeNO may provide additional information on the inflammatory process in the airways.

General discussion

Which pathophysiological mechanisms are involved in the development of persistent airflow limitation?

Persistent airflow limitation is a common finding in patients with difficult-to-treat asthma (1). Cross sectional analysis of our cohort showed a prevalence of 49% when persistent airflow limitation was defined as a postbronchodilator FEV_1 (forced expiratory volume in one second) or FEV_1/FVC (forced vital capacity) <75% predicted with TLC (total lung capacity) >75% predicted (2). Patients with persistent airflow limitation suffer from persistent symptoms of dyspnoea, reduced exercise tolerance and impaired quality of life. In order to identify a treatment strategy for these patients or, more important, to prevent an accelerated decline in lung function, we need to clarify the mechanisms of a more than physiological decline in lung function that may eventually result in persistent airflow limitation.

Structural changes in the airways, referred to as remodelling, are currently thought to affect lung function in asthma. These structural changes might be the result of ongoing inflammation in the airways, however**,** since structural changes can already be seen in early childhood it is questionable whether these two phenomena have a causal relationship or occur at the same time (3). Until today, it is not known which components of airway remodelling contribute to fixed airway obstruction. One of the components that may be responsible is hypertrophic or hyperplastic airway smooth muscle, since recent studies have demonstrated a relationship between increased airway smooth muscle mass, more severe asthma and lower FEV_1 (4;5).

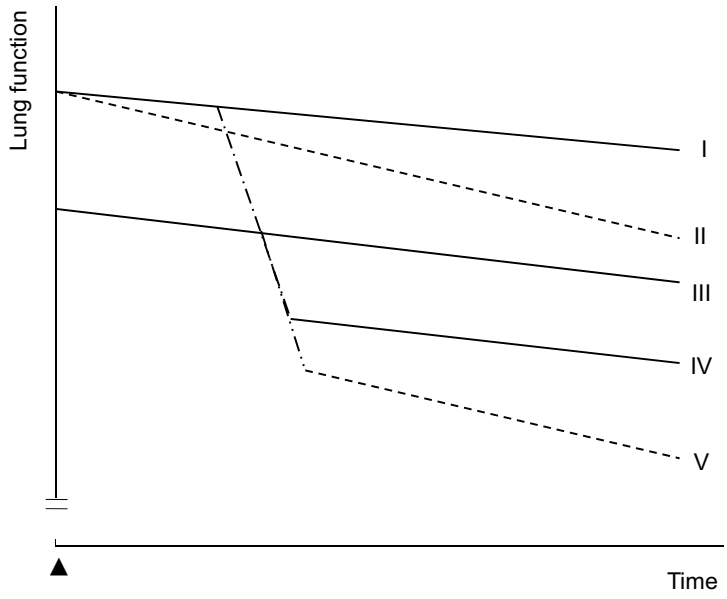
Lung function loss can occur at different stages in life. It has been demonstrated that lung function impairment can take place already in childhood, resulting in an impaired lung function at the start of adulthood (6). Another possibility is that patients with asthma suddenly experience a loss of lung function during adolescence or adulthood after a respiratory tract infection, for example with *Chlamydia pneumonia.* Chronic *Chlamydia pneumoniae* infection, diagnosed by serological testing, was found to accelerate the loss of lung function significantly in subjects who newly contracted nonatopic asthma (7). A third possibility is that lung function gradually deteriorates during adulthood (e.g. due to smoking or recurrent exacerbations (8)) or (fourth option) that a combination of events occurs during life (Figure 1).

The risk factors of an accelerated loss of lung function probably differ within the different time-points in life at which they occur. Most likely, several risk factors together, such as genetic factors, e.g. ADAM 33 (9;10), environmental factors e.g. exposure to cigarette smoke

or infection with intracellular pathogens (7;11;12) and intrinsic factors (e.g. the presence or absence of atopy) lead to the development of structural alterations in the airway wall that ultimately cause fixed airway obstruction (13). In this thesis potential mechanisms that may explain the development of persistent airflow limitation have been investigated.

One of the mechanisms that can cause persistent airflow limitation is deficiency of alpha-1-antitrypsin (AAT). Patients with a deficiency of AAT, especially when they are ZZ homozygote, have an increased risk of developing chronic airflow limitation and lung emphysema (14). In our cohort of patients with difficult-to-treat asthma we could not find any association with heterozygote phenotypes of AAT and persistent airflow limitation. Although our group of patients was rather small and the results, therefore, need confirmation in longitudinal studies with larger cohorts, at present alpha-1-antitrypsin deficiency does not seem to be an important contributor to persistent airflow limitation in this group of patients. In the recommendations of the American Thoracic society and European Respiratory Society of 2003 (15) AAT screening is recommended in patients with asthma and persistent airflow limitation. However**,** the results of our study at least question the routine assessment of alpha-1-antitrypsin levels in this group.

Figure 1. Possible patterns of lung function decline in asthma.



▲: start adulthood; I: normal physiological decline, II: gradual accelerated decline III: low lung function at start of adulthood due to loss in childhood, IV: sudden decline, V: combination of patterns.

Ongoing, inadequately treated, (eosinophilic) inflammation in the small airways might be another mechanism that can result in persistent airflow limitation. Inflammation and dysfunction of the small airways (< 2 mm diameter) has been related to unstable asthma (16), therapy resistance (17) and excessive airway narrowing (18). Although the contribution of small airway pathology to the manifestation of obstructive airway disease has been studied for decades, it appears extremely difficult to develop a precise and noninvasive tool to measure abnormalities in the peripheral airways, the so called “silent zone” of the lung (19).

In 1978, a study of Cosio et al. (20) demonstrated, for the first time, a relationship between small airway function, described by the slope of the nitrogen washout curve, and inflammation in resected lung tissue. The discovery of the possibility that this noninvasive test reflects small airway inflammation, was a great break-through. In the years after, several other noninvasive techniques have been developed to measure ventilation heterogeneity and airway closure such as: multiple breath nitrogen washout test (21), forced oscillation technique (22), high resolution CT scanning (23) and hyperpolarized helium MRI (24).

In 1998, a mathematical model has been developed (25) that could distinguish between nitric oxide deriving from large airways (bronchial NO) and small airways (alveolar NO), thereby providing another noninvasive tool to assess small airway inflammation. Clinical studies, using this model, showed that alveolar NO was associated with eosinophilia in bronchial alveolar lavage (26) and that alveolar NO levels were increased in nocturnal asthma (27). These results raised the question whether alveolar NO would be associated with measures of peripheral airway dysfunction. This was investigated in the study of chapter 4. In addition, we compared the degree of peripheral airway dysfunction and -inflammation between patients with mild-to-moderate and severe asthma.

The results of this study demonstrated a close relationship between alveolar nitric oxide and parameters of peripheral airway dysfunction in severe, but not in mild-to-moderate asthma. Patients with severe asthma did have a higher dN2, pointing towards more ventilation inhomogeneity, and a higher ΔFVC, reflecting more airway collapse during bronchial provocation. Alveolar NO was not different between patients with mild-to-moderate and severe asthma, which might be explained by the large variation in the measured levels. Analysis of a subgroup of severe asthma patients, those who were oral-steroid dependent, revealed that these patients had higher alveolar NO levels as compared to other patients with severe asthma and those with mild-to-moderate disease. This was rather surprising since one would expect that oral corticosteroids in these patients would have reduced the inflammation of the small airways, because of their systemic effect. A later study by

Brindicci et al. also demonstrated higher alveolar NO levels in patients with oral steroid dependent asthma (28). Apparently small airway involvement can be so pronounced or relatively therapy resistant that it cannot be fully controlled by oral corticosteroids and that higher oral doses, parenteral administration or other therapeutic strategies are needed. It has been shown that parenteral administration of high doses of triamcinolon in patients with severe asthma and 'persistent' eosinophilia can reduce sputum eosinophils and results in an improvement of FEV_1 possibly by reducing small airway pathology (17).

The measurement of alveolar NO is an easy to perform, noninvasive tool to assess the peripheral airways. Further theoretical studies have tried to optimize the mathematical model (29) and in clinical studies lower exhalation flows are now being used. This is certainly recommended since higher flows (>250ml/sec) are more difficult to perform and have a poorer signal/noise ratio. In conclusion, the role of small airway disease in the clinical manifestation of asthma remains intriguing. The measurement of alveolar nitric oxide provides us with a new tool to study peripheral airway inflammation and to evaluate the effect of modified therapies such as small-particle inhaled steroids (30).

Which patients are at risk of developing persistent airflow limitation? : predictors of an accelerated decline in lung function.

In 1998 Lange et al. (11) demonstrated that subgroups of patients with asthma, especially male smokers, have an accelerated decline in FEV_1 over time as compared to other asthmatic patients and healthy individuals. This accelerated decline in lung function can result in persistent airflow limitation as has been discussed in the previous chapter. In order to treat patients who may develop persistent airflow limitation at an early stage, it is of paramount importance to detect those patients that are at risk. Apart from determining clinical risk factors, studies have focused on biomarkers that might be associated with an increased risk of (developing) persistent airflow limitation. Cross-sectional studies demonstrated that eosinophilia in blood (31), sputum (2) and bronchial biopsies (32) are associated with persistent airflow limitation. Longitudinal studies have shown a relationship between CD8-positive T-cells in bronchial biopsies and annual decline in FEV_1 (33).

In chapter 6 we studied the decline in FEV_1 over a period of 5-6 years in patients with difficult-to-treat asthma. Potential risk factors (age of asthma onset, duration of asthma, atopy, airway hyperresponsiveness, eosinophils in blood and sputum and the fraction of nitric oxide in exhaled air (FeNO) were assessed at baseline, and the relationship with the change in FEV_1 over 5-6 years was investigated. The median decline in FEV_1 was 12.6 ml/year; 39% of patients had an accelerated decline in FEV_1 (≥ 25 ml/year). Among all parameters that were studied, only FeNO was associated with a decline in FEV_1. Patients with a FeNO ≥

20ppb had a 2 times greater risk of an accelerated decline in FEV_1, which further increased to a relative risk of 3.1 for those who had a normal FEV_1 at baseline. Although previous cross-sectional studies showed a relationship between eosinophilic inflammation and persistent airflow limitation we could not confirm this in our longitudinal study. This despite the fact that sputum eosinophils and FeNO showed a modest correlation on both time points. The association between sputum eosinophils and decline in FEV_1 was, however, so weak that a lack of power cannot be regarded a satisfactory explanation for the discrepancy in findings between previous cross-sectional studies and our longitudinal analysis. Apparently, the information provided by exhaled nitric oxide and sputum eosinophils is not simply interchangeable. In the next paragraph the similarities and dissimilarities between sputum eosinophils and exhaled nitric oxide will be further discussed.

The finding that the association between FeNO and lung function decline is most prominent in those who have a normal lung function at baseline is intriguing and cannot be easily explained. One might speculate that the patients with $FEV_1 \geq 80\%$ of predicted represent a more homogenous group than the patients with an already impaired lung function. This latter group may consist of patients who have suffered from insufficient lung growth as a child, have had accelerated decline in lung function in the past but are now stable, are not optimally controlled at the time of the assessment, or are still experiencing an increased decline in lung function. These four groups probably behave differently: no or minor change in lung function in the first two groups, an improvement in the third group, and a further decline in the fourth. This heterogeneity in the group of patients with already impaired lung function might have biased the relationship between FeNO and decline in lung function in the group of patients with an $FEV_1 < 80\%$ of predicted at baseline. In addition, the function of nitric oxide in the airways is far from clear as it can have pro-inflammatory as well as anti-inflammatory effects and can enhance as well as attenuate smooth muscle contraction. One can only speculate that a high FeNO in those with preserved as compared to those with impaired lung function should be interpreted differently.

Is exhaled nitric oxide more than a surrogate marker of eosinophilic inflammation?

Around 1990 several studies showed that the concentration of nitric oxide (NO) was increased in the exhaled air of patients with asthma (34;35). NO and its related compounds (NO- and NO+) are synthesized by a variety of airway residential cells (epithelial, endothelial and neuronal cells) and inflammatory cells. Nitric oxide is produced from arginine by NO synthases (NOS) which occur in three isoforms: constitutive neuronal nNOS, constitutive endothelial eNOS and inducible iNOS. Inducible NOS can be induced by several cytokines, chemokines, and mediators.

The exact function of nitric oxide in the lung is still far from clear; it can have protective effects (smooth muscle relaxation, attenuation of airway hyperresponsiveness) as well as deleterious effects (pro-inflammatory activities, increased airway secretions, necrosis). NO can be regarded as a free radical that interacts with oxygen, to form reactive nitrogen species. This so called 'nitrosative stress' can eventually induce tissue damage in the airways (36). In addition to the finding that exhaled nitric oxide levels are increased in asthma, different clinical studies have shown that exhaled NO increases during asthma exacerbations, decreases after treatment with inhaled corticosteroids and is associated with eosinophilic airway inflammation (37).

In the last ten years several studies have investigated the clinical application of sputum eosinophils and exhaled nitric oxide in asthma management. Since the assessment of sputum eosinophil percentages was introduced years before nitric oxide measurements became available, most hypotheses are studied with sputum eosinophils first, later followed by studies who used exhaled nitric oxide as a tool. Several clinical applications have been evaluated in these studies:

- *Diagnosis of asthma*: It has been shown that in adults with symptoms suggestive of asthma the presence of sputum eosinophilia (>3%) as well as a FeNO >20ppb are helpful in differentiating between patients with and without asthma, with similar sensitivities (86-88%) (38).

- *Predicting loss of asthma control*: Several studies have looked at the value of biomarkers in predicting loss of control after steroid withdrawal. Although there are conflicting results, sputum eosinophils seem to be more accurate in predicting loss of control or in predicting whether patients require ongoing steroid treatment. However, in view of the availability in the clinic measurement of FeNO can be an alternative (37;39-42)

- *Guidance of treatment decisions*: two studies showed that management strategies based on sputum eosinophil counts reduced the number of asthma exacerbations significantly (43;44). Similar studies with FeNO based treatment strategies did not result in a reduction in exacerbations, although one study did achieve a reduction in inhaled steroid dose in the FeNO group, possibly the result of more individualized therapy (45-47).

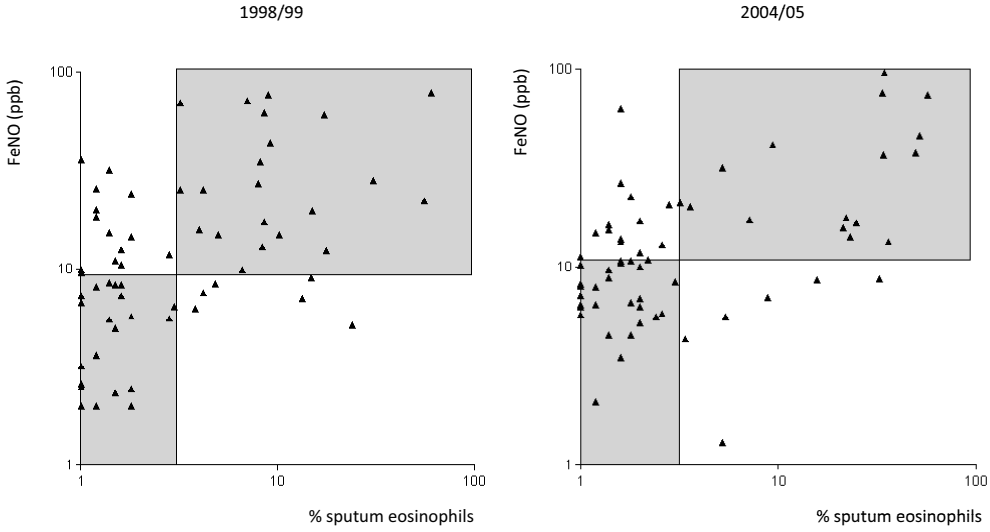
- *Predicting loss of lung function*: as has been shown in chapter 6, FeNO, but not sputum eosinophils, predict lung function decline in patients with difficult-to-treat asthma. However, in cross-sectional studies, sputum eosinophil percentage and not FeNO is associated with FEV_1. No other studies at present have investigated the value of FeNO and sputum eosinophils in predicting lung function loss in asthma.

What can we conclude from all these studies? First of all, that sputum eosinophils and FeNO do not provide the same information about inflammatory or other processes in the airways. This may have to do with the more or less arbitrarily chosen cut-off points for both FeNO and sputum eosinophils that are not necessarily biologically identical. In the study described in chapter 6 we showed a positive association between sputum eosinophils and FeNO at both study time points (as many other studies did before), but one cannot deny that this correlation is far from perfect.

Figure 2 demonstrates that there are many patients (30%) showing discordance between FeNO levels and sputum eosinophil percentages: either they have high FeNO and low eosinophils (21%) or the other way around (9%). This discrepancy has also been discussed by others (45). What can be the explanation for this? Some believe that this discordance is probably caused by the inconsistence of FeNO measurements because this measurement is much more influenced by external factors than the assessment of eosinophil numbers (48). However, short-term repeatability of FeNO levels has shown to be very good and even in the long run, as shown in chapter 7, Figure 3, FeNO levels are consistent. Another option can be that FeNO might be better correlated to eosinophils in another compartment of the airway as has been demonstrated by the study from Lemiere et al. (49) A third explanation is that FeNO is not such a good marker of eosinophilic inflammation after all. However, one could also argue that FeNO is more than a surrogate marker of eosinophilic inflammation. Yes, it gives information about eosinophilic inflammation, but FeNO is also a biomarker for the metabolism of physiologically relevant nitrogen oxides. Reactive nitrogen species formed by the interaction of NO with oxygen radicals, can induce airway damage and possibly be involved in lung function impairment (50).

The role of eosinophils in the pathogenesis and manifestation of asthma remains unclear. Initial studies that used anti-interleukins such as anti-IL5 (51), demonstrated a clear reduction of tissue eosinophils towards normal values but this was not accompanied by an effect on either lung function or airway hyperresponsiveness. The dissociation between the effects of these drugs on eosinophils on one hand and methacholine responsiveness on the other suggests that eosinophils may not mediate methacholine responsiveness in asthma. Sputum eosinophilia seems to be a marker of uncontrolled disease, rather than a

Figure 2. Relationship between sputum eosinophil percentage and FeNO.



Boxes represent patients with high FeNO and high eosinophils or low FeNO with low eosinophils, other patients have discordant data. Cut-off points on x-axes: 2% sputum eosinophils, on y-axes median FeNO. X- and Y-axes are logarithmic. X-axes represent sputum eosinophil percentages +1; n=63 in both graphs.

cause of it. However, more recent studies have investigated the effect of Mepolizumab, a humanized monoclonal antibody against interleukin-5, on patients with refractory asthma and persistent eosinophilic inflammation (52-54).

These studies showed a reduction in exacerbations, an improvement in asthma quality of life, a reduction in blood and sputum eosinophils and allowed prednisone sparing in this specific subgroup of patients. However there was no effect on lung function, airway hyperresponsiveness or exhaled NO levels, again confirming that the information provided by exhaled NO and eosinophils is not congruent. These new studies confirm the hypothesis that some therapies may have effect only in a very well characterized and thus very small subgroup of patients with difficult-to-treat asthma such as those with persistent eosinophilic inflammation (see also discussion of chapter 7).

In conclusion, for some clinical purposes, such as the diagnosis of asthma or predicting loss of asthma control, sputum eosinophils and exhaled nitric oxide seem to be both applicable in the clinic, whereas exhaled nitric oxide measurements have the advantage of being noninvasive and to provide immediate results. Concerning biomarker-based

treatment strategies, sputum eosinophils are definitely on the lead. Whether exhaled NO has more potential as a marker of progressive loss of lung function, as our results of chapter 6 suggest, needs to be confirmed in the future by larger studies with serial lung function measurements. After this, the next challenge will be to evaluate the effect of nitric oxide reducing therapeutic strategies on lung function decline.

Phenotypes of difficult-to-treat asthma, are they persistent over time?

A phenotype can be defined as "the visible properties of an organism that are produced by the interaction of genotype and the environment" (55). It is now more than clear that difficult-to-treat asthma is a heterogeneous condition and we need to define different phenotypes. This can improve our understanding of the underlying mechanisms, natural history and prognosis of this disease; it can help to guide current and possibly future treatment and may provide clues for novel therapeutic interventions(56).

Several phenotypes have already been determined, based on *clinical characteristics* such as atopic vs. non-atopic asthma, early vs. late onset, stable *vs*. frequent exacerbations; on *physiological parameters*; normal lung function *vs.* fixed airflow limitation; on *specific triggers*; e.g. aspirin or NSAID; and on *pathological findings*: eosinophilic, non-eosinophilic, neutrophilic and pauci-granulocytic asthma (57). In order to evaluate the effect of a phenotype specific asthma drug, the phenotype should be consistent over a period of time.

In this thesis we have investigated two specific asthma phenotypes: the obese- (chapter 4) and the eosinophilic phenotype (chapter 6). We demonstrated that obese patients with difficult-to-treat asthma had less signs of airway inflammation (lower sputum eosinophils and lower FeNO) than nonobese patients with difficult-to-treat asthma. Obese patients had reduced lung volumes (FRC/TLC) and more co-morbid factors as compared to nonobese patients. These results fit in with the hypothesis that obese patients with asthma may become (more easily) difficult-to-treat because of the unfavorable effect of adipose tissue on lung function and an increased number of asthma aggravating co-morbid factors such as gastroesophageal reflux and sleep apnoea. Recent studies support this conclusion: obese people with asthma have poorer asthma control (assessed by an asthma control questionnaire) than nonobese asthmatics despite similar symptom perception (58). The bronchial and systemic inflammatory characteristics and the specific pattern of pulmonary function changes in the obese subjects suggests a different phenotype of asthma in this group of patients (58). A study using cluster analysis to define asthma phenotypes identified the obese, non-eosinophilic phenotype as one of the four clusters in refractory asthma(59). Treatment of patients with the obese asthma phenotype should not only consist of anti-inflammatory drug therapy, but need to be focused on weight reduction and adequate

control of asthma aggravating factors. Longitudinal studies following patients with asthma who become obese can further elucidate the role of obesity in the clinical manifestation of asthma and the effect of weight loss on asthma control (60;61).

In chapter 7 we showed that the eosinophilic phenotype is a consistent feature in the majority of patients over a period of five years. Extensive sinus disease is the only independent factor associated with permanent sputum eosinophilia. This implies that patients with difficult-to treat asthma measured on one occasion are likely to keep this phenotype over time especially when they have extensive sinus disease. These are the patients that should be selected when further novel therapies are being studied that aim for reducing sputum eosinophilia to improve asthma outcome. Recently, two separate clinical trials have demonstrated that administering anti-interleukin-5 to a strictly selected subgroup of patients with refractory asthma and eosinophilia does result in positive clinical effects (52-54).

An overview of the prevalence and consistency of different phenotypes in our cohort of patients with difficult-to-treat asthma is shown in Table 2 and 3. The presence of the different phenotypes could not be studied in all patients; therefore the data should be interpreted with caution. The presence (or absence) of sputum eosinophilia seems to be the most stable phenotype. Frequent exacerbations and oral steroid dependence show more inconsistency over time, whereas on the other hand persistent airflow limitation shows more consistency.

Table 2. Prevalences of asthma phenotypes.

	Prevalence at baseline (n=136)	Prevalence at follow-up (n=101)
frequent exacerbations	28.7	34.6
persistent airflow limitation	48.5	41.8
oral steroid dependent	32.3	24.5
eosinophilic inflammation	46.2	36.9
neutrophilic inflammation	50.8	66.2
no clinical phenotype	16.2	26.5
no inflammatory phenotype	27.7	16.9
no phenotype	1.0	2.0

Frequent exacerbations (≥ 3 prednisone courses/yr), n=45; persistent airflow limitation (FEV_1 or FEV_1/VC <75% pred and TLC >75%pred), n=97; oral corticosteroid dependent (chronic oral steroid use), n=100; eosinophilic inflammation (sputum eosinophils≥ 2%), n=44; neutrophilic inflammation (sputum neutrophils ≥ 65%), n=44.

Table 3. Persistence of asthma phenotypes.

	Persistence of phenotype (%)	Persistence of phenotype absence (%)	Concordance(%)
frequent exacerbations	54	95	71
persistent airflow limitation	72	84	78
oral steroid dependent	54	90	79
eosinophilic inflammation	70	96	84
neutrophilic inflammation	74	52	64

Frequent exacerbations (≥ 3 prednisone courses/yr), n=45; persistent airflow limitation (FEV_1 or FEV_1/VC <75% pred and TLC >75%pred), n=97; oral corticosteroid dependent (chronic oral steroid use), n=100; eosinophilic inflammation (sputum eosinophils≥ 2%), n=44; neutrophilic inflammation (sputum neutrophils ≥ 65%), n=44.

Some patients demonstrated an improvement in their asthma during the study. After 5-6 years of follow-up 22% of our patients were no longer classified as difficult-to-treat. At this second visit, fifteen patients had intermittent (n=1), mild (n=4) or moderate asthma (n=10). Three patients had high doses of inhaled or oral steroids and no exacerbations or persistent airflow limitation. According to the ATS definition these three patients meet the criteria for refractory asthma, but they do not meet the initial inclusion criteria of difficult-to-treat asthma of our cohort. One patient was diagnosed with and treated for pulmonary emboli. He stopped all asthma drugs and was symptom free.

Many asthma phenotypes have been defined in the literature, but the main question is which of them are helpful in determining the prognosis of the disease, or the response to (novel) therapy. Will it be possible to characterize all patients with difficult-to-treat asthma in the future? When regarding the most commonly used phenotypes at present, around 74% of the patients that were studies in this thesis fitted in (at least) one of the three clinical phenotypes, and 83% in one of the two inflammatory phenotypes. Nearly hundred percent of patients met the criteria for either a clinical or an inflammatory phenotype (Table 2). However, phenotypes based on a single characteristic do not seem to be the most optimal phenotypes. In order to find key phenotypes that will make individualized asthma treatment possible, characterization of asthma patients should combine genetic, clinical, environmental and inflammatory features.

It is not unlikely that in the future phenotyping of the individual severe asthma patient in the clinic will determine the correct add-on treatment, after, for example, a combination therapy of inhaled steroids and long-acting beta agonist has failed. It is now the challenge to explore which patients benefit most from current and newly developed therapies, such as macrolides, anti-IgE and drugs that target specific cytokines (e.g. IL4, IL5, TNF-alpha), inflammatory cells (TH2 cells, eosinophils) or inflammatory mechanisms (e.g. via p38 map-kinase) (60;62).

Implications for clinical practice

The articles in this thesis have focused on mechanisms and risk factors of difficult-to-treat asthma. Various biomarkers have been investigated to evaluate whether these measurements have any diagnostic or prognostic value. The question is which of these biomarkers has the potential, now or in the future, to be of clinical significance and become part of daily clinical practice.

Body mass index is a composite, simple to determine biomarker, which every doctor should take into account when treating a patient with asthma. It is important to realize, as discussed in chapter 5, that obese patients are prone to have specific asthma aggravating factors due to their overweight. Treatment of these obese asthma patients should therefore consist of proper evaluation and treatment of these factors as well as weight reduction in addition to regular asthma treatment.

A few years ago, two studies demonstrated that sputum eosinophil counts are useful in guiding steroid therapy in order to prevent exacerbations (43;44). In this thesis we demonstrated that sputum eosinophilia in patients with difficult-to-treat asthma is a consistent phenotype over time (chapter 7). Research trials evaluating new asthma drugs should focus on patients with well determined consistent phenotypes who are likely to benefit most. The advantage of this strategy has recently been demonstrated in two studies that investigated the effect of humanized anti-interleukin-5 in asthma patients with persistent sputum eosinophilia (52-54). Although determination of sputum eosinophils seems to have clinical value there is a main disadvantage. The induction of sputum is time-consuming and processing of sputum requires proper equipment, expertise, and also a significant amount of time. As long as this process cannot be shortened it does not seem to have a chance to become part of daily clinical practice.

Numerous studies have evaluated nitric oxide in exhaled breath as a surrogate marker of eosinophilic airway inflammation. However, exhaled nitric oxide levels and sputum eosinophil counts are not simply interchangeable, as has been discussed in the previous

paragraphs. Studies using exhaled nitric oxide to titrate steroid therapy have all been rather disappointing so far, as they have not been able to show the same results as for sputum eosinophils (41;45;46). Although treatment algorithms based on exhaled nitric oxide levels have been proposed (37) and nitric oxide devices appear in more and more clinics nowadays, there is no agreement as yet what should be the cut-off points for increasing or decreasing steroid treatment. However, there seems to be enough reason to assume that low exhaled nitric oxide levels are reassuring and allow steroid reduction when patients are stable. High or intermediate levels remain difficult to interpret, in particular in patients who are on steroid treatment. Several studies have now importantly reported normal values for exhaled nitric oxide (63;64). A study that investigates the value of nitric oxide measurements in successfully tapering oral steroid treatment in difficult-to-treat asthma is now underway (65).

In this thesis another application of exhaled nitric oxide has been investigated. In chapter 3 we showed that alveolar nitric oxide may give information about inflammation in the distal airways, a source of inflammation which is difficult to investigate (66). Patients with distal airway inflammation might benefit from systemic anti-inflammatory therapy or small particle inhaled steroids, although so far it has not been proven that small particle steroids are more effective in small airway disease than conventional inhaled steroids (67). The measurement of alveolar nitric oxide is noninvasive, real-time and easy, especially when using expiratory flows in a range from 50-250 ml/sec (26;28).
Exhaled nitric oxide levels may also help the clinician to identify patients who are at risk of an accelerated decline in lung function as has been demonstrated in chapter 6. However, these results are preliminary and will need confirmation by larger studies. Other possible applications of FeNO measurements are for the diagnosis of asthma (38) and the diagnosis of ‘eosinophilic bronchitis’ (68).
With the current evidence, one cannot state that a nitric oxide analyzer is essential in the diagnosis or treatment of patients with asthma. However, if one uses exhaled nitric oxide values as an extra, noninvasive tool that complement conventional clinical tests, it may help to guide treatment decisions for the individual asthma patient(37).

Directions for future research

Difficult-to-treat asthma is a disease that affects only a small percentage of the asthma population, but unfortunately has a major impact on quality of life and asthma related costs (69). In order to improve the health of these patients either through prevention of asthma, non-drug related health advice, or drug therapy we need to acquire a lot more knowledge about the pathophysiological mechanisms that are involved in the development of certain phenotypes of this disease. In this thesis an effort is made to solve part of the puzzle of understanding difficult-to-treat asthma in all its perspectives.

Apart from defining phenotypes based on clinical, physiological, or pathological parameters, alternative approaches, that take the multidimensionality of asthma into account, are now being studied. These complex statistical approaches such as cluster or factor analysis (59) use multiple parameters to identify clinical asthma phenotypes (56;59). New techniques, such as the pattern analysis of volatile organic compounds in exhaled air by an electronic nose, can further contribute to the discrimination of asthma subgroups (70).

As all researchers know, research always raises more questions than it can answer. Future possible research questions are therefore summarized below:

- Which pathophysiological mechanisms are responsible for an accelerated decline in lung function and the development of persistent airflow limitation?
- Is treatment of (a subgroup of) patients with difficult-to-treat asthma with small particle-inhaled steroids more effective than treatment with conventional inhaled steroids?
- What is the etiology of high exhaled nitric oxide levels in patients with difficult-to-treat asthma who are on high dose inhaled corticosteroids, and is it useful or possible to lower these levels by therapeutic strategies?
- Will measurements of exhaled nitric oxide help us to detect patients at risk for lung function decline in a large asthma population?
- Will phenotyping of asthma help us to define different pathophysiological mechanisms that determine specific therapeutic responses, thereby improving asthma control and prognosis?
- Is the implementation of a systemic evaluation protocol for patients with difficult-to-treat asthma in the clinic cost-effective and can it improve asthma control?

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