The clinical expression of large and small airway dysfunction in asthma

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Asthma is a chronic airway disease affecting approximately 300 million individuals worldwide (1). It is characterized by airway inflammation and bronchial hyperresponsiveness, leading to recurrent episodes with respiratory symptoms. Nowadays, it is widely accepted that asthma affects the total bronchial tree from the large to the small airways. The small airways are usually defined as those with an internal diameter <2 mm and are located from approximately the 8th generation of the bronchial tree (2,3). For many years the exact site of airway inflammation and bronchoconstriction in asthma was controversial. Several decades ago, the large airways were considered as the important site of airway dysfunction in asthma, whereas the contribution of the small airways was thought to be negligible (4). Nowadays, it seems illogical to focus on the large airways only, and in this way disregarding more than 99% of the airways. However, there were several reasons to believe, though incorrectly, that asthma is an isolated large airway disease.

Asthma, a large airways disease?

The paradigm of asthma as a large airway disease found its origins in the 1960s with the new description of the bronchial tree by Weibel (2). In 1915 Rohrer had underestimated the number of branches counting 86 branches with a diameter of 2 mm, and Weibel found 300 to 400 branches with this diameter (5). The findings of Weibel made clear that the number of small airways was much larger, and therefore the resistance of the small airways much lower than previously thought. The cross-sectional area of the bronchial tree exponentially increases towards the end of the bronchial tree, leading to a lower resistance of the small airways compared to the large airways despite the smaller lumen of the small airways (Figure 1). The latter is in line with a study performed in 1967 by Macklem and Mead (6). They measured small airway resistance with a retrograde catheter wedged in the bronchi of dogs and observed that doubling of the small airway resistance would only add 10% to the total airway resistance. Since the contribution of the small airway resistance to the total airway resistance was so small, Mead declared the small airways to be the "lung's quiet zone"(4). This statement was earlier used by Woolcock and colleagues, who supposed that small airway dysfunction would not affect conventional test results and consequently the small airways would remain clinically silent (7). Due to the scarce availability of accurate tests to assess small airway dysfunction, small airway obstruction could only be detected until it became far advanced. In other words, the lack of accurate small airway dysfunction tests has been an important reason to focus predominantly on the large airways.

Until the introduction of the fiberoptic bronchoscope, data on endobronchial sampling of the small airways were limited (8). Biopsies obtained from the large airways provided direct information about airway inflammation and showed an increased eosinophilic inflammation in patients with asthma compared to subjects without asthma (9-11). Investigation of post-mortem tissue of fatal asthma revealed that inflammation was present throughout the bronchial tree including the small airways (12). However, the relevance of these findings was limited as these studies considered only severe life-threatening asthma patients and data was not obtained from in vivo tissue. For a long time, it remained questionable if the inflammatory process involved not only the large conducting airways in asthma but also the smaller airways.

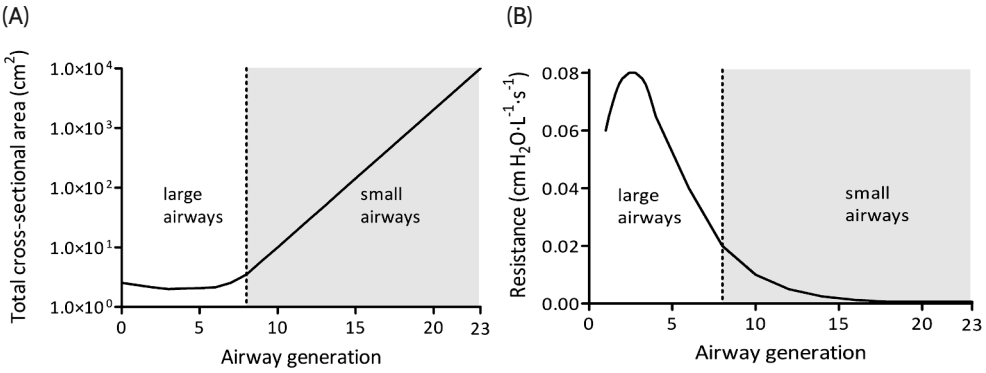

Figure 1. (A) The total cross-sectional area of all the airways in each generation, (B) The total airway resistance of all airways in each generation. This figure is based on the findings of Weibel (2).

Traditionally, asthma has been described as a disease with bronchoconstriction of the large conducting airways, whereas the potency of the small airways to constrict has been doubted for many years (13). This idea was supported by findings of Barnes and colleagues, who showed that muscarinic receptors were abundantly present in the large airways using autoradiographic imaging, whereas muscarinic receptors were nearly absent in the small airways (14,15). They therefore postulated that the greatest bronchoconstriction would occur in the large airways mainly. In addition, Ebina and colleagues investigated post-mortem tissue of asthma patients and showed that smooth muscle hypertrophy was most pronounced in the large airways compared to the small airways (16). This finding supported the idea of Barnes and colleagues that bronchoconstriction would mainly concern the large and not the small airways.

Taken together, because of the minimal contribution of the small airways to the total airway resistance, scarce availability of small airway dysfunction tests, minimal evidence of small airway pathology and the unlikelihood that small airways participate in bronchoconstriction, research of asthma focused predominantly on the large airways and disregarded the role of the small airways (Table 1).

Table 1. Examples of studies supporting the paradigm that asthma was an isolated large airway disease

Asthma, a large airway disease?	
1963 Weibel	Resistance of the small airways is lower than resistance of the large airways
1967 Macklem and Mead	Doubling of small airway resistance adds only 10% to the total airway resistance
1970 Mead	Small airways are lung's "quiet zone"
1983 Barnes *et al*	Muscarinic receptors are nearly absent in the small airways
1990 Ebina *et al*	Smooth muscle hypertrophy of the large airways
1990 Azawwi *et al*	Increased eosinophilic inflammation in bronchial biopsies of asthma patients compared to healthy controls

Small airways in asthma

The interest in the role of the small airways in asthma was renewed with the introduction of new techniques, like the application of peripheral resistance and transbronchial biopsies with the fiberoptic bronchoscopic technique (13). Using fiberoptic bronchoscopy in living humans, Wagner and colleagues observed a sevenfold increase in peripheral airway resistance in patients with mild asthma compared to healthy subjects (17). In line with this, Yanai and colleagues observed that the small airway resistance in patients with asthma contributed to 35-50% of the total airway resistance (18). In a later study by Wagner and colleagues an increased peripheral airway resistance was found in patients with asthma in response to a local challenge of the small airways with histamine (19). Peripheral airway resistance was also shown to be increased in patients with nocturnal asthma compared to patients without nocturnal asthma (20). Overall, these studies suggest that the peripheral airway resistance is increased in patients with asthma and that this increase is related to clinical features such as bronchial hyperresponsiveness and nocturnal asthma.

Pathologic evidence of small airway inflammation has now been provided by studies using transbronchial biopsies (21,22). Kraft and colleagues found an increase in eosinophilic inflammation overnight in the small airways, and not in the large airways, in patients with nocturnal asthma (21). In addition, Wenzel and colleagues observed increased numbers of neutrophils in endobronchial and transbronchial biopsies in patients with severe asthma despite treatment with high dose corticosteroids (22). Extensive research of post-mortem tissue in patients with and without asthma provided additional evidence of small airway inflammation in asthma. Hamid and colleagues investigated the inflammatory process in surgically resected lungs and showed that the number of T-cells, total eosinophils and activated eosinophils was higher in both small and large airway lung tissue of asthma patients compared to controls (23). Additionally, this inflammation was not only present in the inner wall of the airways, between the basement membrane and the smooth muscle, but also in the outer wall, between the smooth muscle layer and the lung parenchyma. Recently, it has been shown by Nihlberg and colleagues and Bergeron and colleagues, using endobronchial and transbronchial biopsies, that the small airways can also be affected by remodeling processes in patients with mild asthma (24,25). Taken together, these pathological studies have shown that inflammatory and remodeling processes affect the total bronchial tree from the large to the small airways.

Nowadays, there is also increasing evidence from numerous clinical and functional studies that the small airways play a role in more severe bronchial hyperresponsiveness. Kaminsky and colleagues performed a local challenge in the small airways with cool dry air using a wedged bronchoscope technique and observed an increased peripheral resistance in patients with asthma after provocation. In line with this, a study of Decramer and colleagues observed an increased peripheral resistance, measured with the FOT, after challenge with cool dry air (26). In addition, Zeidler and colleagues observed increased air trapping in patients with asthma measured with a High Resolution Computed Tomography (HRCT) scan after a cat-room challenge, while there was no fall in FEV_1 (27). These findings suggest that the small airways respond and constrict in response to different environmental stimuli.

The group of Gosens and colleagues has investigated precision cut lung slices of human small airways with videomicroscopy and observed a clear small airway constriction in response to methacholine (unpublished data; personal communication). Application of videomicroscopy and precision cut lung slices enables the direct functional assessment of small airways instead of measuring the presence of muscarnic receptors as performed in previous studies with autoradiographic imaging (14,15). Using videomicroscopy of precision cut lung slices, Brown and colleagues showed that inhibition of muscarinic receptors inhibited smooth muscle contraction of the small airways directly by M3 receptors, and via mediators by M2 receptors in human airways (28). Together, these findings suggest that activation of muscarinic receptors can induce smooth muscle contraction in the small airways.

In summary, recent findings contradict the paradigm that asthma is a large airway disease only and confirm that the inflammatory and remodeling processes in asthma also affect the small airways, and contribute to bronchial hyperresponsiveness (Table 2). The involvement of small airway dysfunction in the clinical expression of asthma is further described in chapter 1.

Table 2. Examples of studies showing that the small airways are importantly involved in asthma

Small airways in asthma!	
1990 Wagner *et al*	A sevenfold increase in small airway resistance in asthma patients compared to healthy controls
1992 Yanai *et al*	Small airway resistance contributes 35-50% of the total airway resistance in patients with asthma
1995 Kaminsky *et al*	Peripheral resistance increases after a local challenge with cool, dry air in patients with asthma
1996 Kraft *et al*	Eosinophilic inflammation increases overnight in transbronchial biopsies of patients with nocturnal asthma
1997 Hamid *et al*	Number of activated eosinophils increases in small airway lung tissue of asthma patients compared to healthy controls
2005 Bergeron *et al*	Airway remodelling is present in the small airways assessed with transbronchial biopsies
2013 Brown *et al*	Cholinergic antagonism of muscarinic receptors in the small airways inhibits smooth muscle contraction
2014 Gosens *et al* (unpublished data)	Small airway smooth muscle cells contract in response to methacholine

Table 3. Tests to assess small airway dysfunction

Test	Parameters of small airways dysfunction	Advantages	Disadvantages
Airway obstruction			
Spirometry	$FEF_{25-75\%}$, $FEF_{50\%}$	▪ Easy to perform ▪ Low costs ▪ Not time-consuming	▪ Low reproducibility ▪ Influenced by large airway obstruction ▪ Not correlated with inflammation (42)
	FVC/SVC	▪ Good detection of BOS after LTX (43)	▪ Not specific
Resistance			
Impulse oscillometry (IOS)	R5-R20, X5, Fres, AX	▪ Easy to perform ▪ Correlates with $FEF_{50\%}$ ▪ Correlates with MCh-induced changes in ventilation heterogeneity (38)	▪ Difficult to interpret ▪ Relationship with severity of disease not known
Air trapping			
Body plethysmography	FRC, RV, RV/TLC	▪ Non-invasive ▪ Correlates with small airway resistance(20) ▪ FRC correlates with number of eosinophils in transbronchial biopsies (42)	▪ Time-consuming test ▪ Influenced by large airway obstruction
Ventilation heterogeneity			
Single breath nitrogen washout test (SBNT)	CV, CV/VC, slope phase III	▪ Non-invasive ▪ Not time-consuming	▪ Low reproducibility
Multiple breath nitrogen washout test (MBNW)	Sacin, Scond	▪ Very sensitive ▪ Good reproducibility ▪ Correlates with FRC (38)	▪ Not widely available ▪ Time consuming in patients with severe ventilation heterogeneity
Imaging			
High resolution computed tomography (HRCT)	Air trapping	▪ Visual information of air trapping ▪ Related to air trapping measured with body plethysmography (44)	▪ Radiation load ▪ High costs

Table 3. Continued

Magnetic resonance imaging (MRI) with hyperpolarized helium	Regional ventilation defects	▪ More detailed information	▪ Technically demanding ▪ High costs
Inflammation			
Bronchoscopy	Transbronchial biopsy	▪ Direct information of inflammation	▪ Invasive
Sputum induction	Late phase sputum	▪ Non-invasive	▪ Little evidence
Exhaled nitric oxide (eNO)	Alveolar eNO	▪ Non-invasive	▪ Influenced by ICS, smoking (45,46) ▪ Time-consuming test

AX: Reactance area, BOS: bronhiolitis obliterans, CV: Closing volume, $FEF_{25-75\%}$: Forced expiratory flow at 25% to 75% of the FVC, $FEF_{50\%}$: Forced expiratory flow at 50% of the FVC, FRC: Functional residual capacity, Fres: Resonant frequency of reactance, FVC: Forced vital capacity, MRI: Magnetic resonance imaging, HRCT: High resolution computed tomography, IOS: Impulse oscillometry, LTX: lung transplantation, MBNW: Multiple breath nitrogen washout test, eNO: Exhaled nitric oxide, R5-R20: Difference resistance of the respiratory system at 5 Hertz and resistance of the respiratory system at 20 Hertz, RV: Residual volume, Sacin: Ventilation heterogeneity generated in the acinar lung zone, SBNT: Single breath nitrogen test, Scond: Ventilation heterogeneity generated in the conductive lung zone, SVC: slow vital capacity, TLC: Total lung capacity, X5: Reactance of the respiratory system at 5 Hertz

Tests to assess small airway dysfunction

Nowadays, there are several tests available that can measure small airway dysfunction in asthma. Table 3 describes the most frequently used tests with their specific advantages and disadvantages (29,30).

Spirometry is a common test to assess severity of asthma and is able to obtain the forced expiratory flow values, i.e. $FEF_{50\%}$ and $FEF_{25-75\%}$, as variable of small airway function. The $FEF_{25-75\%}$ was shown to be closely related with air trapping on an expiratory computed tomography (CT) scan and with ventilation heterogeneity measured with the multiple breath nitrogen washout (MBNW) test (31,32). A disadvantage of the $FEF_{25-75\%}$ is the low reproducibility compared to the FEV_1.

Resistance measurements of the small airways have received renewed interest to assess small airway dysfunction and are performed with the FOT or impulse oscillometry (IOS). The IOS technique enables measurement of large and small airway resistance (R20 and R5-R20), small airway reactance (X5), and reactance area (AX) reflecting small airway function (33,34). Recently, it has been shown that the IOS technique has a good short-term and long-term reproducibility (35). Boudewijn and colleagues compared symptomatic and asymptomatic asthmatic subjects with bronchial hyperresponsiveness using the IOS (36). They observed that symptomatic subjects with asthma had higher R5-R20 and X5 values, reflecting small airway dysfunction, before and after provocation with methacholine than asymptomatic subjects, while there was no difference in R20, reflecting large airway dysfunction. In line with this, Mansur and colleagues showed that the change in small airway reactance (ΔX5) during the methacholine provocation test was

related to methacholine-induced increase in dyspnea, chest tightness and wheezing (37). The spirometric parameters FEV_1 and $FEF_{50\%}$, reflecting the large and small airway respectively, were only associated with methacholine-induced wheezing, suggesting that IOS is a more sensitive test than spirometry to assess changes in asthma symptoms.

Another technique to assess small airway dysfunction, is the relatively new MBNW technique, which measures ventilation heterogeneity of the small conductive and acinar airways (Scond and Sacin). A study using the MBNW showed that the Scond is related to the FRC, which is a measure of air trapping (38). In addition, a higher Scond was also related to a more severe response to methacholine in asthmatic subjects (39). This study showed no association of the response to methacholine with the Sacin, suggesting that the conductive airways reflect a more important lung zone with respect to air trapping and bronchial hyperresponsiveness than the acinar airways in asthma (39).

The use of imaging techniques to assess small airway dysfunction is a new research field. The HRCT scan is a non-invasive method that cannot measure the small airways directly, but can quantify air trapping as reflection of small airway closure (40). The main disadvantage of HRCT scans is the radiation load. Another imaging technique is the magnetic resonance imaging (MRI) with inhalation of hyperpolarized helium. This technique provides a higher resolution than the HRCT and can visualize regional ventilation defects of the total lung (41). It is a promising technique, however at this time data about associations between ventilation defects and small airway parameters or clinical features are limited. The MRI technique is only available in a few specialized centers.

In summary, there are several techniques available to measure small airway dysfunction assessing different aspects like obstruction, ventilation heterogeneity, air trapping and inflammation. Unfortunately, nowadays there is still no cut-off value or gold standard to define small airway dysfunction.

Small particle treatment

The introduction of the new hydrofluoroalkane (HFA) formulation in the 1990s led to inhalation therapy with small particles of 1-2 μm instead of the conventional inhalation therapy with larger particles of 4-6 μm derived from the chlorofluorocarbon (CFC) formulation. The advantage of smaller particles is the higher total lung deposition and especially a higher small airway deposition (47). While inhalation of large particles (6 μm) achieves a total lung and small airway deposition of 46% and 10% respectively, inhalation of small particles (1.5 μm) achieves a total lung and small airway deposition of 56% and 25% respectively.

Several studies now have investigated the effect of small-particle inhaled corticosteroids (ICS) in asthma and have described an improvement in small airway dysfunction with small-particle ICS. For example, Cohen and colleagues observed significant improvements in alveolar nitric oxide and in methacholine-induced air trapping measured with a CT-scan after a 5-week treatment with

small particle ciclesonide (48). In addition, Thongngarm and colleagues observed significantly higher improvements in ventilation heterogeneity of the small airways, as assessed with the single breath nitrogen washout test, in 30 patients with asthma after 3-month treatment with small-particle HFA-beclomethasone than with the conventional CFC-beclomethasone (49). It is important to mention that HFA and CFC formulations not only differ in particle size, but HFA is also delivered with a softer plume, resulting in a lower oropharyngeal deposition compared to CFC formulation (50,51). Particle size is one of the key factors influencing lung deposition of inhalation medication in the lungs, but also the type of the device, formulation of medication, and inhalation flow are factors that contribute importantly to total lung deposition as well as peripheral deposition. Unfortunately, most studies investigating small particle ICS did not control for all these factors, and we cannot be certain whether the observed effects are due to a difference in particle size. Taken together, small particle ICS seems to improve small airway dysfunction and have clinical benefits, whether this is better than large particles has yet not been proven.

A few studies investigated the effect of different particle sizes of β2-sympathicomimetics on airway obstruction. Weda and colleagues compared salbutamol with a content of 15%, 27% and 67% fine particles (<5.9 μm) and found no difference in the efficacy to improve the FEV_1 (52). In addition, Usmani and colleagues investigated the effect of particles of MMAD 1.5, 3.0 and 6.0 μm albuterol and found that the small particles of 1.5 μm were less efficacious to improve the FEV_1 and $FEF_{25-75\%}$ than both the particles of 3.0 and 6.0 μm (47). It was proposed that this difference can be explained by a difference in dose response curve by a shift from a steep dose-response curve to a flat dose-response curve with smaller particles (53). Unfortunately, the majority of the studies investigating the effect of small particle β2-sympathicomimetica included only spirometry and did not include small airway parameters assessed with IOS or MBNW. Further studies are required including a larger panel of small airway dysfunction tests to determine the effect of different particle sizes of β2-sympathicomimetics on both large and small airway obstruction.

Phenotypes of asthma

Asthma is a heterogeneous disease and so far several phenotypes have been discovered (54,55). Presence of small airways dysfunction has been proposed as a distinct phenotype of asthma with a different clinical expression (56). Haldar and colleagues performed a landmark study using an unbiased cluster analysis to identify new clinical phenotypes (57). Their analysis included several clinical, physiological and inflammatory parameters, i.e. sputum cell counts and exhaled nitric oxide, however parameters of small airway dysfunction were not included. One of the phenotypes identified by Haldar and colleagues was the obese non-eosinophilic asthma patient with increased symptoms. This phenotype is discussed in chapter 8.

Aim of the thesis

The first and main aim of this thesis is to assess whether small airway dysfunction contributes to the clinical expression of asthma. To this end, we reviewed the literature and analyzed different asthma populations investigating the relationship between small airway dysfunction and clinical

features of asthma. Secondly, we aimed to develop new tools that can identify patients with small airway dysfunction. We started with a questionnaire to assess small airway dysfunction and a new provocation test with dry powder adenosine.

Outline of the thesis

Chapter 2 gives a systematic overview of studies investigating small airway dysfunction in relation to asthma control, occurrence of exacerbations, nocturnal asthma, bronchial hyperresponsiveness, exercise-induced asthma and allergen exposure. In addition, we explored the relation between small airway dysfunction and exposure to air pollution and described studies that found an effect of treatment on both small airway function and asthma symptoms.

Chapters 3 and 4 investigate the association between small airway dysfunction and specific clinical features of asthma. Chapter 3 focuses on the role of small airway dysfunction in asthma symptoms and bronchial hyperresponsiveness in a study population of 58 patients with mild to moderate-severe asthma, who were extensively characterized with measurements of lung function, impulse oscillometry, exhaled nitric oxide and a methacholine provocation test. Chapter 4 is an observational study in 3,155 asthma patients derived from primary care focussing on the association of small and large airway function with control of asthma, and the response to specific environmental stimuli.

Chapter 5 describes the first step in the development of a small airway dysfunction questionnaire. A new small airway dysfunction tool may help to identify asthma patients with small airway dysfunction. In order to select relevant differences in signs and respiratory symptoms between asthma patients with and without small airway dysfunction, both groups of asthma patients are asked about their perceived asthma symptoms in individual in-depth interviews and in focus groups

Chapters 6 and 7 introduce a new provocation test with dry powder adenosine. The proof of principle in five asthma patients is presented in chapter 6. In chapter 7 we try to challenge the small and large airways selectively with small- and large-particle dry powder adenosine and inhaled with either a low or high flow rate. We hypothesize that a small-particle slow-inhalation provocation test gives a higher small airway deposition and thus a higher response in the small airways than a test with large particles and/or inhalation with a high flow rate. Based on a differential response to the four adenosine challenge tests, we try to identify patients with small airway dysfunction.

Chapter 8 analyzes eosinophils in sputum and bronchial biopsies in obese and nonobese subjects with mild-to-moderate asthma. This study is performed in response to an article of Desai and colleagues showing that eosinophils in biopsies were elevated in obese patients compared to nonobese patients with severe asthma.

Chapter 9 summarises and discusses the results of all articles and gives my future perspectives.

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CHAPTER 2

Small airway dysfunction associates with respiratory symptoms and clinical features of asthma: A systematic review.

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ABSTRACT

Traditionally, asthma has been considered a disease that predominantly involves the large airways. Today, this concept is being challenged, and increasing evidence has become available showing that abnormalities in the small airways also contribute to the clinical expression of asthma. The small airways can be affected by inflammation, remodeling, and changes in the surrounding tissue, all contributing to small-airways dysfunction. In this article we have performed a systematic review of the literature on the association between small-airways dysfunction and clinical signs and symptoms of asthma. This review shows that small-airways dysfunction associates with worse control of asthma, higher numbers of exacerbations, the presence of nocturnal asthma, more severe bronchial hyperresponsiveness, exercise-induced asthma, and the late-phase allergic response. Importantly, small-airways dysfunction can already be present in patients with mild asthma. Our review provides suggestive evidence that a better response of the small airways to inhaled steroids or montelukast associates with better asthma control. For this reason, an early recognition of small-airways dysfunction is important because it enables the physician to start timely treatment to target the small airways. It is important to develop simpler and more reliable tools (eg, questionnaires or bronchial provocation tests with small-particle stimuli) to assess the presence and extent of small-airways dysfunction in daily clinical practice.

INTRODUCTION

Asthma is a chronic inflammatory lung disease affecting the total bronchial tree form the large to the small airways. Four decades ago, it was already suggested that the small airways are involved in asthma. Hogg *et al*, using a retrograde catheter, demonstrated that the resistance of the small airways is increased in patients with chronic obstructive lung disease compared to healthy control subjects (1). However, because the contribution of the small airways to total lung resistance was minimal, asthma was considered a disease mainly of the large airways, and the small airways were labeled the "quiet zone" (1-4).

During the last decade, there has been renewed interest in the role of small airway disease in asthma. The small airways are usually defined as airways with an internal diameter of less than 2 mm, referring to the landmark study of Macklem and Mead, who wedged a retrograde catheter with a diameter of 2 mm in the bronchi to measure airflow resistance (2). The definition is also in line with the findings of Weibel, who found that the total cross-sectional area of the bronchial tree increases exponentially after around the eight-generation airways which have an internal diameter of approximately 2 mm (5). The small airways are difficult to investigate because they are relatively inaccessible. Currently, several tests are available to assess small airway dysfunction. The value and limitations of each test have been extensively reviewed elsewhere (6-8). The conclusion of these reviews is that there exists no gold standard to assess small airway dysfunction, and therefore all parameters are indicative rather than conclusive (6-8).

Recent studies suggest that abnormalities in the small airways can contribute to the clinical expression of asthma (8-10). The small airways can be affected by inflammation, remodeling, and changes in the surrounding tissue, all contributing to small airway dysfunction (9,11-14). The aim of this systematic review is to investigate the association between small airway dysfunction on the one hand and clinical signs and symptoms of asthma on the other hand. To this end, we performed a PubMed search and selected relevant articles based on the following criteria: study population of patients with asthma, measurement of a small airway parameter, and clinical signs or symptoms of asthma (Figure 1). Table 1 shows the small airway parameters that were selected in the current review (6). We did not include magnetic resonance imaging and frequency dependence compliance, since they have not been used in clinical studies relating small airway function to clinical parameters.

We divided the relevant articles in 8 domains possibly associated with small airway dysfunction: symptoms and asthma control, exacerbations, nocturnal asthma, bronchial hyperresponsiveness (BHR), exercise-induced bronchoconstriction, allergen exposure, air pollution, and medication. For each of these domains, relevant articles are further subdivided based on the test used to measure small airway dysfunction according to the following categories: flow, resistance, ventilation, heterogeneity, air trapping. and inflammation (6).

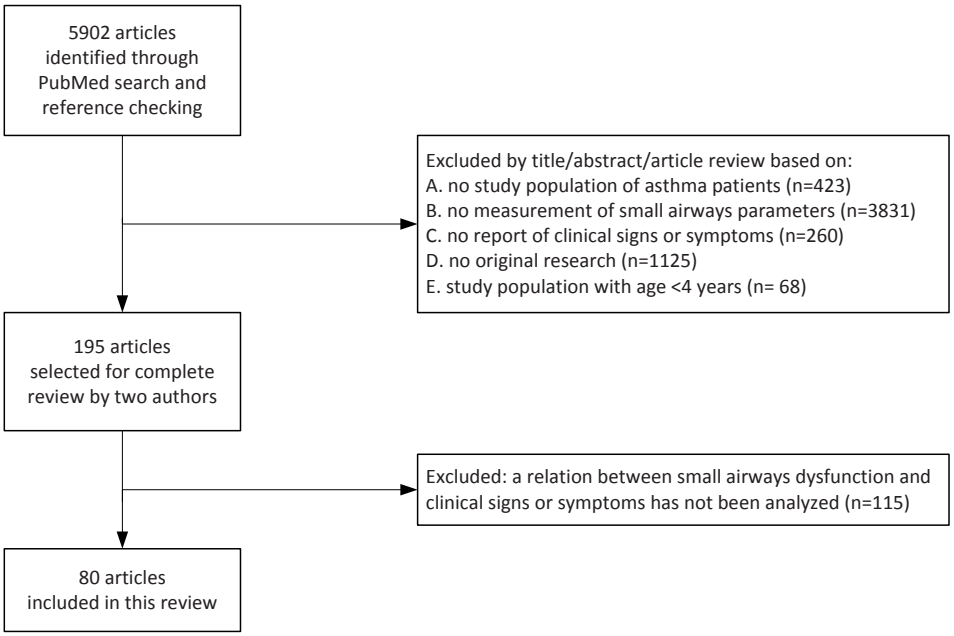

Figure 1. Flowchart of the literature search

A PubMed search resulted in 5902 articles using the following term "Asthma AND (small airway* OR peripheral airway* OR distal airway* OR distal lung OR impulse oscillometry OR alveolar nitric oxide OR exhaled nitric oxide OR nocturnal OR residual volume OR montelukast OR HFA OR hydrofluoroalkane OR extra fine OR transbronchial OR closing volume OR closing capacity OR air trapping OR hyperinflation OR nitrogen OR HRCT OR high resolution CT OR MRI)" limited to the English language and human subjects. Hand searching of the reference lists of retrieved articles and reviews was also undertaken. Titles and/or abstracts and/or full articles were reviewed during the initial search, and 195 articles were selected according to the following criteria: A, a study population of asthmatic patients; B, measurement of small airway parameters; C, reporting clinical signs or symptoms. An article was excluded if it met criteria D, (ie, no original research (review, editorial, case report)) or E, (ie, a study population with age <4 years to exclude transient wheezing). According to these criteria, the relevance of these 224 articles were reviewed by two authors considering whether the relation between small airway dysfunction and clinical signs or symptoms had appropriately been analyzed (clinical symptoms or severity of symptoms were not based on lung function or steroid use). Discrepancies were resolved by means of open discussion with all authors. Using this method, 80 articles were finally selected for extensive review in this article. The search was conducted in October 2012.

Table 1. Parameters to assess small or large airway (dys)function or inflammation

Method	Parameters of small airway (dys)function	Parameters of large airway (dys)function
Flow		
Spirometry	$FEF_{25\%-75\%,}$ $FEF_{50\%}$, FVC/SVC	FEV_1, FEV_1/FVC ratio, PEF
Helium-oxygen flow-volume curves	$FEF_{50\%}$ (no increase)	$FEF_{50\%}$ (increase)
Resistance		
IOS	R5-R20, AX, X5, Fres	R20
Bronchoscopy	Peripheral resistance	
Ventilation heterogeneity		
SBNT	CV, CC; slope phase III	
MBNW-test	Sacin, Scond	
H^3HeMRI	Regional ventilation defects	
Air trapping		
Body plethysmography	FRC, RV, RV/TLC	
HRCT	Air trapping	
Inflammation		
Bronchoscopic biopsy	Transbronchial biopsy	Endobronchial biopsy
Bronchoscopy	BAL	
Sputum induction	Late-phase sputum	Early-phase sputum
Exhaled NO	Alveolar NO	Bronchial NO
Additional		
Frequency dependence of dynamic compliance	Increased respiratory frequency Decreased dynamic compliance	

AX: Reactance area, BAL: Bronchoalveolar lavage, CC: Closing capacity, CV: Closing volume, dN2: Slope of phase III of SBNT, FEF25%-75%: Forced expiratory flow at 25% to 75% of the FVC, FEF50%: Forced expiratory flow at 50% of the FVC, FEV1: Forced expiratory flow in one second, FRC: Functional residual capacity, Fres: Resonant frequency of reactance, FVC: Forced vital capacity, H3HeMRI = Magnetic resonance imaging with inhaled hyperpolarized helium-3 gas, HRCT: High resolution computed tomography, IOS: Impulse oscillometry, MBNW: Multiple breath nitrogen washout test, NO: Exhaled nitric oxide, PEF: Peak expiratory flow, R5: Resistance of the respiratory system at 5 Hertz, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference of R5 and R20, RV: Residual volume, Sacin: Ventilation heterogeneity generated in the acinar lung zone, SBNT: Single breath nitrogen test, Scond: Ventilation heterogeneity generated in the conductive lung zone, SVC: slow vital capacity, TLC: Total lung capacity, X5: Reactance of the respiratory system at 5 Hertz

ASTHMA SYMPTOMS AND CONTROL

Several studies have investigated the association between asthma symptoms or control and small airway dysfunction, as reflected by different parameters of the small airways. Symptoms were assessed with asthma questionnaires or self-reported by the patient.

Takeda *et al* measured large and small airway function with impulse oscillometry in 65 patients with stable asthma and assessed associations with health status, dyspnea, and asthma control, using the St. George Respiratory Questionnaire, the Baseline Dyspnea Index, and the Asthma Control Questionnaire (ACQ), respectively (15). An increase in small airway resistance, as reflected by the total resistance of the respiratory system at 5 Hz (R5) minus the resistance of the respiratory system at 20 Hz (R20; R5-R20), and an increase in large airways resistance, as reflected by the R20 value, were independently associated with a lower health status and more dyspnea. Interestingly, greater small airway reactance (ie. reactance at 5 Hz) was associated with loss of asthma control. Shi *et al* additionally found that dysfunction of the small, but not the large, airways was associated with worse asthma control (16). They found that the R5-R20 and reactance area (AX) values were the only small airway parameters that could discriminate between patients with controlled and uncontrolled asthma, with a high sensitivity and specificity of 84% and 86%, respectively.

Ventilation heterogeneity of the small airways can be investigated with a nitrogen washout test. A higher ventilation heterogeneity is reflected by an increase in the phase III slope. A limitation of this measurement is that the large airways can also contribute to an abnormal phase III slope (17,18). In this context, the multiple-breath nitrogen washout (MBNW) test is an important improvement, because it is able to distinguish between ventilation heterogeneity generated in the conductive lung zone (Scond) and ventilation heterogeneity generated in the acinar lung zone (Sacin), with a cutoff around the 15th generation (19). Farah *et al* demonstrated that patients with poorly controlled asthma have higher ventilation Scond and Sacin values than patients with well-controlled asthma (20). These results are in line with those of Bourdin *et al* who demonstrated that asthmatic patients with more alveolar heterogeneity, as determined with the phase III slope of the single-breath nitrogen test (SBNT), have worse asthma control (Figure 2, A) (21).

Several studies have demonstrated that higher alveolar nitric oxide (NO) concentrations are associated with the presence of symptoms and worse asthma control (22-25). Exhaled NO can be divided into bronchial and alveolar NO based on a mathematic model, assuming that bronchial NO is derived from the proximal large airways and alveolar NO reflects inflammation in the distal small airways (26). Puckett *et al* divided 179 asthmatic children 6 to 17 years of age into 4 groups based on the concentration of alveolar and bronchial NO: (1) normal alveolar and bronchial NO levels; (2) increased bronchial NO levels only; (3) increased alveolar NO levels only; and (4) both increased bronchial and alveolar NO levels (27). Interestingly, even though FEV_1 percent predicted did not differ between the groups, patients with increased alveolar NO levels (groups 3 and 4) had worse asthma control, as assessed by the Asthma Control Test, than patients with normal alveolar and bronchial NO levels or those with increased bronchial NO levels only (groups 1 and 2). In addition, patients with increased alveolar NO levels more frequently had a severe exacerbation.

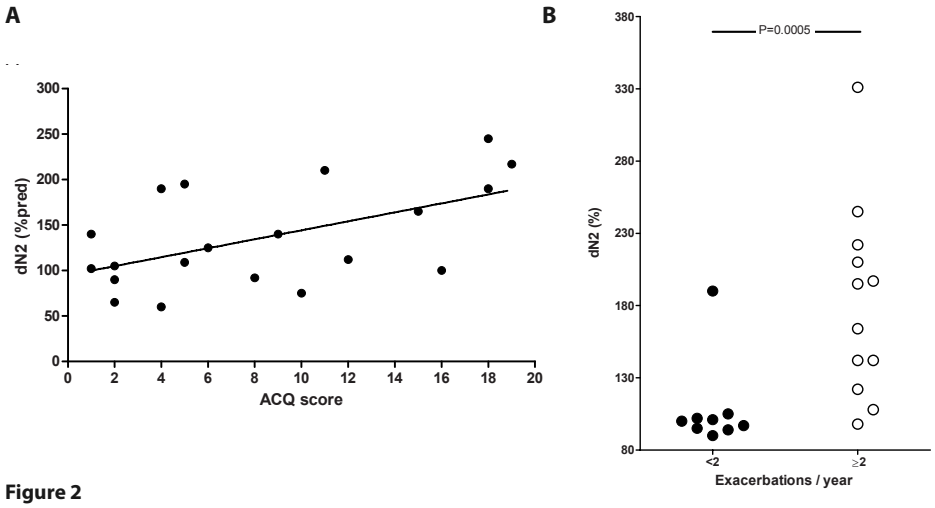

Figure 2

A, Significant correlation between the percent predicted slope of phase III of SBNT (dN2) and the ACQ score (Spearman correlation coefficient: ρ = 0.62, P = .003). B, Significant differences in dN2 values between frequent and infrequent exacerbators (P = .0005). Reproduced with permission from Bourdin *et al.*(21)
SBNT: Single breath nitrogen test; dN2: slope of phase III of SBNT

In contrast with these findings, Mahut *et al* did not observe an association between changes in alveolar or bronchial NO levels over a period of 1 to 12 weeks and change in asthma control in adults and children with asthma (28). In addition, Berry *et al* investigated asthmatic patients using high doses of oral corticosteroid or inhaled corticosteroids (ICSs) and did not observe an association between alveolar NO levels and asthma control (29). A possible explanation for the lack of an association between NO levels and asthma control in the latter 2 studies might have been that the majority of patients used high dose ICSs, which are especially effective in suppressing exhaled NO levels (30). In conclusion, there is some evidence that alveolar NO levels are associated with asthma symptoms. However, it has to be taken into account that both alveolar and bronchial levels are affected by the use of inhaled or oral corticosteroids. Finally, Van Vyve *et al* investigated inflammation in bronchoalveolar lavage (BAL) fluid in relation to the severity of asthma, as defined by the Aas score (31,32). A higher Aas score (ie, more severe asthma) was associated with a higher eosinophil percentage in BAL fluid, suggesting involvement of the small airways.

OCCURRENCE OF AN ASTHMA EXACERBATIONS

Bourdin *et al* showed that frequent (≥2/y) exacerbators have a higher degree of small airway dysfunction as reflected by the SBNT phase III slope than infrequent exacerbators (<2/y), whereas FEV_1 percent predicted values were comparable between these 2 groups (Figure 2,B) (21). These findings are in line with those of in 't Veen *et al,* who demonstrated that frequent exacerbators have a higher SBNT closing volume and closing capacity than infrequent exacerbators (33).

Air trapping can be assessed by using body plethysmography or computed tomographic scanning, which are indirect parameters, to assess small airway dysfunction. Mahut *et al* have compared the presence of air trapping between children with and without a severe asthma exacerbation and with and without symptoms (34). The 108 asthmatic children with a severe exacerbation had more air trapping (ie, a higher residual volume (RV) and RV divided by total lung capacity (TLC; RV/TLC)) than children without exacerbations and mild or no symptoms. In addition, more air trapping, as assessed by using computed tomographic scanning in another study, was associated with asthma-related hospitalizations and a history of pneumonia (35).

Alveolar NO levels were shown to increase during an exacerbation and to subsequently decrease during the resolution, additionally suggesting involvement of the small airways (36). Furthermore, Gelb *et al* showed that an increased alveolar NO level predicts increased asthma exacerbations independently of FEV_1 (37). However, the same was observed with an increased bronchial NO level, and it is questionable whether this finding suggests small airway involvement. In a later study, Gelb *et al* did not find an increase in alveolar NO levels during an exacerbation when they corrected for NO back diffusion from the central to the peripheral airways (38).

In summary, the balance of evidence in the abovementioned studies suggests that a higher degree of small airway dysfunction is associated with more frequent asthma exacerbations, although an influence of large airways dysfunction on these results will also likely play a role.

NOCTURNAL ASTHMA

We identified several studies investigating the association between small airway dysfunction and nocturnal asthma. First, Kraft *et al* showed that peripheral airways resistance, as measured with a wedged bronchoscope, is increased in patients with nocturnal compared with nonnocturnal asthma.(39) A further study investigated both endobronchial and transbronchial biopsy specimens at daytime (4 AM) and nighttime (4 PM) in 11 patients with nocturnal asthma, defined as a 15% or greater decrease in peak expiratory flow (PEF) rate at night, and 10 patients without nocturnal asthma.(40) Although there were no differences in inflammation between day and night in endobronchial biopsy specimens of the large airways, a significant night-time increase in eosinophil counts was observed in the transbronchial biopsy specimens, specifically in patients with nocturnal asthma. These findings suggest that inflammation of the small airways contributes to asthma symptoms and the decrease in lung function at night in patients with nocturnal asthma (Figure 3).

This is in line with the findings of Martin *et al,* who also observed an increased inflammation of the small airways during the night in the BAL fluid of patients with nocturnal asthma.(41) In contrast to the findings of the latter study, Oosterhoff *et al* and Jarjour *et al* did not find an overnight increase in the number of eosinophils in BAL fluid in patients with nocturnal asthma.(42,43) A possible explanation for this discrepancy might be the difference in asthma severity. Martin *et al* investigated predominantly patients with severe asthma (mean FEV_1, 74% of predicted value), whereas Oosterhoff *et al* and Jarjour *et al* investigated patients with milder asthma with a mean

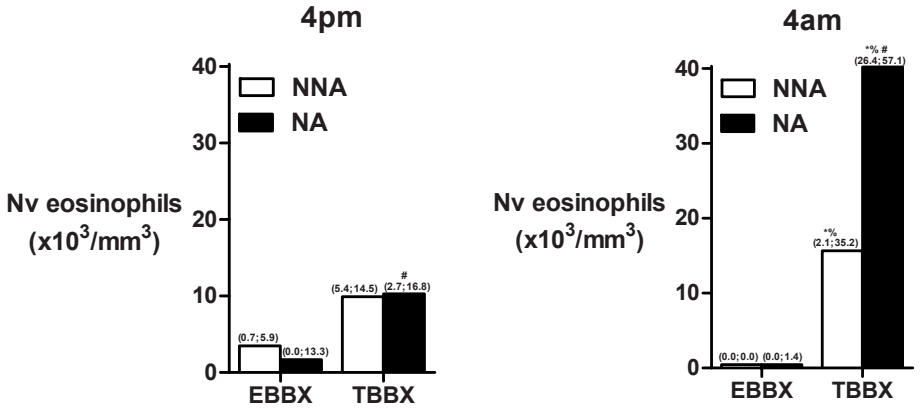

Figure 3

Number per volume (Nv) of eosinophils in patients with nonnocturnal asthma (NNA) and nocturnal (NA) is shown in the endobronchial biopsy specimens (EBBX) and transbronchial biopsy specimens (TBBX) at 4 AM and 4 PM. The open bars represent the nonnocturnal asthma group (n=10) and the solid bars represent the nocturnal asthma group (n=11). Values are expressed as medians with the 25th to 75th interquartile range in parentheses above each bar. #*% $P \leq .05$. The transbronchial biopsy specimens of patients with nocturnal asthma show a significant increase eosinophil numbers overnight. Reprinted with permission from the American Thoracic Society, ©2013, from Kraft *et al.*(40)

FEV_1 of 88% and 89% of predicted value, respectively.(41-43) Taken together, it has been shown that the peripheral airways resistance increased during the night in patients with nocturnal asthma in parallel with an increased small airway inflammation and the occurrence of nocturnal symptoms.

One study compared patients with nocturnal symptoms with patients without symptoms at night using alveolar NO levels.(44) All patients had a recent diagnosis of asthma, were steroid naive and had a comparable lung function and bronchial NO concentration. Interestingly, patients with nocturnal symptoms had higher alveolar NO values than patients without nocturnal symptoms, suggesting that even in patients with mild asthma, nocturnal symptoms are associated with small airway inflammation.

BRONCHIAL HYPERRESPONSIVENESS

Change of small airway function during a provocation test

Two studies have used the wedged bronchoscope technique to investigate the response of the small airways to a provocation test.(45,46) In one study peripheral airways resistance increased faster in patients with asthma than in healthy control subjects after local application of histamine. (45) The other study demonstrated that greater peripheral airways resistance is associated with more BHR to methacholine.(46) Together, these results confirm the sensitivity of the small airways to nonallergic stimuli in asthmatic patients.

This is in line with the findings of Segal *et al,* who performed a methacholine provocation test with both FEV_1 and impulse oscillometry. (47) It was found that both the total and small airway resistance increased in patients with BHR (PC_{20} ≤16 mg/mL) compared with that seen in patients without BHR (PC_{20} >16 mg/mL), whereas large airways resistance was comparable between the groups. Additionally, 9 of 33 patients had symptoms during the challenge, even though their FEV_1 did not decrease. In these patients the total respiratory resistance (R5) increased significantly, predominantly because of an increase in R5-R20 and AX. The latter suggests that the increase in small airway resistance was responsible for the onset of symptoms in these subjects (Figure 4). These findings are in line with those of Mansur *et al,* who showed that a higher small airway reactance is associated with more severe dyspnea, wheezing and chest tightness after provocation. (48) Together, these studies show that the small airways are involved in BHR and that the response in the small airways is associated with the development of symptoms during a provocation test.

Furthmore, several studies have shown that air trapping can occur during methacholine-induced bronchoconstriction.(49-53) For example, Lougheed *et al* showed that 66% of asthma patients hyperinflate to greater than 300 mL at the PC_{20} level, as reflected by a decrease in their inspiratory capacity.(49) Moreover, a higher degree of air trapping was related to increased symptoms of chest tightness and dyspnea. These findings are in line with several other studies showing that a higher degree of air trapping during a methacholine provocation test associates with the severity of dyspnea, even in a multivariate regression analysis after adjusting for the decrease in FEV_1. (50-52) *Vice versa*, the reduction in air trapping after administration of 200 µg of salbutamol at the end of the provocation test was associated with the decrease in the intensity of dyspnea.(51) The mechanism for the increase in air trapping during acute bronchoconstriction is controversial. Possible mechanisms might be expiratory flow limitation of the larger airways, significant intrinsic positive end-expiratory pressure, or closure of the small airways during expiration.(51,54)

Association between small airway dysfunction and severity of BHR

In a retrospective study Drewek *et al* showed that asthma patients with BHR to methacholine have a lower forced expiratory flow at 25% to 75% of forced vital capacity (FEF25%-75%).(55) This is in line with the findings of Currie *et al,* who compared asthmatic patients with moderate-to-severe (PC_{20} ≤1 mg/mL) and borderline (PC_{20} ≥8 mg/mL) BHR to methacholine.(56) Although patients were matched for FEV_1 percent predicted, patients with moderate-to-severe BHR had significantly lower FEF25%-75% values. In addition, Lang *et al* observed a lower forced expiratory flow at 50% of forced vital capacity ($FEF_{50\%}$) and increased BHR in children with severe asthma compared with those with nonsevere asthma, whereas the FEV_1 percent predicted value was comparable between both groups.(57) Furthermore, Backer and Mortensen investigated the airways distribution of radioactive aerosol in children and adults in relation to lung function and BHR.(58) Patients with an irregular deposition of the aerosol had a significantly lower FEF75% value and more severe BHR. Downie *et al* analyzed BHR with the MBNW-test in asthmatic patients. (59) They demonstrated that Scond is associated with the severity of BHR to methacholine. Finally, Pliss *et al* showed that more severe BHR is associated with a more severe small airway inflammation, as reflected by a higher percentage of eosinophils in BAL fluid.(60)

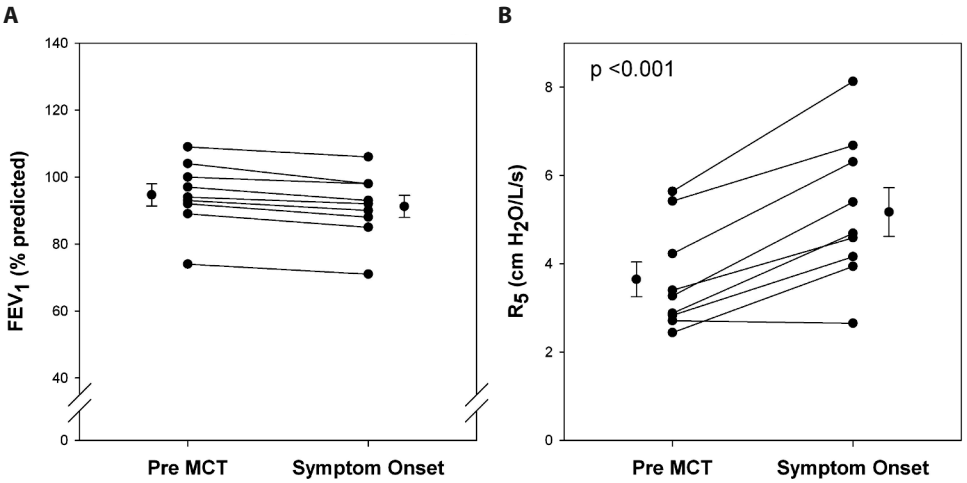

Figure 4

Relationship between onset of respiratory symptoms and changes in FEV_1 (A) and R5 (B) values. Data are illustrated for the 9 of 33 subjects who developed symptoms with minimal change in FEV_1 during the provocation test (mean change, -3.4%). FEV_1: Forced expiratory flow in one second; R5: Resistance of the respiratory system at 5 Hertz; MCT: Methacholine provocation test. Adapted from Segal *et al,* Disparity between proximal and distal airway reactivity during methacholine challenge, COPD, ©2011, Informa Healthcare.(47) Reproduced with permission from Informa Healthcare.

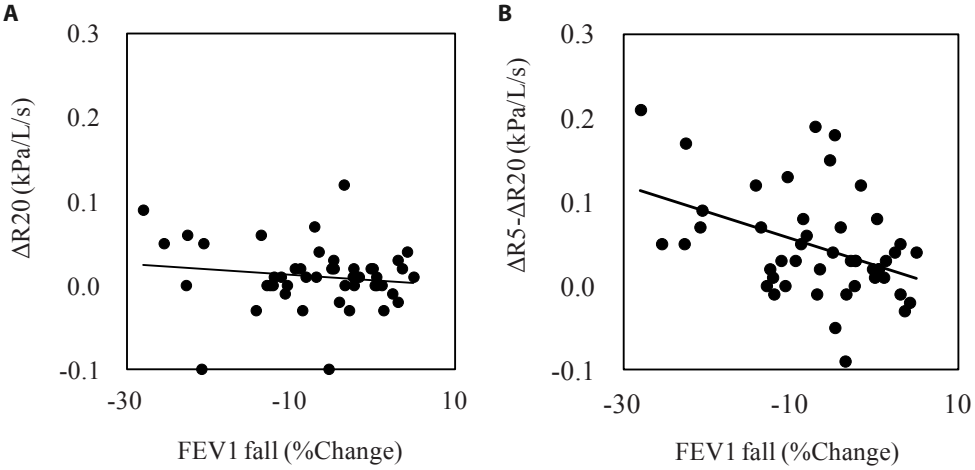

Figure 5

Correlations between the decrease in FEV_1 versus the increasing resistance (R20 (A) and R5-R20 (B)) at 5 minutes after exercise challenge. R5-R20, reflecting resistance of the small airways, is correlated with FEV_1 (ρ = -0.375, P = .009), whereas R20, reflecting resistance of the large airways, did not correlate with FEV_1 (ρ = -0.104, P = .487). Adapted with permission from Lee *et al.*(68)

EXERCISE-INDUCED ASTHMA SYMPTOMS

Involvement of the small airways in the response to exercise

Fonseca-Guedes *et al* found a significant correlation between the exercise-induced decrease in FEF25%-75% and FEV_1, particularly in patients with moderate-to-severe asthma.(61) Interestingly, in patients with mild asthma, a significant decrease in FEF25%-75% (≥26%) was observed, whereas the FEV_1 did not decrease by more than 10%. In addition, Rundell *et al* analyzed lung function and asthma symptoms in ice hockey players before and after exercise and observed a significantly lower baseline FEF25%-75% in subjects with asthma symptoms during or after exercise than subjects without.(62) Kaminsky *et al* performed a bronchoscopy to challenge the small airways locally with cold, dry air.(63) This induced an increase in peripheral airways resistance in asthmatic patients but not in healthy control subjects. In line with this, Decramer *et al* showed that the peripheral resistance, as measured with the forced oscillation technique, increases after a hyperventilation test with cold, dry air.(64) Together, these findings suggest that the small airways are involved in the response to exercise.

Kiers *et al* investigated the role of air trapping in asthmatic patients with a history of exercise-induced asthma.(65) The increase in functional residual capacity was significantly correlated with the exercise-induced decrease in FEV_1. Kosmas *et al* studied the presence of air trapping during exercise in 20 patients with stable asthma and normal lung function at baseline.(66) Exercise-induced asthma, based on a 15% or greater decrease in FEV_1 was only observed in 3 patients, whereas 13 patients had air trapping during exercise. Importantly, the presence of air trapping was associated with reduced exercise capacity. The latter suggests that small airway collapse can occur during and after exercise in patients with stable asthmas without a response in the large airways.

Association between small airway dysfunction and the severity of exercise-induced asthma

Several studies have suggested that small airway dysfunction is associated with more severe exercise-induced bronchoconstriction.(63,67-71) Aronsson *et al* divided 34 asthmatic patients into 2 groups, one with no response and another with a positive response to mannitol, which is another indirect stimulus to measure BHR and closely related to exercise.(72,73) Patients with BHR to mannitol had higher R5-R20 and AX values than patients without BHR. In line with this, Lee *et al* observed that more severe exercise-induced bronchoconstriction is associated with a higher increase in peripheral airways resistance (R5-R20), but not with an increase in large airway resistance (R20) (Figure 4).(68)

Two studies investigated the phase III slope of the single-breath helium and sulfur hexafluoride washout test before and after a cold, dry air hyperventilation test.(69,70) Both studies demonstrated that an increase in the helium and sulfur hexafluoride phase III slopes were associated with the decrease in FEV_1.(69,70) In addition, Ljungberg and Gustaffson showed in asthmatic children that the phase III slope at baseline was correlated with the decrease in FEV_1 after challenge.(70) In contrast, FEV_1 at baseline did not correlate with the decrease in FEV_1 after challenge. This in line with the findings Keen *et al,* who showed that a higher Scond value, as measured with the MBNW test, is associated with the severity of the response to cold, dry air.(71)

THE EFFECTS OF ALLERGEN EXPOSURE ON SMALL AIRWAY DYSFUNCTION

Asthma and allergies are strongly associated, and allergen exposure can provoke asthma symptoms in sensitized subjects. Allergen exposure can result in an immediate airway response, the so-called early asthmatic response, followed by a late-phase response in a subset of asthmatic patients.(74)

The role of the small airways in the allergic response has been investigated by the change in the $FEF_{50\%}$ after breathing a mixture of helium-oxygen compared with room air.(75,76) Because of the lower gas density of helium, it can be assumed that a higher increase in $FEF_{50\%}$ will indicate obstruction in the more proximal airways, which are flow dependent. Metzger *et al* studied the helium-oxygen flow-volume curves in 12 asthmatic patients with both an early- and late-phase allergic response based on a 20% and 10% decrease in FEV_1, respectively.(75) Immediately after the allergen challenge, the $FEF_{50\%}$ increased, suggesting involvement of mainly the large airways. Of interest, the $FEF_{50\%}$ gradually decreased 6 and 24 hours after the allergen challenge, suggesting that the small airways contribute importantly to the late-phase asthmatic response. This is in agreement with the findings of Machado *et al,* who similarly showed an immediate increase in $FEF_{50\%}$ after an allergen provocation followed by a decrease in $FEF_{50\%}$ six hours later. (76) Ahmed *et al* studied the early asthmatic response to ragweed provocation, distinguishing reactors and nonreactors based on a 35% decrease in specific airway conductance.(77) There were no differences between the 2 groups in terms of spirometric results or the phase III slope of the SBNT at baseline. Still, 6 of 10 reactors had an abnormal phase III slope in contrast to 1 of 6 of the nonreactors suggestive for small airway involvement.

Zeidler *et al* investigated 10 asthmatic patients who were exposed to cats until their FEV_1 decreased by 20%.(78) At 6 and 23 hours after this natural cat allergen challenge, FEV_1 had returned to its baseline value. However, they still showed increased levels of air trapping as measured by high resolution computed tomography and SBNT closing volume at both time points. In addition, 6 hours after allergen provocation, FEF25%-75% was decreased compared with baseline values. Together, these observations indicate that the small airways contribute importantly to the late-phase asthmatic response.

Peroni *et al* analyzed air trapping in 18 asthmatic children allergic to house dust mite (HDM). (79) After prolonged HDM avoidance during a stay at high altitude, RV and RV/TLC decreased significantly, yet after subsequent HDM exposure at home, these values increased toward baseline levels, suggesting a small airway response on allergen exposure.

In theory, most particles larger than 10 µm will not enter the airways, and only particles smaller than 5µm will enter the alveoli.(80,81) Most particles of pollen are large with a size of approximately 22-100 µm; however, there are also smaller particles, such as ragweed, with a size of 0.2 to 5.25 µm, which causes symptoms of hay fever.(82,83) Interestingly, pollen can fragment into small respirable particles on hydration by rain or conditions of higher humidity, resulting in an increased number of allergenic aerosols of paucimicronic size that penetrate deep in the lower airways.

(82,84) In this context it is noteworthy that epidemics of asthma attacks have been observed after thunderstorms, especially during the pollen season, suggesting that the small fragments of pollen induce a severe small airway response.(85-88) Another example of allergens with a small size are cat allergens, of which 40% are smaller than 5 µm.(89) Lieutier-Colas *et al* evaluated the effect of provocation with either small particle cat allergens (mass median aerodynamic diameter (MMAD) 1.4 µm) or large particle cat allergens (MMAD 10.4 µm) on the early and late-phase response. (89) Interestingly, the provocative dose (PD) inducing early bronchial symptoms was 20 times smaller for the large than for the small particles. In contrast, 24 hours after provocation with small particles, FEF25%-75% values were significantly lower compared with those after provocation with large particles, the latter being compatible with the notion that the late-phase response is predominantly mediated by the small airways.

ASSOCIATION OF SMALL AIRWAY DYSFUNCTION AND EXPOSURE TO PARTICULATE AIR POLLUTION

Both in children and adults with asthma, higher levels of particulate air pollution have been associated with an increase in respiratory symptoms and use of rescue medication and a decrease in lung function.(90-94) Particulate air pollution can be categorized according to particle size. Particulate matter small than 10µm in diameter (PM10) reflects the coarse particle fraction that will mainly deposit in the larger airways, the particulate matter of less than 2.5 µm in diameter (PM2.5) is referred to as the fine particle fraction, and particles with a diameter of less than 0.1 µm are labeled as ultrafine particles. Fine and ultrafine particles originate to a large extent from incomplete combustion processes, such as those resulting form road traffic and industry. Several studies have investigated the effects of different particulate matter size fractions on respiratory symptoms, medication usage, and lung function.(95-100)

It has been shown by Penttinen *et al* that a higher daily concentration of ultrafine particles, but not PM2.5 and PM10, is associated with a decrease in PEF.(96) These findings are in line with the study of Von Klot *et al,* who found that exposure to a higher concentration of ultrafine particles, but not the coarse particles (PM2.5-10), during 5 and 14 days is associated with increased asthma symptoms, such as wheezing.(97) In addition, the level of exposure to fine and ultrafine particles was associated with increased use of bronchodilators, whereas this association was not find for the level of exposure to coarse particles. In contrast, Maestrelli *et al* observed an association between a higher exposure to coarse particles (PM10) and worse asthma control and quality of life, whereas exposure to fine particles (PM2.5) was not related to these clinical parameters.(98) Small airway function has not been measured in these studies and the contrast in outcomes can perhaps be explained by differences in small airway dysfunction. Taken together, predominantly, the fine and ultrafine fractions contribute to the adverse respiratory health effects of particulate air pollution, probably because of their higher peripheral lung deposition.(101,102) Once deposited in the small airways, fine and ultrafine particles can induce oxidative stress and increase the asthmatic inflammatory response.(103,104) This might explain why Iskandar *et al* did not find an association between ultrafine-particle air pollution and hospital admission in the same week in

a group of asthmatic children because it could be speculated that ultrafine-particle air pollution rather induces an effect in the long term than the short-term.(105)

Two studies have assessed the effects of particulate air pollution on parameters of small airway dysfunction. First, Trenga *et al* found that a higher exposure to fine particles during 24 hours was associated with decrements in $FEF_{50\%}$, but not FEV_1 or PEF, in asthmatic children without anti-inflammatory medication.(99) Next, McCreanor *et al* have compared the effects of high exposure to road traffic air pollution during a 2-hour walk on Oxford Street in London versus low exposure when subjects walked for 2 hours through Hyde Park on a separate occasion.(100) A higher exposure to road traffic-related air pollution, especially the fine- and ultrafine-particle fractions, was accompanied by significant decreases in FEV_1, forced vital capacity, and FEF25%-75%. In summary, especially the fine and ultrafine fractions of particulate air pollution are associated with worsening of asthma control and decreases in parameters of both large and small airway function.

EFFECT OF ASTHMA TREATMENT ON SMALL AIRWAY FUNCTION AND SYMPTOMS

Several studies have investigated the effect of treatment targeting the small airways on asthma control. In a recent study Farah *et al* investigated the predictive value of the change in asthma control after either ICS up-titration in patients with poorly controlled asthma (ACQ>1.5) or those with steroid-naive asthma or ICS down-titration in the case of well-controlled asthma.(106) A higher level of small airway dysfunction, as reflected by higher Sacin and Scond values, was the only independent predictor for either improvement of asthma control after ICS up-titration or loss of asthma control after ICS down-titration. These findings are in agreement with the conclusion that small airway dysfunction is present in asthmatic patients, is related to symptoms, and might require targeted treatment.

Several studies have investigated the efficacy of extra-fine particle pressured metered-dose inhalers with respect to improvement of symptoms and asthma controls.(107-113) Extrafine-particle ICSs an MMAD of approximately 1 µm have a higher lung deposition (50% to 60%) than coarse particle ICSs with an MMAD of 3 to 4 µm (10% to 20%).(114-116) Boulet *et al* compared 3-month treatment with 320 µg hydrofluoroalkane (HFA)-ciclesonide adeministered once daily with 200 µg of dry powder inhaler (DPI)-fluticasone 200 µg administered twice daily in patients with asthma.(107) Although no differences in FEV_1 improvement were observed, improvement in health-related quality of life was significantly higher with HFA-ciclesonide than fluticasone. This is in line with the study of Ohbayashi and Adachi, showing an improvement in the Asthma-related Quality of Life Questionnaire after 3 months' treatment with HFA-beclomethasone compared with DPI-fluticasone together with a decrease in late phase sputum eosinophil counts.108 Furthermore, Huchon *et al* compared the efficacy of 24 weeks' treatment with extrafine fixed combination 200/12 µg of HFA-beclomethasone dipropionate (BDP)/formoterol twice daily versus 500 µg of chlorofluorocarbon (CFC)-BDP twice daily and 24 µg of DPI-formoterol once daily.(109) Although both treatments were equally effective in improving FEV_1, extrafine-particle HFA-BDP/formoterol combination treatment resulted in better asthma control with less symptoms and fewer asthma exacerbations.

More evidence in support of better asthma control with extrafine-particle treatment are derived from a retrospective, observational, real-life study comparing the efficacy of extrafine-particle HFA-beclomethasone (QVAR) to coarse-particle treatment with CFC-beclomethasone.(109) A primary care database was used to identify asthmatic patients who were prescribed either extrafine-particle HFA-beclomethasone or CFC-beclomethasone. Asthmatic patients receiving their first ICS prescription (n = 11,528) or their first increase in ICS dose (n = 774) were included. Extrafine-particle treatment more often resulted in good asthma control, which was defined as no recorded hospital admission or emergency department visits for asthma and no use of oral corticosteroids or antibiotics for respiratory infection of the airways. These results are strengthened by a similar study showing that asthmatic patients treated with extrafine-particle HFA-beclomethasone more frequently achieve asthma control than those treated with coarse-particle treatment with either CFC- or HFA-fluticasone.(111) This is in line with results of 2 further real-life cross-sectional studies showing that the use of extrafine-particle HFA-beclomethasone/formoterol was associated with a higher percentage of patients with well-controlled asthma based on their Asthma Control Test and ACQ scores than the use of fluticasone/salmeterol or budesonide/formoterol combination treatment.(112,113) Taken together, these studies show that extrafine-particle pressurized metered-dose inhalers might have additional clinical benefits in the treatment of asthma compared to coarse-particle treatment.

Several studies investigated the effects of montelukast on the small airways, together with the effects on symptoms or clinical signs. Montelukast is a systematically administered leukotriene receptor antagonist that reaches the small and large airways. Receptors for leukotrienes are expressed at higher levels in fibroblasts derived from the small airways than the large airways, possibly resulting in a predominant effect of montelukast on the small airways.(117) Kraft *et al* studied asthmatic patients with air trapping (RV, >140% of predicted value) and observed a significant improvement in symptoms of wheezing, dyspnea, and cough after treatment with montelukast.(118) Treatment with montelukast resulted in improvements of several lung function parameters; however, only the improvement in RV was associated with less wheezing and chest tightness. These results are similar to those of Zeidler *et al,* evaluating lung attenuation areas with high-resolution computed tomography.(119) An inncrease in lung attenuation was associated with an improvement in the overall mini-Asthma-related Quality of Life Questionnaire.

Spahn *et al* investigated the effect of montelukast on the small airways in children with asthma.(120) RV/TLC improved after treatment with montelukast compared with placebo, whereas FEV_1, FEV_1/forced vital capacity ratio, and FEF25%-75% values did not differ between the groups. These studies demonstrate an association between improvements in symptoms and small airway function after treatment with montelukast, whereas no relation existed with FEV_1 improvement, suggesting that montelukast has beneficial effects, particularly on the small airways.

CONCLUSIONS

This systematic review demonstrates that small airway dysfunction is associated with clinical features of asthma: worse control of asthma,(15,16,20,21) higher numbers of exacerbations,(21,27,33) nocturnal asthma,(40,41,44) more severe BHR,(55,56,59,60) exercise-induced asthma,(61,64,67-71) and the late-phase allergic response(75,76,78,89) (Table 2).

It is important to mention that the data of this review are limited because most of the studies are small and not primarily designed to answer our research question. Another limitation is the lack of a gold standard to assess small airway dysfunction, which made it necessary to mention many types of tests in this review. Obviously, all these tests have specific shortcomings and they frequently cannot rule out an influence of large airways dysfunction. For these reasons, the exact role of the small airways in asthma remains to be elucidated, and more research is necessary to obtain more conclusive evidence.

Notwithstanding these shortcomings, results of the literature provide supportive evidence for a contribution of small airway dysfunction to the clinical expression of asthma. Interestingly, a few studies have shown that small airway dysfunction is not only a feature of severe asthma, but can also be present in patients with mild asthma who have a low level of symptoms and FEV_1 values in the normal range.(15,47,55,61,68) This indicates that the possibility of small airway dysfunction should be considered in the complete spectrum of asthma severity. The latter might be of clinical importance because a better small airway response to treatment with extrafine-particle ICSs or montelukast is accompanied by better asthma control.(112,113,119) For this reason, further research is needed to develop simpler and more reliable tools (e.g. questionnaires or bronchial provocation tests using small particle stimuli) for assessment of the presence and extent of small airway dysfunction in clinical practice. An early recognition of small airway dysfunction enables the physician to start treatment targeting the small airways.

Table 2. Summary of studies investigating the association of small airway dysfunction with the clinical expression of asthma-

	Symptoms and asthma control	Exacerbations	Nocturnal asthma	Bronchial hyperresponsiveness	Exercise
Flow				▪ $FEF_{25\%-75\%}$ is lower in patients with BHR.[55,56] ▪ Children with severe asthma have a lower $FEF_{50\%}$ and severe BHR.[57] ▪ Patients with an irregular aerosol deposition have a lower $FEF_{75\%}$ and more severe BHR.[58]	▪ The decrease in $FEF_{25\%-75\%}$ is correlated with the decrease in FEV_1 in response to exercise.[61] ▪ $FEF_{25\%-75\%}$ was lower in subjects with symptoms in response to exercise than in subjects without symptoms.[62]
Resistance	▪ A higher R5-R20 is associated with a higher BDI score.[15] ▪ A higher X5 is associated with a higher ACQ score.[15] ▪ R5-R20 and AX are discriminating parameters for control of asthma.[16]		▪ Rp values are increased in patients with nocturnal asthma.[39]	▪ Rp values increased more in asthmatic patients compared with healthy control subjects.[45] ▪ A higher Rp value is associated with severe BHR.[46] ▪ R5-R20 values increased more in patients with $PC_{20} \leq 16$ mg/mL than $PC_{20} > 16$ mg/mL during a methacholine provocation test.[47] ▪ An increase in X5 value is associated with an increase in symptoms of dyspnea, wheezing, and chest tightness.[48]	▪ Rp values increased in patients with asthma in response to cold, dry air.[63] ▪ A higher Rp value is associated with more severe BHR.[63,67] ▪ Peripheral resistance measured with FOT increases after a provocation with cold, dry air.[64] ▪ R5-R20 and AX values are increased in patients with BHR to mannitol.[72] ▪ An increase in R5-R20 values is associated with a more severe decrease in FEV_1 in response to exercise.[68]
Ventilation hetero-geneity	▪ Sacin and Scond values are increased in patients with ACQ scores ≥1.5.[20] ▪ An increased dN2 value is associated with a higher ACQ score[21]	▪ dN2 values are increased in patients with ≥2 exacerbations/y.[21] ▪ CV/VC and CC/TLC values are increased in patients with ≥2 exacerbations/ y.[33]		▪ An increased Scond value is associated with more severe BHR.[59]	▪ A higher helium and SF6 phase III slope value is associated with a more severe response to exercise.[69,70] ▪ A higher Scond value is associated with a more severe response to cold, dry air.[71]

Table 2. Continued

Air trapping		▪ RV/TLC values are increased in children with a severe exacerbation.[34] ▪ CT-determined air trapping is related to indicators of exacerbation.[35]		▪ FRC values are increased in patients with chest tightness after a metha-choline provocation test.[49] ▪ The increase in IC values is correlated with a higher Borg score during a provocation test.[50] ▪ Air trapping (ΔIC or ΔRV) predicts the occurrence of dyspnea during a provocation test.[51-53]	▪ An increased FRC value is associated with a more severe response to exercise.[65] ▪ An increased IC value is associated with reduced exercise capacity.[66]
Inflammation	▪ A higher alveolar NO value is associated with more asthma symptoms.[22-25] ▪ Patients with an increased alveolar NO value have worse asthma control, as reflected by a lower ACTscore.[27] ▪ Greater eosinophil numbers are associated with a higher asthma severity score.[31]	▪ Alveolar NO values increase during an asthma exacerbation.[36] ▪ A higher alveolar NO value predicts the occurrence of asthma exacerbation.[37]	▪ Eosinophil numbers in transbronchial biopsy specimens increase overnight in patients with nocturnal asthma.[40] ▪ Eosinophils numbers in BAL fluid increase overnight in patients with nocturnal asthma.[41] ▪ Alveolar NO values are increased in patients with nocturnal symptoms.[44]	▪ A higher percentage of eosinophils is associated with more severe BHR.[60]	

One study found no increased alveolar NO levels during an exacerbation.(38) Two studies demonstrated no association between alveolar NO values and ACQ scores.(28,29) The studies of Oosterhoff *et al* and Jarjour *et al* did not observe an increase in the number of eosinophils overnight measured with BAL in patients with nocturnal asthma.(42,43)

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CHAPTER 3

Effects of small airway dysfunction on the clinical expression of asthma: A focus on asthma symptoms and bronchial hyperresponsiveness

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ABSTRACT

Background: The small airways are an important site of inflammation in asthma. However, the relation between small airway dysfunction and clinical expression of asthma has hardly been studied.

Aim: To investigate the association of small and large airway dysfunction with asthma symptoms and bronchial hyperresponsiveness (BHR).

Methods: Fifty-eight patients with asthma were characterized with spirometry, body plethysmography, impulse oscillometry, alveolar and bronchial exhaled nitric oxide, and a methacholine provocation. Symptoms of nocturnal asthma, exercise-related symptoms, BHR symptoms, and respiratory symptoms were assessed with the Asthma Control Questionnaire and Bronchial Hyperresponsiveness Questionnaire. Perception of dyspnea was rated with the Borg score during the provocation test.

Results: Small and large airway dysfunction did not associate with higher scores for nocturnal, exercise-related, or BHR symptoms. Only higher scores on *wheezing* were significantly associated with higher values of difference between R5 and R20 (R5-R20) ($r = 0.367$, $P < 0.01$) and AX ($r = 0.354$, $P < 0.01$). Lower $FEF_{25-75\%}$ ($P = 0.024$) and higher R5-R20 ($P = 0.003$) values were independently associated with more severe BHR to methacholine, but not FEV_1 or R20 values. The increase in dyspnea during the methacholine provocation was strongly and independently correlated with the decrease in FEV_1 and reactance of the respiratory system at 5 Hertz.

Conclusion: Small and large airway dysfunction poorly associate with asthma symptoms in our patients. However, deteriorations in small airway dysfunction are strongly related to an increase in dyspnea during bronchial provocation with methacholine. Small airway dysfunction contributes also independently to the clinical expression of asthma, as reflected by the severity of BHR.

INTRODUCTION

Traditionally, studies investigating the clinical expression of asthma have focused on the role of the large airways and disregarded a contribution of the small airways. Nowadays, it is accepted that the small airways are an important site of airway inflammation and remodeling in asthma (1,2). In addition, some studies suggest that dysfunction of the small airways is related to more severe asthma symptoms, nocturnal asthma and exercise-induced asthma (3-6). Although the small airways have been subject of investigation during the last years, the relation between small airway dysfunction and asthma symptoms has hardly been studied. A better understanding of how small and large airway dysfunction contribute to asthma symptoms may help to improve asthma management by more targeted therapy.

A few studies have investigated the relation between small airway dysfunction and presence of bronchial hyperresponsiveness (BHR), which is a core clinical feature of asthma (7-9). Wagner *et al* were the first to demonstrate that the small airways are sensitive to provocation with nonspecific stimuli leading to an increased small airway resistance (7). Additionally, Downie *et al* showed that ventilation heterogeneity of the small airways also relates to more severe BHR(9). Together, these findings indicate that the small airways are involved in BHR.

Small and large airway function can be assessed with several tests, for example, spirometry, obtaining the forced expiratory flow at 25% to 75% of the FVC ($FEF_{25-75\%}$) and FEV_1 reflecting small and large airway function, respectively, body plethysmography or exhaled nitric oxide (eNO). As there is no gold standard to specify small or large airway dysfunction, all variables are assumed to reflect either one or the other, and the sensitivity and specificity of the tests concerning small or large airway function are not known (10,11). A recently rediscovered method that is used to asses small and large airway dysfunction is impulse oscillometry (IOS), a simple technique measuring resistance and reactance of the airways (12). Resistance at 20 Hz is considered to reflect the large airways (R20) and resistance at 5 Hz the total airways. Small airway resistance can be calculated with the difference between the resistance at 5 Hz and 20 Hz (R5-R20). Reactance of the respiratory system at 5 Hertz (X5) and total reactance area (AX) are also assumed to reflect small airway function (12,13). Yamaguchi *et al* found significantly higher improvements in IOS parameters (R5-R20 and AX) after treatment with small-particle hydrofluoroalkane-134a beclomethasone dipropionate (HFA-BDP) than after large-particle chlorofluorocarbon (CFC)-BDP (14). In contrast, the responses of spirometric values were comparable between the two treatments, supporting the notion that IOS is a sensitive measurement to assess small airway function.

The aim of this study was to investigate the association of small and large airway dysfunction with asthma symptoms as well as BHR in 58 patients with asthma extensively characterized with respect to small and large airway function.

METHODS

Study design

This cross-sectional study was part of a research project developing a questionnaire assessing small airway dysfunction (NCT01360294). Patients between 18 and 75 years were recruited via general practitioners. Inclusion criteria were a physician's diagnosis of asthma and either a positive response to the methacholine provocation test (provocative concentration causing a 20% fall in FEV_1 (PC_{20}) <39.3 mg/ml) or a maintenance therapy with inhaled corticosteroids (ICS) and presence of asthma symptoms in the last 3 months (Bronchial Hyperresponsiveness Questionnaire (BHQ) symptom score >0). Patients attended the pulmonary outpatient department once and filled in questionnaires and performed lung function measurements. All patients gave written informed consent. The study was approved by the local medical ethics committee.

Symptom assessment

Nocturnal symptoms, exercise-related symptoms, BHR symptoms, and respiratory symptoms were assessed using the Dutch version of the Asthma Control Questionnaire (ACQ) and the BHQ (6,15-17). The ACQ assesses symptoms of last week and the BHQ of the last 3 months. Nocturnal symptoms were measured with the ACQ-1 "*how often were you woken by your asthma during the night*", ACQ-2 "*how bad were your asthma symptoms when you woke up*" and BHQ-2 "*waking up at night due to chest tightness*". Symptoms related to exercise were measured with the ACQ-3 "*how limited were you in your activities*", BHQ-4 "*trouble walking uphill*". The total BHQ-score was used to analyze symptoms of BHR. Respiratory symptoms were investigated with the ACQ-4 "*how much shortness of breath did you experience*", ACQ-5 "*how much of the time did you wheeze*", BHQ-1 "*breathlessness*", BHQ-3 "*shortness of breath*", BHQ-6 "*chest tightness*", BHQ-10 "*acute breathlessness*" and BHQ-11 "*wheezing*".

Assessment of alveolar and bronchial eNO, IOS, spirometry and body plethysmography

Exhaled nitric oxide was measured at multiple flow rates (20, 50, 100, and 200 ml/s) with the NIOX (Aerocrine, Stockholm, Sweden) according to current recommendations (18). The modified mathematical model of Tsoukias and George was used to differentiate between the bronchial and alveolar compartment, respectively, bronchial flux of eNO (JNO) and alveolar concentration of eNO (Calv), and subsequently corrected for axial back-diffusion (19-21). Due to technical problems, eNO measurements were taken in 39 patients. The resistance of the respiratory system was analyzed using impulse oscillometry (IOS masterscreen; E. Jaeger, Wurzburg, Germany) according to standard recommendations (22). Patients performed spirometry and body plethysmography tests (Masterlab, Viasys Healthcare, Höchberg, Germany) according to international guidelines (23-25).

Large and small airway function

Selected parameters to reflect large airway dysfunction were FEV_1, FEV_1/forced vital capacity (FVC), and R20. Parameters for small airway dysfunction were $FEF_{25-75\%}$, residual volume (RV), RV/total lung capacity (TLC) and IOS parameters R5-R20, X5, and AX (10,11). Bronchial and alveolar eNOs were thought to reflect an inflammatory signal from the large and small airways, respectively.

Methacholine provocation test including IOS and Borg scores

A 2-min methacholine provocation test was performed with the Jaeger APS Pro system using a Medic-Aid sidestream nebulizer (Viasys Healthcare) and doubling concentrations of methacholine bromide (0.038-39.3 mg/ml) (26). IOS was measured 30 s after every provocation step followed by an FEV_1 after 90 s. Borg scores, measuring dyspnea, were obtained at baseline and after every step during the provocation test in 37 patients. The PC_{20} was determined by linear interpolation using log-transformed concentrations. Patients not reaching a 20% fall in FEV_1 were assigned twice the highest concentration (78.6 mg/ml).

Statistical analysis

Analyses were performed with SPSS version 20 (IBM, Armonk, NY, USA). Correlations between large and small airway parameters and asthma symptoms were calculated with the Spearman's correlation coefficient. To correct for multiple comparisons between large and small airway parameters and questionnaire scores, we applied the Bonferroni method. Severity of BHR was expressed by the slope of the FEV_1, calculated as the percentage change between the FEV_1 at the last provocation step compared to the FEV_1 at baseline divided by the last given methacholine concentration. The slopes of the IOS parameters (R20, R5-R20, X5) and Borg scores of the provocation test were calculated as the absolute change between the values at the last provocation step minus the values at baseline divided by the last methacholine concentration. Univariate regression of severity of BHR and the slopes were analyzed with the Spearman's rank correlation test.

The slope of the FEV_1 was log-transformed to obtain normal distribution. We performed multivariate linear regression to assess contributing factors to severity of BHR. We included variables with a *P*-value < 0.1 in the univariate regression in our multivariate regression model, with a maximum of one small and one large airway parameter assessed by spirometry and one small and one large airway parameter assessed by IOS. Multivariate regression analysis on BHR was adjusted for age and gender.

RESULTS

A total of 58 patients with mild to severe asthma were included in this study. Their clinical characteristics are presented in **Table 1**.

Asthma symptoms

Higher scores on nocturnal symptoms and exercise-related symptoms were not significantly correlated with more dysfunction of the large or small airways (**Table 2**). Total BHQ score, representing symptoms of BHR, was related to a lower FEV_1 percentage predicted, with a *P*-value approaching statistical significance (r = -0.257, *P* = 0.052). Higher scores on BHQ-11 "*wheezing*", representing respiratory symptoms, were significantly associated with higher R5-R20 and AX values (**Table 3**). Also an unexpected correlation was observed, that is, higher scores on ACQ-3correlated with a higher FEV_1/FVC value, reflecting less obstruction.

Bronchial hyperresponsiveness severity measured with a methacholine provocation test

More severe BHR correlated with several small and large airway parameters **(Table 4)**. Figure 1 shows the correlation between severity of BHR and FEV_1, and R20 (not significant), reflecting the large airways, and $FEF_{25-75\%}$ and R5-R20, reflecting the small airways (**Figure 1**). Multivariate regression analysis of severity of BHR included the large airway parameters, FEV_1% predicted and R20, and the small airway parameters, $FEF_{25-75\%}$ % predicted and R5-R20, with additional adjustment of age, gender, and ICS dose. As severity of BHR was not associated with smoking habits, smoking was not included in the model. Lower age, lower $FEF_{25-75\%}$ % predicted, and higher R5-R20 values were independent predictors of more severe BHR (**Table 5**). FEV_1 and FEV_1/FVC had a comparable correlation coefficient, and when exchanging FEV_1% predicted by FEV_1/FVC only younger age (β = -0.278, *P* = 0.02) and higher R5-R20 (β = 0.299, *P* = 0.02) were independently associated with more severe BHR.

Changes in small and large airway parameters, and dyspnea during methacholine provocation

The slope of the FEV_1 during the methacholine provocation test correlated with the slope of R20, R5-R20 and X5 (**Table 6**). The increase in dyspnea, as assessed with the Borg score, was associated with a deterioration in FEV_1, R5-R20 and X5 but not with the change in R20. Multivariate regression analysis showed that the slope of X5 was significantly associated with the slope of the Borg score independently of the slope of the FEV_1 (X5; β = 0.431, *P* < 0.01 and FEV_1; β = 0.569, *P* < 0.01; R2 = 0.997).

Table 1. Characteristics of the study population

(n=58)		
Gender (m/f)	16/42	
Age (years)	54	(20-75)
BMI (kg/m^2)	29	(21-52)
ICS use (n, %yes)	50	(86)
ICS dose (µg)*	625	(0-3000)
Smoker (%current/ex/never)	7/53/40	
Pack-years (years)	2.5	(0-51)
Asthma control# (%well/partly/uncontrolled)	48/28/24	
Treatment step$ (%step 1-5)	12/14/31/43/0	
FEV_1 (%pred)	109	(66-140)
FEV_1/FVC (%)	78	(52-89)
$FEF_{25-75\%}$ (%pred)	77	(23-154)
RV (%pred)	99	(53-160)
RV/TLC (%)	33	(19-45)
R20 (kPa·s·L^{-1})	0.36	(0.22;0.67)
R5-R20 (kPa·s·L^{-1})	0.09	(-0.03;0.44)
X5 (kPa·s·L^{-1})	-0.12	(-0.49;-0.03)
AX (kPa·s·L^{-1})	0.61	(0.02;4.20)
Alveolar eNO (Calv, ppb) (n=39)	2.9	(-0.3;67.9)
Bronchial eNO (JNo, Pl/s) (n=39)	0.94	(0.24;12.4)
PC_{20} methacholine ≤39.3mg/ml (% yes)	59	
PC_{20} methacholine (mg/ml)	11.6	(0.05-78.6)

Data are presented as median (range) or percentage

*beclomethasone equivalent ; # ACQ score <0.75; ≥0.75 to <1.5; ≥1.5(31); $ asthma severity according to GINA guidelines(32)

BMI: body mass index, ICS: inhaled corticosteroids, FEV_1: Forced expiratory volume in one second, %pred: percentage of the predicted value, FVC: forced vital capacity, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of the FVC, RV: residual volume, TLC: total lung capacity, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between R5 and R20, X5: Reactance of the respiratory system at 5 Hertz, Calv: Alveolar concentration of eNO, JNO: bronchial flux of eNO, PC_{20}: provocative concentration causing a 20% fall in FEV_1, ACQ: Asthma Control Questionnaire, BHQ: Bronchial Hyperresponsiveness Questionnaire

Table 2. Univariate correlations between symptoms of nocturnal asthma and large and small airway parameters (n=58)

	Nocturnal symptoms			**Exercise-related symptoms**		**BHR Symptoms**
	ACQ 1 Nocturnal awakening	*ACQ 2 Symptom severity upon awakening*	*BHQ 2 Waking up at night due to chest tightness*	*ACQ 3 Activity limitation*	*BHQ 4 Trouble walking uphill*	*BHQ Total score*
FEV_1 (%pred)	-0.032 *P* = 0.81	-0.023 *P* = 0.87	-0.095 *P* = 0.48	0.035 *P* = 0.79	-0.183 *P* = 0.17	***-0.257*** ***p=0.05‡***
FEV_1/FVC (%)	0.239 *P* = 0.07	***0.303*** ***P = 0.02***	0.218 *P* = 0.10	**0.354** **p=0.01***	0.185 *P* = 0.17	0.183 p=0.17
$FEF_{25-75\%}$ (%pred)	0.133 *P* = 0.32	0.178 *P* = 0.18	0.115 *P* = 0.39	0.252 *P* = 0.056	0.063 *P* = 0.16	0.046 p=0.73
RV/TLC (%)	-0.148 *P* = 0.27	-0.104 *P* = 0.44	-0.014 *P* = 0.91	-0.223 *P* = 0.09	-0.032 *P* = 0.81	-0.078 p=0.56
R20 (kPa·s·L^{-1})	-0.086 *P* = 0.52	-0.078 *P* = 0.56	0.063 *P* = 0.64	-0.009 *P* = 0.95	0.140 *P* = 0.30	0.085 p=0.53
R5-R20 (kPa·s·L^{-1})	0.069 *P* = 0.61	-0.062 *P* = 0.65	0.181 *P* = 0.18	-0.014 *P* = 0.92	0.214 *P* = 0.11	0.102 p=0.45
X5 (kPa·s·L^{-1})	0.042 *P* = 0.76	0.192 *P* = 0.15	-0.078 *P* = 0.57	0.080 *P* = 0.56	-0.184 *P* = 0.18	-0.102 p=0.45
AX (kPa·s·L^{-1})	0.065 *P* = 0.63	-0.109 *P* = 0.42	-0.153 *P* = 0.26	-0.030 *P* = 0.83	0.200 *P* = 0.14	0.094 p=0.49
Alveolar eNO (Calv, ppb) (n=39)	-0.055 *P* = 0.74	-0.113 *P* = 0.49	-0.267 *P* = 0.10	-0.042 *P* = 0.80	-0.198 *P* = 0.23	-0.062 *P* = 0.7**1**
Bronchial eNO (JNo, Pl/s) (n=39)	-0.062 *P* = 0.71	***-0.370*** ***P = 0.02***	-0.167 *P* = 0.31	0.096 *P* = 0.56	0.124 *P* = 0.45	-0.163 *P* = 0.32

p-values considered significant: p<0.017 for nocturnal symptoms, p<0.025 for exercise-related symptoms, p<0.05 for BHR symptoms. *significant after Bonferroni correction, ‡ p-value = 0.052. (Spearman's correlation coefficient)

ACQ: asthma control questionnaire, BHQ: bronchial hyperresponsiveness questionnaire, FEV_1: forced expiratory volume in one second, %pred: percentage of the predicted value, FVC: forced vital capacity, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of the FVC, RV: residual volume, TLC: total lung capacity, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between R5 and R20, X5: Reactance of the respiratory system at 5 Hertz, Calv: Alveolar concentration of eNO, JNO: bronchial flux of eNO

Table 3. Univariate correlations between respiratory symptoms and large and small airway parameters (n=58)

	Respiratory symptoms						
	ACQ 4 Shortness of breath	*ACQ 5 Wheezing*	*BHQ 1 breath-lessness*	*BHQ 3 Shortness of breath*	*BHQ 6 Chest tightness*	*BHQ 10 Acute breathless-ness*	*BHQ 11 Wheezing*
FEV_1 (%pred)	-0.157	-0.073	-0.169	***-0.295***	-0.019	-0.071	-0.184
	p=0.24	p=0.59	p=0.21	***p=0.03***	p=0.89	p=0.60	p=0.17
FEV_1/FVC (%)	***0.269***	0.191	***0.300***	0.217	0.181	***0.277***	0.134
	p=0.04	p=0.15	***p=0.02***	p=0.10	p=0.17	***p=0.04***	p=0.32
$FEF_{25-75\%}$ (%pred)	0.10	0.086	0.180	0.074	0.134	0.137	0.039
	p=0.44	p=0.52	p=0.18	p=0.58	p=0.31	p=0.30	p=0.77
RV/TLC (%)	-0.106	-0.038	-0.177	-0.107	-0.068	0.015	0.052
	p=0.43	p=0.78	p=0.18	p=0.43	p=0.61	p=0.91	p=0.70
R20 ($kPa \cdot s \cdot L^{-1}$)	0.059	0.121	0.064	0.105	0.004	0.022	0.017
	p=0.66	p=0.37	p=0.64	p=0.44	p=0.98	p=0.87	p=0.90
R5-R20 ($kPa \cdot s \cdot L^{-1}$)	0.054	0.135	0.157	***0.277***	0.007	0.097	**0.367**
	p=0.69	p=0.32	p=0.24	***p=0.04***	p=0.96	p=0.47	**p<0.01*‡**
X5 ($kPa \cdot s \cdot L^{-1}$)	0.039	-0.047	-0.092	-0.206	0.027	-0.052	*-0.249*
	p=0.77	p=0.73	p=0.50	p=0.12	p=0.84	p=0.70	*p=0.06*
AX ($kPa \cdot s \cdot L^{-1}$)	0.041	0.135	0.115	***0.281***	-0.003	0.095	**0.354**
	p=0.77	p=0.32	p=0.40	***p=0.04***	p=0.98	p=0.48	**p<0.01*§**
Alveolar eNO (Calv, ppb) (n=39)	0.084	0.189	-0.147	-0.115	0.076	-0.296	-0.059
	p=0.61	p=0.25	p=0.37	p=0.49	p=0.65	p=0.07	p=0.72
Bronchial eNO (JNo, Pl/s) (n=39)	0.034	-0.034	-0.121	0.050	0.017	-0.007	-0.02
	p=0.84	p=0.84	p=0.46	p=0.76	p=0.92	p=0.97	p=0.88

p-values considered significant: p≤0.007 for respiratory symptoms

* significant after Bonferroni correction, ‡ p-value= 0.005, § p-value= 0.007 for multiple comparisons. (Spearman's correlation coefficient)

ACQ: asthma control questionnaire, BHQ: bronchial hyperresponsiveness questionnaire, FEV_1: forced expiratory volume in one second, %pred: percentage of the predicted value, FVC: forced vital capacity, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of the FVC, RV: residual volume, TLC: total lung capacity, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between R5 and R20, X5: Reactance of the respiratory system at 5 Hertz, Calv: Alveolar concentration of eNO, JNO: bronchial flux of eNO

Table 4. Univariate correlations of severity of BHR, reflected by the FEV_1 slope (n=58)

Variable	Correlation coefficient	P value
Age (years)	-0.142	0.29
BMI (kg/m^2)	-0.017	0.90
ICS dose (µg)*	-0.307	**0.02**
FEV_1 (%pred)	-0.434	**<0.01**
FEV_1/FVC (%)	-0.436	**<0.01**
$FEF_{25-75\%}$ (%pred)	-0.488	**<0.01**
RV (%pred)	0.394	**<0.01**
RV/TLC (%)	0.208	0.12
R20 (kPa·s·L^{-1})	0.228	0.09
R5-R20 (kPa·s·L^{-1})	0.523	**<0.01**
X5 (kPa·s·L^{-1})	-0.382	**<0.01**
AX (kPa·s·L^{-1})	0.466	**<0.01**
Alveolar eNO (Calv, ppb) (n=39)	0.122	0.46
Bronchial eNO (JNO, Pl/s) (n=39)	0.183	0.27

*beclomethasone equivalent (Spearman correlation coefficient)

P-value <0.05 was considered significant.

BMI: body mass index, ICS: inhaled corticosteroids, FEV_1: Forced expiratory volume in one second, FVC: forced vital capacity, %pred: percentage of the predicted value, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of the FVC, RV: residual volume, TLC: total lung capacity, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between R5 and R20, X5: Reactance of the respiratory system at 5 Hertz, Calv: Alveolar concentration of eNO (n=40), JNO: bronchial flux of eNO (n=40).

Table 5. Multivariate linear regression of predictors of BHR severity, reflected by the FEV_1 slope*

	β	P value
Age (years)	-0.366	**0.01**
Gender (m/f)	0.043	0.69
ICS dose (µg)	-1.47	0.20
FEV_1 (%pred)	0.349	0.12
$FEF_{25-75\%}$ (%pred)	-0.484	**0.02**
RV (%pred)	0.174	0.21
R20 (kPa·s·L^{-1})	-0.021	0.85
R5-R20 (kPa·s·L^{-1})	0.439	**<0.01**

$R^2 = 0.52$

*the FEV_1 slope was log-transformed

P-value <0.05 was considered significant.

M:male, f: female (is coded 1), ICS: inhaled corticosteroids, FEV_1: Forced expiratory volume in one second, %pred: percentage of the predicted value, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of the forced vital capacity, RV: residual volume, R5-R20: difference between resistance at 5 Hertz and 20 Hertz, R20: resistance of the respiratory system at 20 Hertz

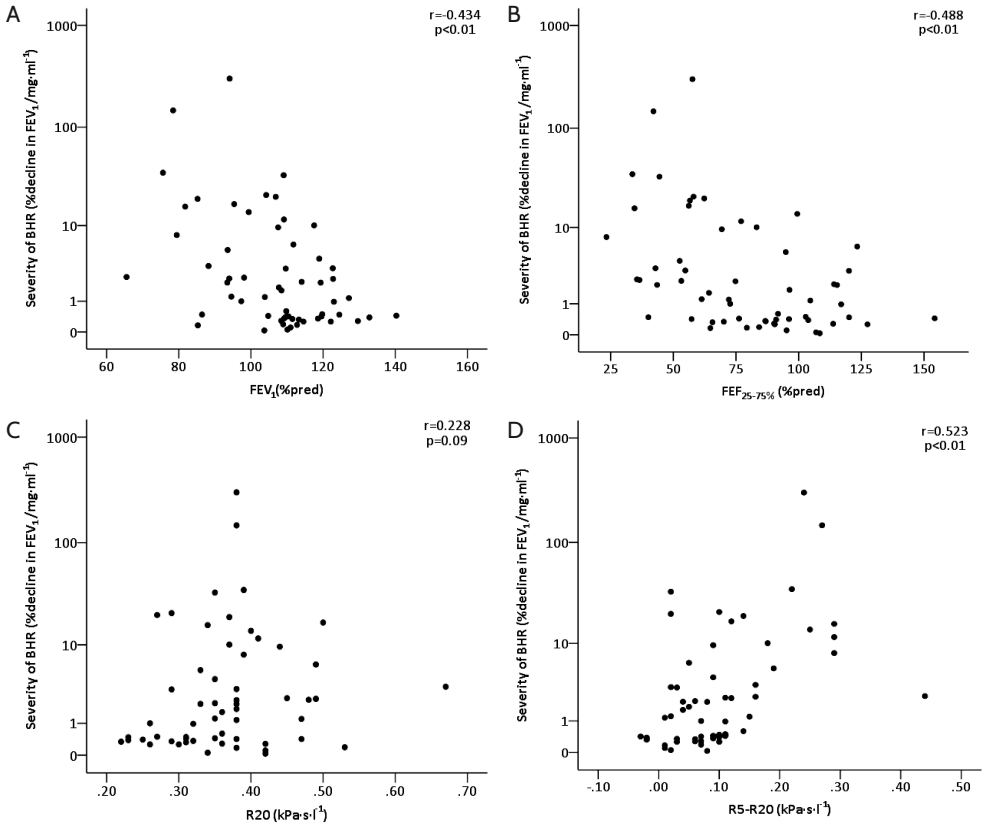

Figure 1: Correlation plot of the severity of BHR, that is, slope of the FEV_1, (%decline in FEV_1/(mg/ml)) with (**A**) the FEV_1 (%pred), (**B**) the forced expiratory flow at 25% to 75% of the FVC ($FEF_{25-75\%}$) (%pred), (**C**) the resistance of the respiratory system at 20 Hertz (R20) (kPa·s·L-1), and (**D**) the difference between R5 and R20 (R5-R20) (kPa·s·L-1).

Table 6. Univariate correlations of the change in FEV_1 and IOS parameters with the change in dyspnea score during a provocation test, reflected by the slopes of the FEV_1, R20, R5-R20, X5 and Borg score.

	Slope of R20	Slope of R5-R20	Slope of X5	Slope of Borg
Slope of FEV_1	**0.315, (p=0.02)**	**0.846, (p<0.01)**	**0.763, (p<0.01)**	**0.902, (p<0.01)**
Slope of R20	-	**0.311, (p=0.02)**	0.183, (p=0.17)	0.146, (p=0.39**)**
Slope of R5-R20	-	-	**0.865, (p<0.01)**	**0.872, (p<0.01)**
Slope of X5	-	-	-	**0.730, (p<0.01)**

(Spearman's correlation coefficient)

Analysis included data of 58 patients with respect to the slope of the FEV_1, R20, R5-R20, X5, and data of 37 patients with respect to the slope of the Borg score.

P-value <0.05 was considered significant.

FEV_1 slope(% decline in FEV_1/ mg·ml-1); slopes of the R20, R5-R20 and X5 (kPa·s·L-1/mg·ml-1); Borg slope (Borg score/mg·ml-1) FEV_1: Forced expiratory volume in one second, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between R5 and R20, X5: Reactance of the respiratory system at 5 Hertz

DISCUSSION

This study provides new insights into the role of small airway dysfunction in relation to asthma symptoms and BHR. Small airway dysfunction associates poorly with patient-perceived symptoms, as only higher small airway resistance and reactance were related to symptoms wheezing. However, when measuring BHR to methacholine, we found small airway dysfunction, that is, a lower $FEF_{25-75\%}$ and a higher R5-R20, to be independently associated with more severe BHR to methacholine, whereas large airway dysfunction as reflected by lower FEV_1 or higher R20 was not.

In the present study, large airway dysfunction was not significantly related to patient-perceived nocturnal symptoms, exercise-related symptoms, BHR symptoms, and respiratory symptoms. This is in line with previous studies showing that asthma symptoms poorly correlate with the FEV_1 (27,28). In contrast to our expectations, small airway dysfunction also poorly correlated with asthma symptoms. We found that small airway dysfunction was only associated with wheezing, when small airway dysfunction was measured with IOS. These results contrast with the findings of our systematic review where we found suggestive evidence for a relation between small airways dysfunction and respiratory symptoms (6). Although only a few studies have directly investigated this relation (3,15). Takeda *et al* found that the small airway parameters R5-R20 and X5 were associated independently of the large airway parameters FEV_1 and R20 with either Asthma Quality of Life Questionnaire, St. George's Respiratory Questionnaire, ACQ or Baseline Dyspnea Index (3). Next, Bourdin *et al* investigated 21 patients with asthma and found that a higher ACQ score was correlated with a steeper slope of the single-breath nitrogen washout test and higher RV/TLC values, while the ACQ score did not correlate with FEV_1 and $FEF_{25-75\%}$ values (29). In addition, Mansur *et al* found that the increase in X5 following a methacholine provocation test was correlated with the increase in dyspnea, tightness, and wheezing. The latter study investigated the change in symptoms during a provocation test and not the severity of symptoms at baseline as our study did. Analysis of the change in symptoms during provocation in the present study also showed a strong correlation between methacholine-induce deteriorations in large and small airway dysfunction and the concomitant increase in dyspnea. Taken together, it remains uncertain whether both small and large airway dysfunction associate with asthma symptoms at baseline and, if it is present, the association appears to be weak and more frequently related to IOS than spirometric parameters. As small airway dysfunction was not taken into account with the development of these questionnaires, it is probable that the ACQ and BHQ do not include symptoms related to small airway dysfunction. For this reason, we think we need a new questionnaire to detect symptoms related to especially small airway dysfunction.

When we tested severity of BHR by a methacholine provocation test instead of by the patient-perceived BHQ score, severity of BHR was associated with small but not large airway dysfunction. This finding is in line with a study of Telenga *et al* showing that the forced expiratory flow at 50% of the FVC ($FEF_{50\%}$) is an independent predictor of the provocative dose of histamine causing a 20% fall in FEV_1 (PD20) (30). We extended these findings by including IOS, thereby showing that small airway resistance, that is, R5-R20, is an additional independent predictor of more severe

BHR. We did not find any relationship of severity of BHR with bronchial and alveolar eNO, which are supposed to reflect central and peripheral airway inflammation, respectively. However, this can be due to the lower number of patients in whom eNO measurement was taken.

Besides measuring the fall in FEV_1 during the methacholine provocation test, the airway response was also measured with IOS parameters and dyspnea Borg scores. The change in FEV_1 was closely related to the changes in R5-R20 and X5; however, it correlated weakly with the change in R20. This was an unexpected finding as FEV_1 and R20 are both considered to reflect large airway function. We suggest that the FEV_1 not only reflects changes in the large airways, but is also affected by changes in small airway function. Although it is not exactly known which compartment of the bronchial tree is measured with the R20, we speculate that it mainly reflects the resistance of the trachea and cartilage-containing large central airways which hardly narrow in response to provocation.

A strength of our study is the use of a heterogeneous study population, containing current, ex-, and never smokers, obese as well as non-obese patients, steroid-naïve patients and steroid users with asthma. This reflects the real-life situation, and our conclusions may be translated to the broad spectrum of asthma patients. A limitation of our study was the relatively small sample size of 58 patients. Future studies with a larger sample size are needed to confirm the poor relationship between asthma symptoms and small airway dysfunction as found in our asthma population.

In conclusion, our results suggest that small airway dysfunction poorly associates with asthma symptoms. However, small airway dysfunction contributes importantly to the clinical expression of asthma as reflected by the severity of BHR when measured with a methacholine provocation test and by the increase in dyspnea during bronchial constriction. For this reason, small airway dysfunction should not be disregarded in asthma management. Moreover, treatment of the small airways may improve patients' well-being with respect to BHR and accompanying dyspnea.

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CHAPTER 4

Small and large airway dysfunction in relation to asthma control and the response to environmental stimuli;

An observational study in 3,155 asthma patients from primary care

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Submitted

ABSTRACT

Background: The clinical relevance of small airway dysfunction in asthma is a topic of debate. So far, most studies exploring the relation between the clinical expression of asthma and small airway dysfunction investigated small subgroups of asthma patients.

Objective: To investigate whether asthma control and responses to environmental stimuli associate with large and/or small airway dysfunction in a large unselected asthma population derived from primary care.

Methods: We selected 3,155 patients with a doctors' diagnosis of asthma. Patients performed spirometry before and after a bronchodilator. FEV_1 and $FEF_{25-75\%}$ reflecting large and small airway function respectively, were used in the analyses. Primary outcomes were asthma control, assessed with the asthma control questionnaire (ACQ) and respiratory responses to environmental stimuli, assessed by a tick-list with several stimuli, e.g. animals or dust.

Results: Patients with uncontrolled asthma had significantly lower pre-bronchodilator FEV_1 and $FEF_{25-75\%}$ values than the group with controlled asthma. Separate multivariate regression models showed that a higher ACQ score was associated with both a lower FEV_1 and $FEF_{25-75\%}$, independently from age, gender, BMI, smoking habits, inhaled corticosteroid use, and FVC. Different environmental stimuli were associated with FEV_1 or $FEF_{25-75\%}$ values, i.e. responses to fog and exercise with lower FEV_1 values, and responses to animals with lower $FEF_{25-75\%}$ values.

Conclusion: This large study indicates that both large and small airway dysfunction contribute to the clinical expression of asthma, as represented by asthma control and responses to different environmental stimuli.

INTRODUCTION

Asthma is a chronic inflammatory airway disease with a prevalence around 8% (1). It is characterized by variable airway obstruction and bronchial hyperresponsiveness leading to episodes with respiratory symptoms. Still, the exact contribution of airway obstruction to the severity of asthma symptoms, and particularly the contribution of small airway obstruction, has not been elucidated.

For a long time research on asthma focused on the role of the large airways and disregarded the small airways. Nowadays, several studies have shown that the small airways, similar to the large airways, are involved in the inflammatory and remodeling processes underlying asthma (2,3). In addition, it has been proposed that small airway dysfunction contributes to the clinical expression of asthma, e.g. to worse asthma control, presence of nocturnal asthma and more severe bronchial hyperresponsiveness (4-8). For example, Telenga and colleagues showed that a lower $FEF_{50\%}$ value, reflecting small airway obstruction, was associated with more severe bronchial hyperresponsiveness independently of the FEV_1, a predominantly large airway parameter (9). Furthermore, Fonseca-Guedes and colleagues investigated asthmatic children and observed that the $FEF_{25-75\%}$, representing the small airways, could fall in response to exercise without a fall in the FEV_1 (10). These results suggest that the small airways are involved in the clinical expression of asthma.

Most studies investigating small airway dysfunction and the clinical expression of asthma have focused on subgroups of asthma patients and studied relatively small numbers of patients with sample sizes ranging between 10 to 100 asthmatics (4,11). Population studies have not yet been performed. Investigating specific subgroups of asthma patients, like those with exercise induced asthma or severe asthma, provide an incomplete overview of small airway dysfunction. Studies using a large representative asthma population may thus reveal better insights in the role of small airway dysfunction in asthma and will be relevant for a broad range of asthma patients (12,13).

A representative asthma population has recently been investigated by Price and colleagues showing that the chance to achieve good asthma control is higher in patients receiving small particle Hydrofluoroalkane (HFA)-beclomethasone compared to coarse particle fluticasone (14). Since inhaled corticosteroids (ICS) with small particles are considered to have a larger deposition in the small airways than coarse particle ICS, these results suggest that the small airways are important for asthma control. However, this study did not include lung function measurements, and therefore it is not clear whether improvement of small and/or large airway dysfunction contributed to a better asthma control.

The aim of the present study is to investigate whether asthma control and responses to environmental stimuli, reflecting hyperresponsiveness of the airways, associate with large and/or with small airway dysfunction in a large and representative sample of asthma patients derived from primary care.

METHODS

Study design

Data was collected by the "asthma/COPD service" in the northern part of the Netherlands (Certe laboratory, Groningen, The Netherlands). This service supports the general practitioner in diagnosing and treating patients with asthma and COPD. The service assesses lung function and takes questionnaires in local laboratories. Pulmonologists inspect the data online and subsequently advise in diagnoses and therapy or suggest referral to a pulmonologist. The setup and feasibility of the asthma/COPD service has been described by Metting and colleagues (15). Analyses were performed with data of baseline examinations collected between the start of the asthma/COPD service in 2007 until 2011. By then, the database contained data from 3369 adult asthma patients, derived from 308 participating general practitioners. All patient data was made anonymous. The scientific board of the asthma/COPD service approved use of the data for this study.

Study population

Patients with a physicians' (pulmonologist) diagnosis of asthma and aged ≥18 years were included. Gender, age, height, and weight were assessed by the pulmonary function technician. Subjects were divided into current smokers (smoked during a period >12 months and stopped <12months), ex-smokers (smoked during a period >12 months in the past and now stopped ≥12 months), and never smokers (never smoked or smoked during a period ≤12 months). Pulmonary medications were written out completely by the pulmonary function technician and entered into the database.

Asthma control and respiratory response

Asthma control was assessed with the asthma control questionnaire (ACQ), consisting out of 6 questions, i.e. nocturnal awakening, symptom severity upon awakening, activity limitation due to asthma, shortness of breath due to asthma, wheezing and use of reliever inhaler within the past week (16). Asthma control was divided into two groups based on the total ACQ score, i.e. controlled asthma (ACQ <0.75) and uncontrolled asthma (ACQ ≥0.75) (17).
Respiratory responses to environmental stimuli were assessed by a tick-list of having either chest-tightness, breathlessness or wheezing with exposure to the following stimuli: animals, (house) dust, grasses, trees, cold air, fog, exercise, cigarette smoke, baking smell, paint smell and perfume.

Large and small airway function

Large and small airway function were measured with spirometry (Welch Allyn™, spirometer) before and 15 minutes after inhalation of 400 µg salbutamol. The forced expiratory volume in the first second (FEV_1) was considered to reflect large airway function; the forced expiratory flows at 25% to 75% ($FEF_{25-75\%}$) of the forced vital capacity (FVC) was considered to reflect small airway function (**Table 1**) (18). Spirometry was conducted by a trained lung technician according to the criteria of the American Thoracic Society (ATS) (19).

Statistical analysis

Reversibility of spirometric parameters was calculated by the difference between the post- and pre-bronchodilator values divided by the pre-bronchodilator value.
Differences between the asthma control groups were tested with the student's T-test or Mann Whitney U test for normally or non-normally distributed parameters, respectively. Differences between the categorical variables were calculated with the Chi-square test. Univariate correlations with the ACQ score were calculated with Spearman's correlation coefficients. Multivariate linear regression has been used to calculate the correlations of FEV_1 and $FEF_{25-75\%}$ %predicted with ACQ, independent from gender, age, body mass index (BMI), smoking habits, ICS use, ICS dose and FVC. Because of multicollinearity between the FEV_1 and the $FEF_{25-75\%}$ %predicted, we made separate regression models for both parameters. Correlation between the FEV_1 and the $FEF_{25-75\%}$ was determined with the Pearson correlation coefficient.
To identify independent associating respiratory stimuli with the FEV_1 or the $FEF_{25-75\%}$ multivariate regression analysis was performed adjusting for gender, age, height, smoking habits, and ICS use. The absolute pre-bronchodilator values of FEV_1 and $FEF_{25-75\%}$ were used in the multivariate regression as theses values were the dependent variables in the linear regression model. Regression models with the $FEF_{25-75\%}$ were also adjusted for FVC. All analyses were performed with SPSS version 20.

RESULTS

Patients with complete data of pre-bronchodilator spirometry values were included in this study (n=3,155). Of these patients 3143 had complete data for the ACQ score.

Differences in large and small airway dysfunction between asthma control groups

In total 33% patients had controlled and 67% uncontrolled asthma according to the total score of the ACQ (**Table 1**). The asthma patients with uncontrolled asthma had significantly lower pre-bronchodilator FEV_1 and $FEF_{25-75\%}$ %predicted values than asthma patients with controlled asthma. In addition, the degree of FEV_1 and $FEF_{25-75\%}$ reversibility was significantly higher in patients with uncontrolled asthma than patients with controlled asthma (**Figure 1**).

Association of large and small airway dysfunction with asthma control and with asthma symptoms

Lower values of the pre-bronchodilator FEV_1 and $FEF_{25-75\%}$ %predicted were significantly associated with a higher total ACQ score, reflecting less asthma control (**Table 2; Figure 2**). Lung function parameters were also correlated with the separate ACQ items (**Table 2**). Lower values of the FEV_1 %predicted were significantly associated with higher scores on all items. Lower values of the $FEF_{25-75\%}$ were significantly associated with higher scores on wheezing and use of reliever inhaler.

Table 1. Patient characteristics divided controlled and uncontrolled asthma (total numbers)

	(n)	Controlled asthma (n=1048)		Uncontrolled asthma (n=2095)		p-value
Female, n (%)	(3143)	608	(58)	1347	(64)	0.001
Age (years)	(3143)	49	(17)	46	(16)	0.001
BMI (kg/m^2)	(3143)	26	(5)	28	(6)	<0.001
Smoking (%curr, nev/ex)	(3132)		17/51/32		25/41/34	<0.001
ICS use, n (%yes)	(3020)	611	(60)	900	(45)	<0.001
ICS dose (µg/day) ‡#	(1300)	500	(75-2000)	1000	(50-4000)	<0.001
FEV_1/FVC pre (%)	(3143)	75	(9)	75	(9)	0.785
FVC pre (%pred)	(3143)	104	(14)	101	(16)	<0.001
FEV_1 pre (%pred)	(3143)	94	(15)	91	(16)	<0.001
$FEF_{25-75\%}$ pre (%pred)	(3143)	68	(26)	66	(26)	0.038
Reversibility FEV_1 (%)	(3078)	5.7	(7)	7.2	(11)	<0.001
Reversibility $FEF_{25-75\%}$ (%)	(3051)	19.5	(23)	21.7	(28)	0.028

*Differences between groups are tested with the student's t-test, Mann Whitney U test or chi-square test as appropriate. Data is shown as mean (SD) or as median (range), or as percentage.
‡ beclomethasone equivalent, #only patients with ICS (2.6% using small particles ICS)

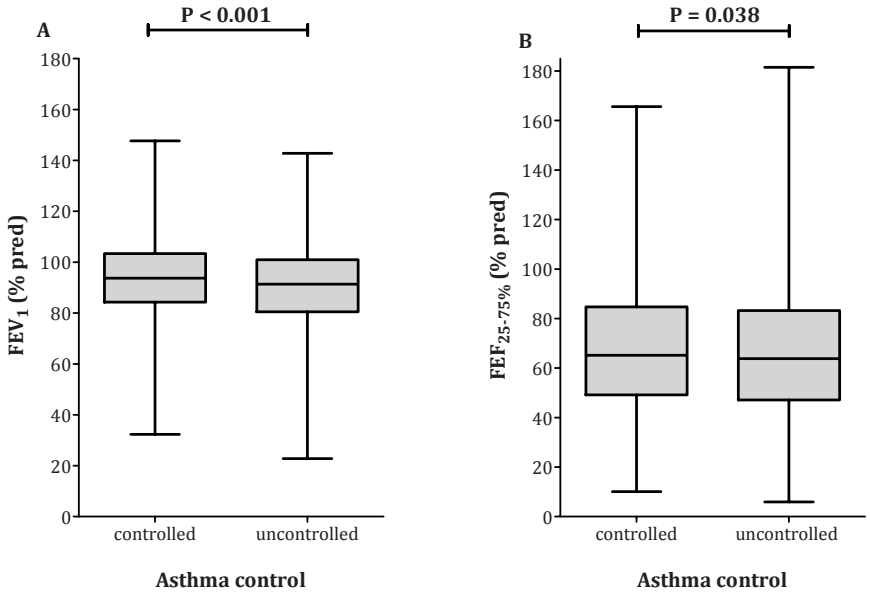

Figure 1. Difference between asthma control groups for (**A**) the FEV_1 %predicted and (**B**) the $FEF_{25-75\%}$ %predicted

Table 2 Associations between ACQ scores and pre-bronchodilator lung function parameters

	FEV_1 (%pred)	$FEF_{25-75\%}$ (%pred)
ACQ total score	-0.131**	-0.044*
Nocturnal awakening	-0.058**	-0.011
Symptom severity upon awakening	-0.068**	0.002
Activity limitation	-0.085**	0.009
Shortness of breath	-0.083**	-0.002
Wheezing	-0.195**	-0.155**
Use of reliever inhaler	-0.117**	-0.088**

Spearman's correlation coefficient; *p-value <0.05, **p-value <0.01

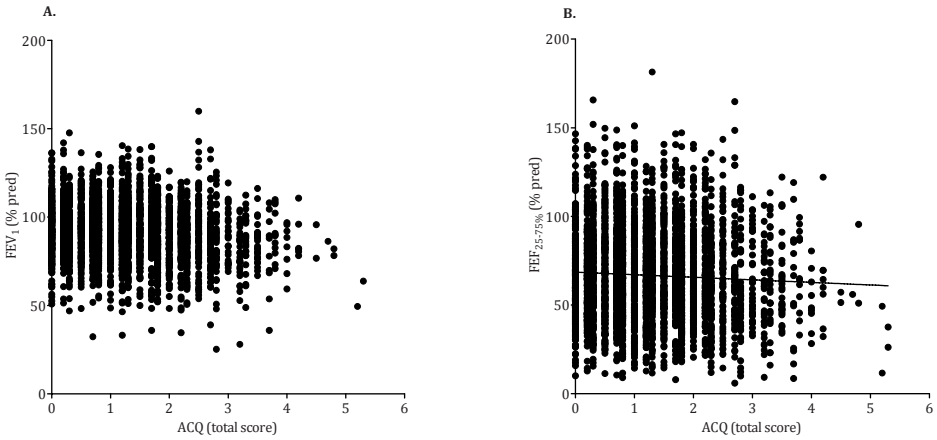

Figure 2. Correlation plot of (**A**) the FEV_1 %predicted (r=-0.131, p<0.001) and (**B**) the $FEF_{25-75\%}$ %predicted (r=-0.044, p=0.015) with the ACQ score.

Table 3. Multivariate linear regression for ACQ total score including (**a**) a large airway parameter, the FEV_1 pre-bronchodilator and (**b**) a small airway parameter, the $FEF_{25-75\%}$ pre-bronchodilator.

3.a	B	β	P value
FEV_1 (%pred)	**-.005**	**-.081**	**<0.01**
Age (years)	-.007	-.129	<0.01
Gender (f)	.149	.077	<0.01
BMI (kg/m^2)	.022	.136	<0.01
Smoking (never/current)	.356	.158	<0.01
Smoking (never/ex)	.101	.051	0.01
ICS use (yes)	-.474	-.251	<0.01
ICS dose (μg)	$2.83 \cdot 10^{-4}$	.167	<0.01
FVC (%pred)	-.004	-.061	0.03

$R^2 = 0.112$

3.b	B	β	P value
$FEF_{25-75\%}$ **(%pred)**	**-.002**	**-.047**	**0.01**
Age (years)	-.008	-.133	<0.01
Gender (f)	.154	.079	<0.01
BMI (kg/m^2)	.022	.135	<0.01
Smoking (never/current)	.356	.158	<0.01
Smoking (never/ex)	.101	.050	0.01
ICS use (yes)	-.474	-.251	<0.01
ICS dose (µg)	$2.82 \cdot 10^{-4}$	.167	<0.01
FVC (%pred)	-.007	-.114	<0.01

$R^2 = 0.111$

Table 4 Multivariate linear regression models with (**a**) FEV_1 pre-bronchodilator and (**b**) $FEF_{25-75\%}$ pre-bronchodilator as outcome variable for each environmental stimulus

4.a FEV_1	B	SE	P value
Animals	-.021	.021	.318
(House) dust	-.008	.019	.675
Grasses	.022	.021	.292
Trees	.053	.023	**.036**
Cold air	-.002	.021	.918
Fog	-.069	.020	**<.001**
Exercise	-.079	.024	**.001**
Cigarette smoke	-.008	.020	.686
Baking smell	-.052	.034	.127
Paint smell	-.033	.021	.108
Perfume	-.017	.021	.431

4.b $FEF_{25-75\%}$	B	SE	P value
Animals	-.152	.039	**<.001**
(House) dust	-.068	.036	***.055***
Grasses	.024	.039	.531
Trees	.047	.046	.313
Cold air	.039	.038	.308
Fog	***-.069***	***.036***	***.055***
Exercise	-.055	.044	.216
Cigarette smoke	.010	.037	.792
Baking smell	.008	.062	.896
Paint smell	.018	.038	.627
Perfume	-.001	.039	.983

Models are adjusted for age, gender, height, ICS use and smoking status (r square of model 3.a 0.657-0.659; model 3.b 0.373-0.374).

Multivariate regression analysis for asthma control

Since FEV_1 and $FEF_{25-75\%}$ values were strongly correlated (r=0.717, p<0.001), their independent association with the ACQ score could not be assessed (**Figure 3**). Separate multivariate regression models showed that higher FEV_1 and $FEF_{25-75\%}$ values were associated with a lower ACQ score, independently from age, gender, BMI, smoking habits, ICS use, ICS dose, and FVC (**Table 3**).

Association of large and small airway dysfunction with respiratory response to environmental stimuli

FEV_1 and $FEF_{25-75\%}$ were associated with various environmental stimuli adjusted for all demographic variables. Responses to fog and exercise were significantly associated with a lower FEV_1 value and on the other hand a response to trees was significantly associated with a higher FEV_1 value. Responses to animals were significantly associated with a lower $FEF_{25-75\%}$, whereas the association of responses to dust and fog reached almost statistical significance (p=0.055 and p=0.055 respectively; **Table 4**).

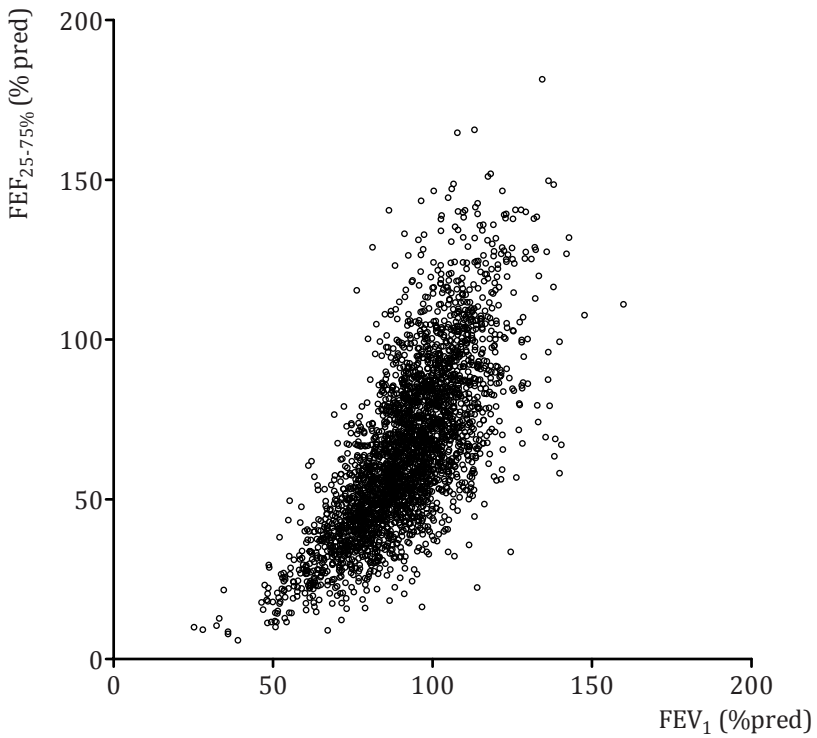

Figure 3. Correlation plot of the FEV_1 %predicted with the $FEF_{25-75\%}$ %predicted (r=0.717, p<0.001). Pearson correlation coefficient with pre-bronchodilator values.

DISCUSSION

Both large and small airway dysfunction are associated with worse asthma control in this large cohort of asthma patients with a wide range of asthma severity and control. Next, large and small airway dysfunction are associated with symptoms after exposure to different environmental stimuli.

To our knowledge, this is the first study investigating small airway dysfunction in relation to the clinical expression of asthma in a large unselected asthma population. The availability of both large and small airway function parameters, questionnaire scores, smoking habits, and, ICS use and dose, in such a large data set is unique. The inclusion of a large real life population is another strength, which makes it possible to generalize conclusions to a broad spectrum of asthma patients. There are also some remarks that could be made to our study. Its design was limited as potential confounding factors like comorbidity or therapy adherence were not recorded. Additionally, we were unable to determine the contribution of small airway dysfunction to asthma control independently of large airway dysfunction and vice versa, since they were strongly interrelated. It could be proposed that the FEV_1 and $FEF_{25-75\%}$ are so closely related that they do not provide differential signals. However, the correlation graph of the lung function variables shows a curved line with more variability in the range of higher FEV_1 and $FEF_{25-75\%}$ values, suggesting that both parameters reflect a distinct signal (**Figure 3**).

Asthma control

We observed a significant difference in both large and small airway dysfunction for pre-bronchodilator values and reversibility when comparing groups with controlled and uncontrolled asthma. Previous studies with small sample sizes have shown conflicting results whether there is a relation of large and small airway dysfunction with asthma control, using different small airway tests, i.e. impulse oscillometry (IOS), multiple-breath nitrogen washout (MBNW) test (20-22). In line with our study, Takeda and colleagues found asthma control to be associated with large and small airway dysfunction in 65 asthma patients using IOS instead of the spirometric values reflecting small airways (20). Farah and colleagues observed only an association of asthma control with small airway dysfunction, represented by the ventilation heterogeneity generated in the conductive lung zone of the small airways (Scond) measured with the MBNW technique in a group of 105 asthmatics (Scond with ACQ score r=0.26) (21). In contrast, Gonem and colleagues could not find an association between asthma control and small airway dysfunction, using IOS and MBNW in a group of patients with more severe asthma (74 patients, 14 patients using oral prednisone). They did observe a relation between asthma control and large airway dysfunction (FEV_1 with ACQ r=-0.29) (22). Together, none of the previous studies, including our study, observed strong correlations of large or small airway dysfunction with asthma control. In our study, lung function values were weakly correlated with separate ACQ items. Interestingly, the FEV_1 values were associated with all six ACQ items, while the $FEF_{25-75\%}$ values were only associated with wheezing and use of reliever inhaler medication, suggesting that a higher ACQ score, i.e. worse asthma control, is related to both large and small airway dysfunction, but probably more closely to large airway dysfunction. The weak association of small airway dysfunction with the ACQ may be explained by the larger

variability of the $FEF_{25-75\%}$ compared to the FEV_1, as reflected by the coefficient of variation of 39% versus 17%. Another interpretation for the weak association between small airway dysfunction with the ACQ could be that the small airways contribute less importantly to asthma control than the large airways. Alternatively, small airways dysfunction could contribute importantly to asthma control, but the ACQ may be not sensitive to pick up small airway dysfunction. Although this interpretation may seem far-fetched, it should be noted that the ACQ has been developed using the conventional large airway parameters FEV_1 and peak expiratory flow values without taking small airway parameters into account (16). For this reason, it would be of interest to develop a specific small-airway questionnaire, which is validated using small airway measurements.

Response to environmental stimuli

Stimuli of fog and exercise eliciting respiratory symptoms in asthmatics were associated with large airway function, while stimuli of animals were significantly associated with small airway function. These findings suggest that these stimuli have differential effects, i.e. nonspecific stimuli in the large airways and allergic stimuli in the small airways, or they suggest a different deposition. For example, large fog particles with a size ranging between 1 to >10 µm may deposit in the large and small airways, but probably predominantly in the large airways (23,24). On the other hand small particle cat allergens may deposit substantially in the small airways (25). Interestingly, Zeidler showed that exposure to cats provoked a significant response in the small airways, as assessed with the $FEF_{25-75\%}$ and with air trapping measured with High Resolution Computed Tomography (26). Unexpectedly, a response to trees was associated with a higher FEV_1. We do not have an explanation for this finding as sensitization to trees has been reported to be related to more severe asthma (27). Also in contrast with recent findings in the literature, we did not observe a relation between a response to exercise and small airway function, but a relation between exercise and large airway function (10,28). Indeed, we did not specify the response to exercise as exercise-induced asthma, and this response may also be related to non-pulmonary causes like heart failure or a poor cardio-circulatory condition in this population from which a quarter is aged over 60. Since we did not explore the exercise response in detail, or explore the response to allergens with a blood or skin prick test we cannot draw firm conclusions about their relation to large or small airway dysfunction. Nevertheless, the search for differential responses to various stimuli with large and small airway dysfunction is novel and promising. If we understand whether stimuli have a differential effect or selective deposition in the large or small airways, we can develop new strategies to improve patients well-being.

Our results call for further studies since the observation that large and small airway dysfunction is related to worse asthma control and presence of respiratory symptoms may have clinical consequence for management of asthma. Our results support the findings of Price and colleagues showing that treatment with small particles ICS was related to better clinical outcomes and a better chance to achieve asthma control than treatment with large particle ICS in a large unselected population (14). Since small particle ICS will potentially reach not only the large but also the small airways, it may improve asthma control by an effect on large and small airway dysfunction (29,30). In contrast, large particle ICS will only deposit in the large airways and achieve probably no effect

on small airway dysfunction. We were not able to analyze differences in asthma control between subjects with and without asthma control, as only 2.6% of the subjects used small particle ICS. Longitudinal studies investigating the change in asthma control in relation to the change in large and small airway dysfunction are needed to confirm our results. Future studies should also consider to include new questionnaire, as a specific small-airway questionnaire to assess symptoms and control in subjects with asthma. Our results suggest that stimuli may have differential effect on large or small airway function. Future studies should further investigate the relationship between small airway function and allergen sensitization, using specific Immunoglobulin E or skin-prick tests or the response to a allergen provocation test to verify the putative link found in our study. It would also be of interest to perform non-allergic bronchial provocation tests with small and large particles and investigate whether this type of response is related to large or small airway dysfunction.

Both large and small airway dysfunction are related to the clinical expression of asthma as reflected by worse asthma control, suggesting that treatment of both large and small airway dysfunction may lead to clinical benefits. In addition, we observed that large and small airway dysfunction are related to different environmental stimuli, i.e. a response to fog and exercise but not trees, and a response to animals, respectively. The latter suggests that inhaled stimuli may have differential effects on the large and small airways.

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CHAPTER 5

Development of a tool to recognize small airway dysfunction in asthma (SADT)

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ABSTRACT

Background: Small airway dysfunction (SAD) contributes to the clinical expression of asthma. The identification of patients who suffer from SAD is important from a clinical perspective, as targeted therapy may improve patients' well-being and treatment efficacy.

Aims: We aimed to realize the first step in the development of a simple small airway dysfunction tool (SADT) that may help to identify asthma patients having SAD.

Methods: Asthma patients with and without SAD were interviewed. Patients were selected to participate in this study based on $FEF_{50\%}$ and R5-R20 values from spirometry and impulse oscillometry respectively.

Results: Ten in depth interviews and two focus groups revealed that patients with and without SAD perceived differences in symptoms and signs, habits and health related issues. For example, patients with SAD reported more wheeze, were unable to breathe in deeply, mentioned more symptoms related to bronchial hyperresponsiveness, experienced more pronounced exercise-induced symptoms and more frequently had allergic respiratory symptoms after exposure to cats and birds. Based on these differences, 63 items were retained to be further explored for the SADT.

Conclusions: The first step of the development of the SADT tool shows that there are relevant differences in signs and respiratory symptoms between asthma patients with and without SAD. The next step is to test and validate all items in order to retain the most relevant items to create a short and simple tool, which should be useful to identify asthma patients with SAD in clinical practice.

INTRODUCTION

Asthma is one of the most common chronic diseases in people of all ages in developed countries (1). Frequently reported symptoms are breathlessness, chest tightness, wheeze, cough, limitation of physical activity, and nocturnal awakening. Large airway obstruction due to inflammation and remodeling was traditionally thought to be the origin of these symptoms. However, there is growing consensus that the small airways are also affected, and play a role in the clinical expression of asthma (2,3). A recent systematic review showed that small airway dysfunction (SAD) is associated with worse asthma control, a higher number of exacerbations, the presence of nocturnal asthma, more severe bronchial hyperresponsiveness (BHR) and exercise-induced asthma (4). Moreover, clinical studies have shown that small particle treatment with inhaled corticosteroids reduces the number of exacerbations and improves asthma control(5-7). Thus, it has become increasingly important to identify those asthma patients in whom SAD is present. Several tests are available to assess SAD in patients with asthma, like the forced expiratory flow rates at 50 or at 25 to 75% of the forced vital capacity ($FEF_{50\%}$ or $FEF_{25-75\%}$) which can easily be assessed with spirometry (8-10). This FEF is closely related to air trapping on an expiratory CT-scan (11,12). In addition, impulse oscillometry (IOS) has been used as an easy tool to measure the resistance of the small and large airways (10).

Another method that could help to assess the presence of SAD in asthma patients is by identifying symptoms associated with SAD. These could then be used in a questionnaire to assess both the probability of SAD and the burden of symptoms associated with SAD. So far, it has not been studied whether small or large airway obstruction in asthma generates different symptoms. This may well be the case, since small airways have a smaller lumen size than large airways and lack cartilage. Therefore, smooth muscle contraction may lead to a collapse of the small airways, contributing to air trapping and the perception of chest tightness (13). Additionally, there is a difference in vagal innervation between the large airways and deeper lung structures, including the small airways (14,15). Finally, not all environmental stimuli are able to reach the small airways. This depends on the particle size, aerodynamic properties and local airway flow characteristics. For instance, cat allergen may reach the peripheral airways, whereas most pollen will never do so because of their large particle size (16,17).

Thus, this study aims to determine which self-reported differences in symptoms might potentially differentiate between asthma patients with SAD and without SAD. In the future, these items might be used to create a tool to recognize SAD in asthma in daily clinical practice, the SADT.

METHODS

Selection of study population

The participants were selected out of a database of patients attending the Primary Care asthma/COPD service of "Certe Laboratory" (The Netherlands)(18). This database contains 3,721 patients with a doctor's diagnosis of asthma. We selected asthma patients aged between 18 and 75 years with spirometry according to the American Thoracic Society (ATS) criteria available (Figure 1). This group (n=1,578) was divided into three groups (tertiles) based on their post-bronchodilator $FEF_{50\%}$ percent predicted, in order to select patient populations with and without probable SAD. The 33% patients (n=526) with the lowest $FEF_{50\%}$ represented the group of asthma patients with probable SAD and the 33% patients (n=526) with the highest $FEF_{50\%}$ the group (probably) without SAD. Since a restrictive lung function may lead to low $FEF_{50\%}$ predicted values and the false interpretation of existing SAD, we decided to exclude patients with an FVC <90% predicted, leaving 398 patients in the group with SAD and 491 patients in the group without SAD. Characteristics of the source population are shown in Table 1.

General practitioners were contacted and asked for permission to contact their patients eligible for this study. Patient recruitment continued until saturation with respect to content was reached. Finally, 120 patients from the lowest tertile and 150 patients from the highest tertile were invited to participate in this study and a total of 65 patients accepted to participate. They completed the asthma control questionnaire (ACQ), the Clinical COPD Questionnaire (CCQ) and the Bronchial Hyperresponsiveness Questionnaire (BHQ), and performed measurements of spirometry, impulse oscillometry and a methacholine provocation test (methods of the measurements are described in the appendix). Four patients were unable to perform spirometry and were excluded. Patients with BHR (n=34), i.e. a provocative concentration causing a 20% fall in FEV_1 (PC_{20}) methacholine bromide ≤39.3 mg/ml, were included for further analysis.

In-depth interviews and focus groups

First, explorative interviews were performed aiming to collect topics for the focus group interviews. For these in-depth interviews, 7 patients with and 3 without SAD were selected, based on current $FEF_{50\%}$ values lower or higher than the lower limit of normal (LLN). One patient from the highest tertile $FEF_{50\%}$ had an $FEF_{50\%}$ of 45% predicted with current spirometry and switched to the group selection with SAD.
Patients were asked about their symptoms, age of asthma onset, possibly related illnesses or allergies and worsening factors or situations such as exercise, weather conditions, psychological stress, and physical fatigue. After 10 in-depth interviews, saturation of topics was attained.

Subsequently, 6 patients with and 5 patients without SAD were identified to attend the focus group interviews. Patients were selected based on a combination of spirometry and IOS measures (figure 1). Presence of SAD was defined as both $FEF_{50\%}$ < LLN and R5-R20 >0.10 kPa/L/s. Absence of SAD was defined as both $FEF_{50\%}$ > LLN and R5-R20 ≤0.10 kPa/L/s. The cut-off value for R5-R20 was based on a study population of 110 healthy, never or currently smoking subjects, age 18-73 years (NCT00848406; (19)). Of these subjects, 90% had an R5-R20 <0.10 kPa/L/s.

Table 1. Characteristics of source population divided into the lowest and highest $FEF_{50\%}$ tertile

	Lowest $FEF_{50\%}$ tertile (n=398)		Highest $FEF_{50\%}$ tertile (N=491)	
Age (years)	54	(12)	48	(13)
Gender (%female)	72		62	
BMI (kg/m²)	28	(5)	29	(6)
Smoking (%current/ex/never)	26/37/37		15/42/43	
ICS (%yes)	53		50	
FEV_1 (%predicted)	89	(9.8)	109	(12)
FEV_1/FVC (%)	70	(5.7)	83	(4.3)
$FEF_{50\%}$ (%predicted)	51	(9.9)	104	(17)
ACQ total score	1.3	(0.9)	1.2	(0.9)

Data presented as mean (SD) or percentage.

BMI = body mass index, ICS= inhaled corticosteroids, FEV_1 = Forced expiratory flow in one second, FVC = forced vital capacity, $FEF_{50\%}$ = forced expiratory flow at 50% of the FVC, ACQ= Asthma Control Questionnaire

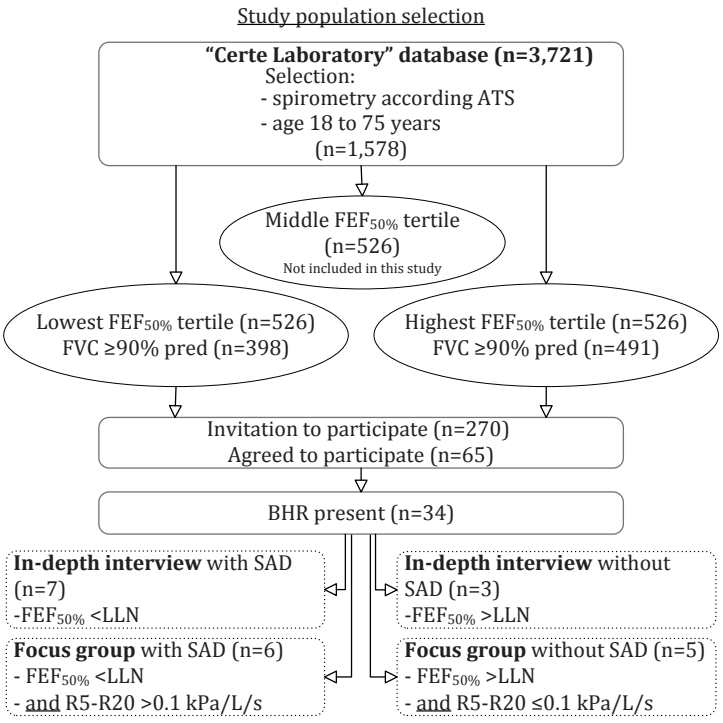

Figure 1: Flowchart of the study with the selection of the study population

ATS: American Thoracic Society, BHR: Bronchial hyperresponsiveness, FEV_1 = Forced expiratory flow in one second, FVC = forced vital capacity, $FEF_{50\%}$ = forced expiratory flow at 50% of the FVC, LLN: lower limit of normal, R5-R20: Difference between the resistance at 5Hz and 20Hz

Table 2. Patient characteristics participating in the focus groups

	In-depth interview group					Focus group				
	With SAD (n=7)		Without SAD (n=3)			With SAD (n=6)		Without SAD (n=5)		
	median	(range)	median	(range)	p-value	median	(range)	median	(range)	p-value
Age (years)	45	(25-73)	52	(20-63)	0.833	54	(25-73)	47	(36-51)	0.247
Gender (female/male)	5/2		2/1		1.000	5/1		4/1		1.000
BMI (kg/m^2)	28	(23-39)	36	(32-38)	0.117	29	(23-52)	32	(24-38)	0.792
Smoking (current/ex/never %)	1/4/2		1/1/1		0.728	0/5/1		0/1/4		0.080
ICS (yes/no)	5/2		3/0		1.000	4/2		3/2		0.497
FEV_1 (%predicted)	79	(66-104)	99	(94-123)	0.117	81	(76-88)	112	(97-127)	0.004
FEV_1/FVC (%)	62	(52-73)	83	(81-89)	0.017	64	(52-77)	83	(80-88)	0.004
$FEF_{50\%}$ (%predicted)	37	(23-62)	107	(89-115)	NA	40	(23-55)	114	(82-122)	NA
RV (%predicted)	120	(88-160)	72	(71-89)	0.033	120	(88-160)	88	(77-98)	0.017
IOS R20 (kPa/l/s)	0.39	(0.29-0.50)	0.33	(0.32-0.40)	0.517	0.39	(0.34-0.67)	0.38	(0.26-0.49)	0.429
IOS R5-R20 (kPa/l/s)	0.27	(0.10-0.44)	0.19	(0.11-0.25)	0.383	0.25	(0.14-0.29)	0.05	(0.01-0.08)	NA
IOS X5 (kPa/l/s)	-0.22	(-0.49;-0.12)	-0.23	(-0.26;-0.11)	1.000	-0.23	(-0.49;-0.22)	-0.07	(-0.12;-0.06)	0.004
PC_{20} methacholine[#] (mg/ml)	1.1	(0.1-6.4)	3.5	(1.3-23.9)	0.183	1.13	(0.14-5.55)	9.8	(2.8-20.9[@])	0.017
ACQ (total score)	0.5	(0.0-1.5)	2.0	(0.0-2.2)	0.383	0.6	(0.0-1.3)	1.0	(0.3-2.2)	0.177
BHQ symptoms (score)	0.4	(0.0-2.5)	1.8	(0.3-3.1)	0.383	1.3	(0.0-2.7)	1.9	(1.3-2.7)	0.329
BHQ stimuli (score)	1.2	(0.0-4.3)	1.8	(0.0-4.3)	0.833	2.5	(0.1-3.8)	2.5	(0.9-4.1)	0.662

[@]Patient with a PC_{20} 20.9 mg/ml methacholine used 800µg ICS. [#]Values were log transformed. Differences were tested with a non-parametric test. For ordinal variables differences were tested with the Fisher's Exact test or Chi-square test as appropriate.

BMI = body mass index, ICS= inhaled corticosteroids, FEV_1 = Forced expiratory flow in one second, FVC = forced vital capacity, $FEF_{50\%}$ = forced expiratory flow at 50% of the FVC, RV = residual volume, IOS = impulse oscillometry, R20: Resistance of the respiratory system at 20 Hertz, R5-R20: Difference between the resistance at 5Hz and 20Hz, X5 = Reactance of the respiratory system at 5 Hertz, PC_{20} = provocative concentration causing a 20% fall in FEV_1, ACQ= Asthma Control Questionnaire, BHQ = Bronchial Hyperresponsiveness Questionnaire

Methods of interviews and focus groups

All useful topics (items) were selected to be further discussed during the focus group interviews. Discussions in the focus groups were literally and fully transcribed by two authors of this article (LSG and EvdW). Qualitative data management software (NVivo 9 (20)) was used. All items mentioned in the in-depth and focus group interviews were organized in groups of items of interest. Then, a list of all items that differed between SAD and non-SAD patients of the focus groups was created. Afterwards, the list was back- and forward translated by a native English speaking pulmonology expert and two Dutch bilingual primary care researchers with knowledge of pulmonology.

The study and procedures were approved by the medical ethics committee of the University Medical Center Groningen and all patients gave written informed consent.

RESULTS

The clinical characteristics of the 7 asthma patients with SAD and 3 patients without SAD participating in the individual in-depth interviews are presented in table 2. Both groups of patients were comparable for most clinical parameters, except FEV_1/FVC ratio and residual volume (RV). A total of 6 asthma patients with and 5 without SAD participated in the focus groups (table 2). No differences between the group with and without SAD were observed in most parameters, except for lung function parameters. Four patients of the SAD focus group had also participated in the individual in-depth interviews. Patients participating in the in-depth interview or focus group did not use small particle inhalation medication.

Item organization and selection

All items that appeared to be different between the two groups were selected and a total of 63 items was retained. All items were phrased in a positive way (for example "I have an immediate reaction to cats"). Of these phrases, 21 phrases were in line with symptoms of patients with SAD and not of patients without SAD (e.g. "When I feel asthmatic, I feel it in my chest."), whereas 41 phrases were in line with symptoms of patients without SAD (e.g. "I frequently have a hoarse or husky voice"). In addition one open question ("At what age did you first suffer from asthma symptoms?") was added. The resulting 63 items are shown in table 3 and are divided in the following 10 domains:

1. The *Asthma Symptoms* domain (13 items); patients with SAD reported to wheeze more often and more easily and were not able to breathe in deeply when having asthma symptoms.
2. The *Ears, Nose and Throat* domain (10 items); these symptoms were only mentioned by patients without SAD.
3. The *Localization of Symptoms* domain (6 items) includes items concerning the exact spot of pain and other signs when feeling asthmatic. SAD patients for example mentioned an oppressive feeling and pain in the chest, whereas patients without SAD felt bloated and

sometimes had pain in the middle or the upper part of the back.

4. The *BHR to Exercise* domain (8 items); patients with SAD mentioned problems with regular physical activities, while those without SAD were rarely hindered in their activities.
5. The *BHR to Allergens* domain (4 items) shows that patients with SAD react to birds and cats, while those without SAD cannot stand wool or down.
6. The *BHR to Weather Changes* domain (6 items) reflects that both groups seem to react to weather changes, but in a different way.
7. The *Stress and Fatigue* domain (7 items); patients with SAD reported more asthma symptoms in relation to stress, while patients without SAD reported more asthma symptoms related to periods of fatigue.
8. The *Gastrointestinal Complaints* domain (3 items); Patients without SAD mentioned to suffer sometimes from gastrointestinal problems related to their asthma, but asthma patients with SAD did not.
9. The *Skin problems* domain (3 items); only patients with SAD reported eczema related to asthma.
10. The last domain (3 items) was named *Miscellaneous*. Patients without SAD reported often getting car-sick and having asthmatic relatives. Patients with SAD reported to be somewhat older when their first symptoms appeared. These items did not combine with any other domains and were thus placed in this last domain.

Table 3. 63-items of the SADT

	Without SAD	With SAD
Domain 1: Asthma symptoms	Concentrating on my breathing helps me when I feel asthmatic. People often tell me they can hear me breathing, even in a calm situation. I only wheeze when I feel very asthmatic. I often cough unexpectedly. I often cough superficially (tickling cough) before I get bothered by coughing more deeply. I can see it coming when I get my asthma. I often have a period without feeling asthmatic and without needing rescue puffs.	I'm not able to breathe in deeply when I feel asthmatic. I sometimes wheeze when I'm at ease or in rest. When I'm physically active (like walking the stairs), I sometimes wheeze. I can feel suddenly asthmatic without having any other symptoms. I almost always feel slightly asthmatic and I take a rescue puff regularly. I have suffered from bronchitis.
Domain 2: Ear/nose/ throat complaints	My asthma symptoms are preceded by the flu or a cold. When I feel asthmatic, I almost always have symptoms comparable to a cold. I usually get a cold first, and thereafter start coughing. I often suffer with my ears. I often have runny or painful eyes without having hay fever. I frequently have a hoarse or husky voice. When I feel asthmatic, it often comes with symptoms of my throat, nose, ears or eyes. When I feel asthmatic, I often, also suffer from a sore throat. My tonsils or adenoids have been removed.	I usually have runny or painful eyes when I have hay fever.
Domain 3: Localization of symptoms	When I feel asthmatic, I feel it in the middle of my back. When I feel asthmatic, I feel it in the top of my back. When I feel asthmatic, I feel a stab or a sting in my back or my ribs. When I feel asthmatic, I sometimes feel bloated.	When I feel asthmatic, I have a pressing and oppressive feeling. When I feel asthmatic, I feel it in my chest.

Domain 4: BHR to exercise	As a child, I always participated in all games and sports. I am able to walk a long distance without resting. When I'm not ill, I can easily do physical activities such as walking the stairs. When I feel asthmatic when exercising, it is very often due to the environment (grass, trees, flowers…). When I feel asthmatic when exercising, it is very often due to the weather. Sometimes I go running or jogging.	Actually, I cannot perform strenuous exercise or sport, because I will become asthmatic. Physical activities always make my asthma worse.
Domain 5: BHR to allergens	I cannot stand woolen blankets or clothes. I cannot stand the down filling in pillows.	I have an immediate reaction to birds. I have an immediate reaction to cats.
Domain 6: BHR to weather changes	I tire more rapidly due to weather changes. My breathing becomes easier in cold air. I always sleep with an open window, otherwise I feel asthmatic.	My asthma worsens in autumn. I feel asthmatic more rapidly due to weather changes. I feel asthmatic when I suddenly enter a cold environment.
Domain 7: Stress and fatigue	I rapidly get tired due to my asthma symptoms. Feeling tired is as much part of my asthma as feeling short of breath. I tire more rapidly due to my asthma symptoms. When I'm feeling tired I will probably get asthmatic in a few days. When I feel asthmatic, I often, also have a headache.	In stressful situations, I get particularly asthma symptoms. In stressful situations I have physical symptoms such as complaints of the nose, throat or voice.
Domain 8: Gastro-intestinal tract	Sometimes I feel asthmatic or out of breath because of heartburn. Sometimes, when I'm short of breath, it can be a relief to burp. Sometimes I have stomach problems which can make me feel asthmatic.	
Domain 9: Skin		I get eczema because of weather changes. I get skin problems (like eczema) when touching some kinds of food (e.g. fruit or vegetables). My asthma symptoms and eczema alternate.
Domain 10: Miscellaneous	I often get car sick or travel sick. I have more than three close relatives suffering from asthma or comparable illnesses.	
	At what age did you first suffer from asthma symptoms?	

DISCUSSION

This is the first phase of the development of a questionnaire aiming to help identifying asthma patients with SAD based on self-reported symptoms in clinical practice. Based on the differences that we found after ten in-depth interviews and two focus groups in patients with and without SAD, we identified a total of 63 items that may help to differentiate between patients with and without SAD. In short, we found that patients with SAD reported more wheeze, were unable to breathe in deeply, mentioned more symptoms related to BHR, experienced more pronounced exercise-induced symptoms and more frequently had allergic respiratory symptoms after exposure to cats and birds.

Interestingly, a number of the observed differences between patients with and without SAD, are supported by recent observations reported in the literature (21-31). Our finding that patients with SAD were unable to breathe in deeply and reported more wheeze may reflect hyperinflation, compatible with small airway closure. Mansur *et al* also found a relationship between more symptoms of wheezing and more severe small airway dysfunction during a methacholine provocation test (21). In addition, patients with SAD indicated that they had suffered from bronchitis at least once in their life, whereas patients without SAD did not. More frequent symptoms and need of rescue treatment are compatible with worse asthma control and the occurrence of asthma exacerbations, findings that have been related to SAD in asthma in previous studies as well (22-24).

Patients with SAD also reported more frequently symptoms of BHR to exercise, allergens and weather changes (domains 4, 5 and 6). This is compatible with earlier studies on BHR showing that hyperresponsiveness is associated with small airway obstruction in asthma (25-27). Interestingly, Zeidler *et al* showed that patients with allergic asthma exposed to cat allergens have predominantly a response of the small airways measured by HRCT scan (28). Our asthma patients with SAD reported an immediate and strong response to cats. Indeed, these allergens can be found on rather small particles (diameter < 2.5 μm), probably affecting asthma patients with SAD more than patients without SAD (29). In contrast, patients without SAD reported to respond strongly to wool and down. This might be related to house dust-mite excretion in these tissues, which is found on larger particles (10 μm) (30). Exercise induced symptoms were predominantly mentioned by patients with SAD, which is in line with a study of Lee *et al*. showing an association between the severity of exercise-induced response and an increased resistance of the small airways (31).

For some of the observed differences between patients with and without SAD we have not found corresponding findings in the existing literature. These novel findings might be interesting to study in a more systematic way in further research regarding symptoms of SAD in asthma.

Our patients represent the asthma population as present in the original primary care database of the asthma COPD service from "Certe Laboratory". Included patients had a median age of 51 years and a median BMI of 30 kg/m^2, 77% of the patients being female, whereas the asthma-population of the primary care database had a mean age of 53 with a mean BMI of 29 kg/m^2 and consisted of 68% females. The smoking habits of our study population, i.e. 12/47/41% current/never/ex

smokers, were comparable with the asthma-population of the primary care database .Thus the final list of 63 items is not limited to non-smokers. This is of importance since a few studies have suggested an important effect of smoking on SAD(32,33). With exclusion of smokers we could have missed signs and symptoms important for SAD.

The study has some limitations. The scientific community has so far not provided an agreed gold standard for the diagnosis of SAD in asthma. We did not use Computed Tomography (CT scan) or multiple breath nitrogen washout tests to assess the presence of small airway disease, yet we used a very precise way of selecting and reselecting patients with and without SAD, based on a combination of both $FEF_{50\%}$ and R5-R20 values. These parameters are frequently described as a reliable way to assess SAD(8,10). The division in patients below and above the LLN of the $FEF_{50\%}$ has also been used in a previous study to compare patients with and without SAD(26). For the R5-R20 we used a cutoff value that was based on a group of 110 well selected and well characterized healthy controls(19).
In summary, the present study is a first step in the development of the SADT. We generated 63 items for the new small airway dysfunction tool, the SADT, which aims to identify patients with SAD. The items that were identified cover a broad area of asthma symptoms related to airway hyperresponsiveness, response to allergens and physical exercise. All generated items will be further tested and validated in a large asthma population with a wide spectrum of severity during a multinational longitudinal study that will start in the near future. The next study will retain the most relevant items with which a short and simple tool to determine SAD in asthma patients will be realized.

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CHAPTER 6

Adenosine dry powder inhalation for bronchial challenge testing: Proof of concept in asthmatic subjects

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ABSTRACT

Adenosine is an indirect stimulus to assess bronchial hyperresponsiveness (BHR) in asthma. Bronchial challenge tests are usually performed with nebulised solutions of adenosine 5'-monophosphate (AMP). The nebulised AMP test has several disadvantages, like long administration times and a restrictive maximum concentration that does not result in BHR in all patients. In this study, we investigated the applicability of dry powder adenosine for assessment of BHR in comparison to nebulised AMP. Dry powder adenosine was prepared in doubling doses (0.01–80 mg) derived from the nebulised AMP test with addition of two higher doses. Five asthmatic subjects performed two bronchial challenge tests, one with nebulised AMP following the 2-minute tidal breathing method; the second with dry powder adenosine administered with an investigational inhaler and single slow inhalations (inspiratory flow rate 30–40 L/min). All subjects reached a 20% fall in FEV_1 with the new adenosine test (PD20) compared to four subjects with the AMP test (PC_{20}). Dry powder adenosine was well tolerated by all subjects and better appreciated than nebulised AMP. In conclusion, this new bronchial challenge test appears to be a safe and convenient alternative to the nebulised AMP test to assess BHR in asthmatic subjects.

INTRODUCTION

Bronchial challenge tests are used to measure bronchial hyperresponsiveness (BHR), a hallmark of asthma. These tests are usually performed with methacholine, which acts directly on the airway smooth muscle cells (1). However, it has been described that BHR in response to indirectly acting stimuli, such as adenosine, may better reflect bronchial inflammation than BHR to methacholine (2,3). Recently, it was investigated whether bronchial challenge testing with small and large particles aerosolised adenosine 5'-monophosphate (AMP) can discriminate between asthmatic subjects that respond well to treatment with either small or large particles inhaled corticosteroids (ICS) (4). A significant improvement was observed in the provocative concentration causing a 20% fall in FEV_1 (PC_{20}) with the small particles AMP in subjects receiving small particles ICS, whereas there was no improvement in subjects receiving large particles ICS. We therefore believe that the concept of identifying asthmatics with small airway dysfunction by challenging them with small particle AMP is valid. However, because the bronchial challenge with small particles AMP led to a 20% fall in FEV_1 in only 60% of the subjects, we clearly need to further optimise the technical (administration-related) aspects of this test.

Although adenosine is the agent that ultimately leads to smooth muscle constriction, solutions of AMP are being used because of its higher solubility compared with adenosine. Historically, BHR challenge tests have mostly been performed with provoking agents diluted in 0.9% saline that are administered by nebulisation. Doubling concentrations of AMP up to a maximum of 300–400 mg/mL are administered, following methacholine bronchial challenge test protocols (5). Such high AMP concentrations have been shown to greatly affect nebuliser performance in terms of droplet size and output rate, leading to smaller (but relatively heavier) droplets and a lower output rate at higher concentrations (6). The differences in droplet size imply that the site of deposition differs between low and high AMP concentrations, whereas the effect on output rate results in differences in administered volume (dose). Therefore, differences in response to low and high concentrations cannot be assigned to concentration alone.

Another disadvantage of the current test is that tidal breathing with small volumes (±500 mL) is not a very effective method to wash-in relatively large functional residual capacity (FRC) volumes (±2000 mL), resulting in long administration times, which are burdensome to both the patient and the lung function lab. In addition, the use of solutions of the stimulus leads to stability concerns upon storage (7).

All of the issues described above can be addressed by replacement of the nebulisation procedure by dry powder inhalation. With a dry powder inhaler (DPI), adenosine can be used instead of its precursor AMP as solubility is no longer an issue. Adenosine is stable in the dry state, so DPI formulations can be stored for relatively long periods of time. Additionally, a DPI enables the administration of a dose in one single inhalation, which significantly reduces the administration time. Moreover, by using an effective inhaler, the particle size is independent of the drug dose leading to similar deposition for both low and high doses. Consequently, regional targeting can

be facilitated using different particle sizes for the stimulus and controlling the flow rate at which the particles are administered (8).

We have developed a dry powder test concept for bronchial challenge testing with adenosine. The development and *in vitro* performance of this test have been described in the first paper of this series (9). The test consists of doubling dose steps of adenosine starting from 0.01 mg, and we could show that the fraction of the dose delivered and the particle size distribution of the aerosol are both independent of the dose (9). In this pilot study, we aimed to investigate whether this new dry powder adenosine bronchial challenge test can induce a 20% fall in FEV_1 in subjects with asthma that is comparable to the fall induced with the nebulised AMP test. Secondarily, we wanted to investigate the applicability of this new test concept, with respect to both its safety and patient comfort.

SUBJECTS AND METHODS

Subjects

Five subjects with a doctor's diagnosis of asthma, 18–65 years, were included in this pilot study. The study protocol was approved by the local Medical Ethics Committee (METc number 2012.057, University Medical Center Groningen, The Netherlands) and written informed consent was obtained from all participants.

Study design

The participating subjects attended the clinic on two days with an interval of one month maximally. On each day, a bronchial challenge test using either nebulised AMP or dry powder adenosine was performed. Subjects had to withhold their bronchodilating medication (short-acting β2-agonists for 6 hours, ICS and long-acting β2-agonists for 12 hours).

Adenosine dry powder

The novel adenosine dry powder test that was investigated in this study consisted of doubling dose steps in a range of 0.01–80 mg. In order to cover the entire expected dose range, three powder formulations were prepared by spray drying, which consisted of either pure (100%) adenosine, or adenosine and lactose as diluent (1% and 10% adenosine) (9). Adenosine and lactose (both *Ph.Eur.* quality) were obtained from BUFA Spruyt Hillen (IJsselstein, The Netherlands). Spray drying was performed in the hospital pharmacy under Good Manufacturing Practice conditions using a Büchi B290 Mini Spray Drier (Büchi Labortechnik, Switzerland).
The doses were provided in individually sealed aluminium blisters. The 40 mg and 80 mg doses consisted of respectively two and four blisters each containing 20 mg adenosine. The dose range was derived from the regular AMP test, with addition of two higher dose steps (Table 1). The powder was administered using an investigational inhaler especially designed for the dispersion of the adenosine powder formulations used in this study (Figure 1). Its dispersion principle is

Table 1: Conversion of AMP to adenosine

AMP		Adenosine
Concentration (mg/mL)	Dose (mg)	Dose (mg)
0.04	0.004	-
0.08	0.007	-
0.16	0.014	0.01
0.32	0.028	0.02
0.64	0.057	0.04
1.25	0.11	0.08
2.5	0.22	0.16
5	0.44	0.32
10	0.88	0.64
20	1.8	1.25
40	3.5	2.5
80	7.1	5
160	14	10
320	28	20
-	-	40
-	-	80

The conversion is based on the estimated delivered doses of AMP, which were calculated by multiplying the AMP concentrations by the calibrated nebuliser output rate (0.13 mL/min), nebulisation time (2 min) and a duty cycle of 0.34.[9]

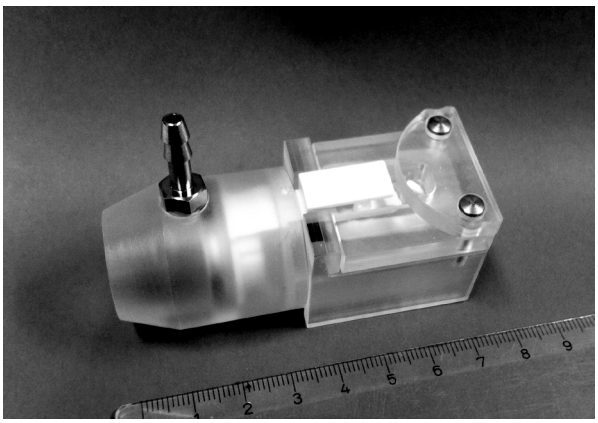

Figure 1: The investigational inhaler used to administer dry powder adenosine. The outlet on the mouthpiece is used for measuring the pressure drop across the inhaler during inhalation, from which the inspiratory flow rate is calculated.

based on air classifier technology (10), which is also present in the Novolizer® (MEDA) and Twincer™ (11) DPIs. The dry powder adenosine aerosol had a mass median aerodynamic diameter (MMAD) of 2.6–2.9 µm and a geometric standard deviation (GSD) of 1.6 over the entire dose range when dispersed with the investigational inhaler.

Bronchial challenge test with nebulised AMP

The AMP challenge test was performed using the two-minute tidal breathing method (12,13). After a safety step with nebulised 0.9% saline, subsequent doubling AMP concentrations of 0.04–320 mg/mL were inhaled for 2 min, followed by a 3 min interval between the nebulisation steps. Ninety seconds after every step, an forced vital capacity (FVC) manoeuvre was performed, obtaining the FEV_1, FVC, and forced expiratory flow at 50% of the forced vital capacity ($FEF_{50\%}$).

Bronchial challenge test with dry powder adenosine

The adenosine challenge test was performed by inhalation of subsequent doubling adenosine doses of 0.01–80 mg with a single slow inspiratory manoeuvre, followed by a 3 min interval between the inhalations (the 40 and 80 mg doses were administered using two and four inhalations respectively). The subjects were instructed to exhale completely, to subsequently inhale as long as possible at a flow rate of 30–40 L/min, and finally to hold their breath for 10 s at maximal inspiration. To control and record the inspiratory flow rate during inhalation, the inhaler was connected to a flow measurement device with a visual feedback system, showing the actual flow rate of the subject's inhalation on a computer screen. The 40 mg and 80 mg doses were respectively inhaled in two and four consecutive inhalations. Also in this test, an FVC manoeuvre was performed 90 s after every dose step.

Borg dyspnoea scores

Dyspnoea was scored with the Borg scale, ranging from 0 to 10 (no to maximal breathlessness) (14), before the first administration and after each administration of AMP or adenosine, as well as at the end of the test.

Data analysis

Reference values for spirometry were obtained from Quanjer *et al* (15). The PC_{20} (for AMP) and PD_{20}values (for adenosine) of the bronchial challenge tests were determined by log-linear interpolation between the second-to-last and last FEV_1 value (13). Converting the AMP PC_{20} value into the corresponding PD_{20}value for adenosine (following Table 1) allowed for a direct comparison of the provocative doses. $FEF_{50\%}$ values at PD_{20}were calculated by interpolating between the last and second-to-last value. Wilcoxon-signed rank tests were performed to test for differences between the nebulised AMP test and dry powder adenosine test, concerning the PD_{20}values, $FEF_{50\%}$ at PD_{20}values, and changes in Borg dyspnea scores.

RESULTS

Subjects

Five asthmatic subjects were included in the study, whose demographic characteristics are shown in Table 2. All had a baseline FEV_1 > 80% predicted on both test days. After instruction and practicing, all subjects performed technically satisfactory inhalations. They generated sufficiently high flow rates and inhaled volumes through the adenosine DPI for complete dose release from the blisters and good dispersion of the powder.

Comparison of BHR to adenosine and AMP

All subjects reached a 20% fall in FEV_1 with the new adenosine test (PD20), whereas only four subjects reached this threshold with the AMP test (PC_{20}) (Table 3). Subject 3 reached a PD_{20}after inhalation of 80 mg of adenosine, which was two dose steps higher than the highest concentration of AMP according to the calculated dose range for dry powder adenosine. After inhaling 320 mg/mL AMP, the fall in FEV_1 was 9% in this subject.

The numbers of administered doses were comparable for the two tests, as a result of leaving out the two lowest dose steps in the new dry powder test (Table 3). PD_{20}AMP and PD_{20}adenosine were not significantly different (P = 0.144). In Figure 2, the courses of the FEV_1 (A and B) during both tests are given for all individual subjects.

All subjects showed a more than 20% fall in $FEF_{50\%}$ with the dry powder adenosine test compared to four subjects with the AMP test (Figure 2, C and D). The $FEF_{50\%}$ at 20% fall of the FEV_1 (Table 3, n=4) appeared to be lower after challenge with dry powder adenosine, but this difference did not reach statistical significance (P = 0.068).

Table 2: Subject demographic characteristics

Subject No.	Sex	Age (years)	Smoking (pack years)	FEV_1 (% predicted)	FEV_1/FVC (%)	Medications
1	Male	38	Never	107	76	Alvesco 160 µg b.i.d., formoterol 12 µg b.i.d.
2	Female	35	Ex (5.25)	84	74	No medication
3	Male	28	Ex (3)	91	78	Symbicort 400/12 µg b.i.d., Levocetirizine 5 mg q.d.
4	Female	44	Current (20.25)	81	63	Symbicort 400/12 µg prn (twice/week)
5	Female	47	Ex (0.11)	117	72	Salbutamol prn

Table 3: Comparison of the responses to AMP and adenosine per individual subject

Subject No.	AMP					Adenosine			
	PC_{20}	PD_{20} adenosine	$FEF_{50\%}$ at PD_{20}(L/s)	Δ Borg	No. doses	PD_{20}	$FEF_{50\%}$ at PD_{20}(L/s)	Δ Borg	No. doses
1	10.52	0.93	4.47	-	10	1.53	2.70	-	9
2	29.97	2.65	1.85	0.5	11	2.18	1.64	2	10
3	NA	-	-	2	14	62.12	2.41	5	14
4	1.94	0.17	1.43	0.5	7	0.68	1.33	0	8
5	5.16	0.46	1.72	3	9	1.25	1.69	5	9

PD_{20}adenosine: PC_{20} AMP converted into the corresponding PD_{20}adenosine

Δ Borg: change in Borg dyspnea score (highest concentration AMP/adenosine - baseline)

NA: no 20% fall in FEV_1 was obtained

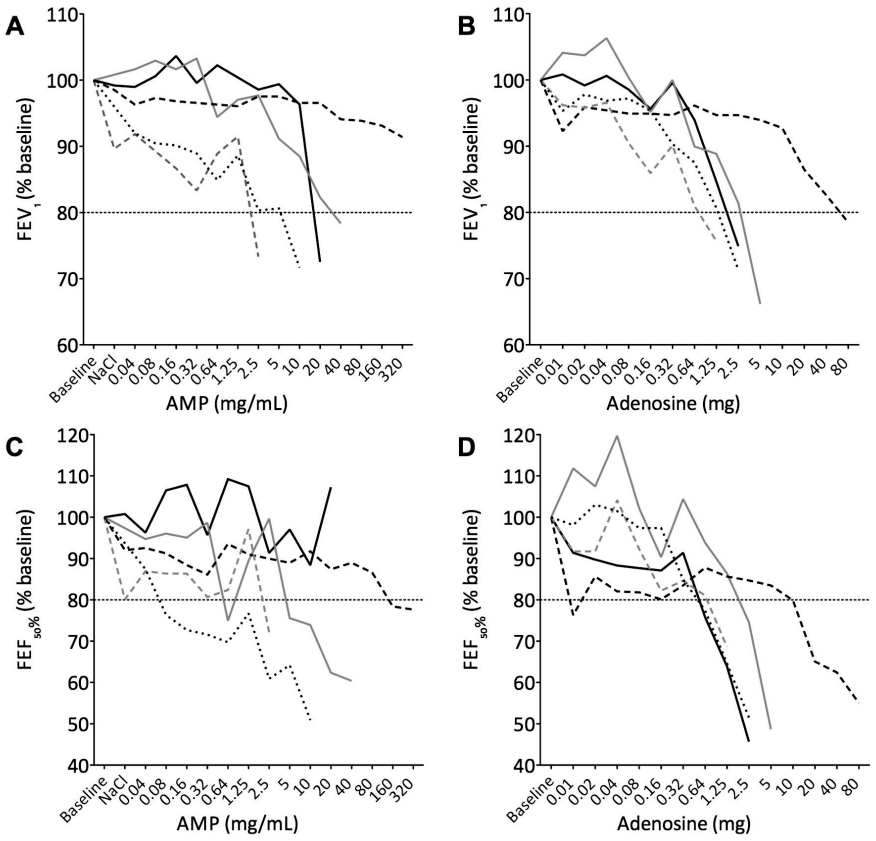

Figure 2: Values for FEV_1 (A and B) and $FEF_{50\%}$ (C and D) relative to baseline measured at the consecutive dose steps per individual subject.

Safety and subject experience of the dry powder adenosine test

The deepest declines in FEV_1 measured in this study were 28.3% on AMP and 33.8% on adenosine. All subjects recovered to 90% of baseline FEV_1 within 15 min after administration of salbutamol 400 µg. No safety issues and no serious adverse events were encountered during or after the challenges. The dry powder adenosine was well tolerated by all subjects. The inhalation of dry powder adenosine did not appear to induce more coughing than the inhalation of nebulised AMP. A bitter taste was sometimes reported for the doses that consisted of pure adenosine (> 2.5 mg), whilst at the lower doses, the sweetness of lactose predominated.
Maximum Borg scores reported for the challenges with AMP and adenosine were 3 and 5 respectively. Overall, the scores were not significantly different (P = 0.144). The entire dry powder adenosine test (including recovery) could be finished within 60 min, compared to 90 min for the nebulised AMP test. All subjects expressed their preference for the dry powder adenosine test over the nebulised AMP test, mostly because it was faster.

DISCUSSION

In this proof-of-concept study, we investigated the efficacy, acceptance, and safety of a new adenosine bronchial challenge test in a small number of asthmatic subjects. The results demonstrate that a dry powder system of adenosine is suitable for the assessment of bronchial hyperresponsiveness in asthmatic subjects. Dry powder adenosine induces a response in both the FEV_1 and $FEF_{50\%}$ suggesting bronchoconstriction of both large and small airways. This can be explained by the use of an aerosol with a relatively small particle size in combination with a low inspiratory flow rate, a combination that allows for substantial deposition of the stimulus in the periphery of the lungs and thus for a small-airway response (8). Inevitably, a part of the aerosol is deposited in the upper and central airways too, so a response of these airways is to be expected as well. Further research is needed before we can discriminate between small airway dysfunction and large airways dysfunction based on challenge tests with small and large adenosine particles respectively. The next step is therefore to compare different particle sizes and inhalation flows in a group of asthmatic subjects who are extensively characterised with respect to small and large airways dysfunction.
The provocative doses causing a 20% fall in FEV_1 did not differ significantly for the two tests and were less than two doubling doses apart for each of the four subjects who reached the 20% threshold in both tests. BHR can vary each day due to environmental stimuli and a normal variability includes 1.5 dose step within two days for 90% of the patients (13). The dose range that was calculated for dry powder adenosine thus correlates well with the concentration range of AMP. Moreover, with the new test we were able to administer higher doses than the highest AMP concentration, leading to a response in all subjects, including the AMP-negative subject. Based on these results, we consider further development of the test with the current dose range justified.
In this pilot study, no difficulties were encountered with the inhalation manoeuvre. The medium-high resistance of the inhaler facilitated the subjects in attaining (and retaining) the desired low inspiratory flow rate, as well as in extending their inhalations over several seconds. These long

inhalations may have an additional beneficial effect on the peripheral deposition of the aerosol particles. After release and inhalation of the aerosol in the first part of the inspiratory manoeuvre, additional (clean) air is inhaled for further transport of the aerosol towards the more distal regions of the lungs.

Importantly, no adverse events were encountered either. Some coughing was reported in response to dry powder adenosine, but not to a larger extent than to nebulised AMP. The low inspiratory flow rate aids in keeping throat deposition to a minimum. The bitter taste that was experienced especially after inhalation of the 20 mg doses did not impede continuation of the test.

This small pilot study has some minor drawbacks. Firstly, the timelines of the AMP and adenosine protocol were not completely exchangeable, because tidal breathing took two minutes and the slow IVC manoeuvre approximately 10–20 seconds. Although the shorter duration of the adenosine test was considered a benefit by the participating subjects, it may have implications for the response to the stimulus. Secondly, the IVC manoeuvres and 10-second breath hold may have led to smooth muscle relaxation and bronchoprotection, which is probably not present after two minutes of tidal breathing. This bronchoprotective effect may even be greater after inhaling the highest doses of adenosine, since these dose steps consist of two or four blisters. On the other hand, only one subject reached the highest adenosine dose that required four blisters, but still showed a 20% fall in FEV_1.

In conclusion, this study demonstrates that bronchial challenge testing using inhaled dry powder adenosine is feasible. The new dry powder adenosine test has several improvements compared to the AMP test, most importantly the possibility to administer higher doses of the stimulus and the lower burden on the patient. Further studies will be performed with this test concept to study the influence of particle size and inspiratory flow rate on the airway response to dry powder adenosine.

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CHAPTER 7

Targeting the small airways with dry powder adenosine: A challenging concept

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Submitted

ABSTRACT

Introduction: Recent studies suggest that treatment with small-particle inhaled corticosteroids has clinical benefits. However, we lack adequate diagnostic tools to identify asthma patients with small airway dysfunction. We hypothesized that we could identify these patients by selectively challenging the small and large airways. We tested this by challenging the airways with small- and large-particle dry powder adenosine, inhaled with a high or low flow rate.

Methods: Asthma subjects performed four dry powder adenosine tests, with either small (MMAD 2.7 µm) or large (MMAD 6.0 µm) particles, inhaled once with a low flow rate (30 L/min) and once with a high flow rate (60 L/min). Spirometry and impulse oscillometry were performed after every provocation step.

Results: The adenosine tests induced a response in the parameters FEV_1, $FEF_{25-75\%}$, R5-R20 and X5 in the majority of the 11 asthma subjects who participated. No significant differences were found between the four tests with respect to the threshold values of FEV_1 (p=0.12), $FEF_{25-75\%}$ (p=0.37), R5-R20 (p=0.60) or X5 (p=0.46). Both small-particle and large-particle adenosine induced a response in the small airways in 41 out of all 42 tests.

Discussion: This study shows that a dry powder adenosine challenge is able to induce a response in the large and small airways. In contrast to our hypothesis, all four adenosine tests provoked a response in the small airways, even the test with large particles and a high inhalation flow rate. These findings are compelling and show we need to reappraise the underlying mechanisms of bronchial hyperresponsiveness.

INTRODUCTION

Recent technological improvements have led to new pharmaceutical formulations with small particles for inhalation therapy. An advantage of small-particle instead of large-particle inhaled treatment is a higher total lung deposition in addition to better peripheral deposition in the small airways (1,2). Recent studies in asthma have shown that treatment with small-particle inhaled corticosteroids (ICS) associates with improved asthma control and less small airway dysfunction (3-5). However, we are currently not able to specifically diagnose asthma patients with small airway dysfunction, who will probably benefit most from the inhalation of small-particle medication.
Cohen and colleagues tried for the first time to identify responders and non-responders to treatment with small-particle inhaled corticosteroids (ICS) using an indirect provocation test with large and small particles of dissolved adenosine 5'-monophosphate (AMP) (6). The 20% fall in FEV_1 (PC_{20}) after provocation with small-particle AMP improved significantly in asthma subjects after a 4-week treatment with ciclesonide, a small-particle ICS. In contrast, there was no improvement in the PC_{20} provoked with large-particle AMP. Interpretation of this study was hampered by the fact that only 60% of the subjects reached a 20% fall in FEV_1 with the small-particle AMP test. The low response rate was partly attributed to the very small mass median aerodynamic diameter (MMAD) of 1.1 µm, which is probably too small for effective deposition in the airways with the tidal-breathing method. Furthermore, the challenge with nebulized AMP has a number of other disadvantages, i.e. it is impossible to dissolve AMP at higher concentrations than approximately 320 mg/mL, the particle size distribution and nebuliser output rate are not consistent over the entire concentration range, and good clinical manufacturing of sterile, diluted agents is more complicated than manufacturing of most dry powder formulations (7).

Provocation with dry powder may help to overcome the above-described disadvantages of AMP nebulisation (8). Dry powder provocation with mannitol (MMAD 3 µm) acting as an indirect stimulus similar to AMP, has shown to produce bronchoconstriction in subjects with asthma, but responses of the small airways to mannitol have not yet been investigated (9-11).

Usmani and colleagues used a bronchodilator instead of a bronchoconstrictor and investigated by two-dimensional scintigraphic imaging, deposition of monodisperse (geometric SD ≤1.2) salbutamol aerosol after one single inspiration (1). Deposition in the small airways improved significantly by inhaling smaller particles, i.e. a small airway deposition of 10%, 17% and 25% in case of particles with an MMAD of 6, 3 and 1.5 µm respectively. These findings of Usmani and colleagues show that inhaled small particles deposit in the small airways after a slow inspiratory manoeuvre, whereas large particles deposit more centrally in the large and intermediate airways.

In the current pilot study, we aimed to challenge the small and large airways selectively with dry powder adenosine, using the findings of Usmani and colleagues. To this end, asthmatic subjects were challenged with small (MMAD 2.7 µm) and large (MMAD 6.0 µm) particle adenosine, inhaled with one slow (30-40 L/min) and one fast (60-70 L/min) inspiration. We hypothesized that a small-particle slow-inhalation provocation test gives a higher deposition and thus a higher response in the small airways than a test with large particles and/or inhalation with a high flow rate (Figure 1).

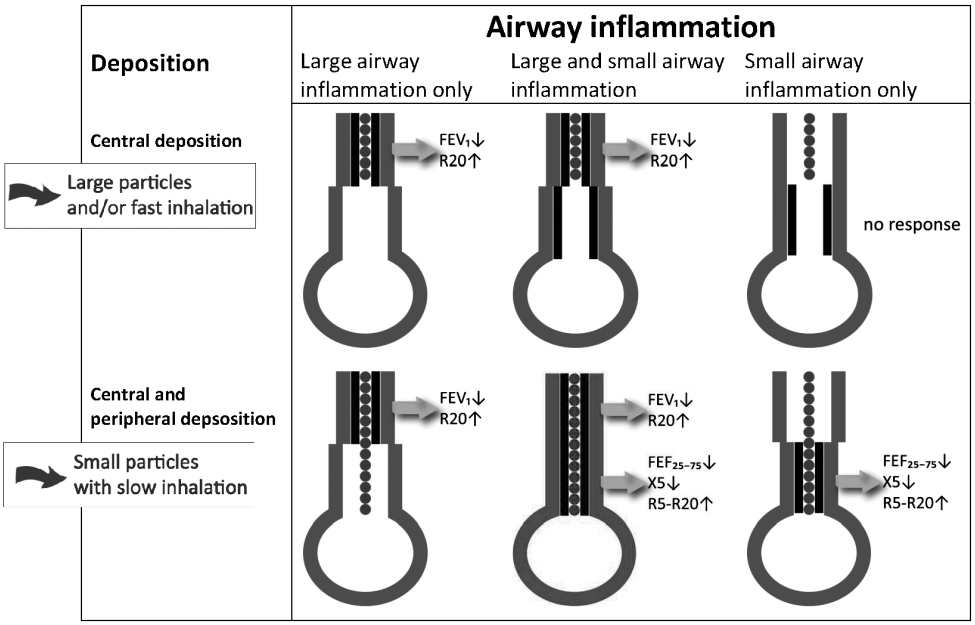

Figure 1. Hypothetical response patterns with dry powder adenosine

This simplified figure shows the hypothetical response patterns upon provocation with dry powder adenosine in the present study. The first assumption is that there are three sites of inflammation: isolated large airway inflammation (left picture), both large and small airway inflammation (middle picture), isolated small airway inflammation (right picture). The second assumption is that airways only obstruct if adenosine particles (circles) are deposited in inflamed airways (black layer). The third assumption is that large airway dysfunction is reflected by FEV_1 and R20, and small airway dysfunction by $FEF_{27-75\%}$, R5-R20 and X5. The fourth assumption is that large (MMAD 6.0 µm) particles, or particles inhaled with a high flow rate (60-70 L/min) deposit in the central airways (upper row), whereas small (MMAD 2.7 µm) particles inhaled with a low flow rate (30-40 L/min) deposit in the central and peripheral airways (lower row).

In this study we had no information about the site of airway inflammation, nor the site of adenosine deposition. If the above-described assumptions are correct there are four potential response patterns (Table 2b). Because we included subjects based on a positive AMP test with a 20% fall in FEV_1, it is unlikely that we included subjects with isolated small airway inflammation (right column).

METHODS

Study design

In this randomized cross-over study subjects performed four adenosine provocation tests on separate days in randomized order. Dry powder adenosine provocation tests were carried out with either small (MMAD 2.7 µm) or large particles (MMAD 6.0 µm). Both tests were performed once with a slow inhalation (30-40 L/min) and once with a fast inhalation (60-70 L/min) with a breath-hold time of 10 s. These adenosine provocation tests were preceded by a baseline visit assessing questionnaires and measuring a multiple breath nitrogen washout (MBNW)test,

impulse oscillometry (IOS), spirometry, body plethysmography and a conventional nebulized AMP provocation test. Details of the performed measurements are presented in the online supplement.

Subjects

Subjects with a doctor's diagnosis of asthma, never smokers, 18-65 years old were selected. Subjects were steroid naïve or stopped steroids 4 weeks before the first visit. Exclusion criteria were a recent exacerbation (<2 months), upper respiratory tract infection (<2 weeks), FEV_1 <50% predicted or <1.2 L, pregnancy, or a diagnosis of another pulmonary disease. Subjects with a positive AMP response (PC_{20} ≤320 mg/mL) on visit 1 were included. This pilot study aimed to include 10 subjects, with replacement of individual subjects in case of withdrawal. We expected that 10 subjects can demonstrate the potential of the principles analyzed in this study. The Medical Ethics Committee of the University Medical Center Groningen approved the study and all subjects gave written informed consent.

Adenosine dry powder provocation test

The adenosine dry powder provocation test was performed with doubling adenosine doses ranging from 0.04 to 20 mg. Adenosine provocation took place with a time interval of 3 minutes and measurements of IOS at 30s and FVC at 90s similar to the conventional AMP provocation test. The provocation test was stopped when the FEV_1 fell ≥20% compared to baseline. Details are presented in the online supplement. The pharmaceutical manufacturing of doubling doses dry powder adenosine with a small particle size and the first clinical pilot in five asthma patients have been described previously (8,12).

Small and large airways parameters

FEV_1 and R20 were considered as large airway parameters, $FEF_{25-75\%}$, R5-R20 and X5 as small airway parameters (13).

Small and large airway response

A positive response to the provocation test was defined as a 20% fall (PD_{20}) in the FEV_1 or $FEF_{25-75\%}$ or a 40% increase (PD_{40}) in any of the IOS parameters R20, R5-R20 or X5. The 40% increase in IOS parameters was based on older studies using a threshold of 40% increase in resistance measured with the forced oscillation technique or 40% decrease in specific airway conductance measured with body plethysmography (14-16). Further details are presented in the online supplement.

Statistical analysis

The Friedman test was used to test for differences between the four adenosine provocation methods with respect to eliciting a bronchoconstrictive response in the large and small airways and the absolute change in Borg score. Linear mixed effect models were used to estimate the effect of particle size and inhalation flow rate on $PD_{20}$and PD_{40}. In addition, linear mixed effect models were used to make pairwise comparisons between the four tests. Spearman's rank correlation test was used to calculate associations between baseline lung function parameters and the different $PD_{20}$and PD_{40} values for the tests applying small particles with a slow inhalation and applying large particles with a fast inhalation, as most extreme variants. Associations with a p-value <0.2 were included in the multivariate regression analysis. Backward linear regression method was used to determine the parameters that independently predict a small or large airway response. Subsequently, forward linear regression analyses were performed to verify the predictors. Analyses were performed with SPSS version 20.

Table 1 Characteristics of study population (n=11)

	Median	(IQrange)
Age (years)	22	(20;40)
Gender (n, female)	7	
ICS use (n, yes)	10	
ICS dose (µg)*	500	(0;1500)
ACQ score (total score)	1.0	(0.3;2.7)
BHQ score (total score)	1.4	(0.2;2.9)
FEV_1 (%pred)	92	(86;113)
FEV_1/FVC (%)	76	(65;97)
$FEF_{25-75\%}$ (%pred)	62	(49;120)
RV (%pred)	92	(35;131)
R20 (kPa/L/s)	0.37	(0.27;0.50)
R5-R20 (kPa/L/s)	0.04	(-0.02;0.32)
X5 (kPa/L/s)	-0.1	(-0.25;-0.05)
AX (kPa/L)	0.23	(0.07;2.73)
Sacin (L^{-1})	0.074	(0.045;0.144)
Scond (L^{-1})	0.026	(0.012;0.080)
PC_{20} AMP (mg/mL)	15.3	(1.51;34.8)

*beclomethasone equivalent

ACQ: asthma control questionnaire, AMP: adenosine 5'-monophosphate, AX: reactance area, BHQ : bronchial hyperresponsiveness questionnaire, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of FVC, FEV_1 : forced expiratory volume in 1 second, FVC: forced vital capacity, ICS: inhaled corticosteroids, R5-R20: difference between the resistance of the respiratory system at 5 Hz and 20 Hz, R20: resistance of the respiratory system at 20 Hz, RV: residual volume, PC_{20}: provocative concentration causing a 20% fall in FEV_1, Sacin: ventilation heterogeneity of the acinar lung zone, Scond, ventilation heterogeneity of the conductive lung zone, X5: reactance at 5 Hz.

RESULTS

Characteristics of study population

A total of 26 subjects gave informed consent and 11 subjects were included in the study. Fifteen subjects were excluded due to either a severe (PC_{20} < 0.04 mg/mL) or no response to AMP. One of the included subjects dropped out because of increased breathlessness. This subject performed only two large-particle adenosine tests. All obtained data have been used in the analyses. Baseline characteristics are shown in Table 1.

Adenosine dry powder tests

After instruction and practicing, all subjects were able to attain the defined flow rates through the test inhaler. A second inhalation was required for 10% of the blisters to achieve full dose release (Supplementary Table E1). The four different adenosine dry powder tests were well tolerated by all subjects. Cough was evoked in response to all four tests in one subject, and in response to the fast-inhalation provocation tests in two subjects. Nevertheless, all subjects were able to finish the tests.

Large and small airway response to adenosine tests

The large and small airway responses to the four adenosine tests are illustrated in Figure 2 showing one subject as typical example. The small-particle slow-inhalation test induced in the majority of the subjects responses in the parameters FEV_1, $FEF_{25-75\%}$, R5-R20 and X5 (Table 2a). The small-particle slow-inhalation test induced a response in R20 in only 2 out of 10 subjects and a response in R20 was present in only 11 out of all 42 tests. As a result, $PD_{40}R20$ was not included in further analyses. The small-particle fast-inhalation test induced in 10 out of 10 subjects a response in the FEV_1 and $FEF_{25-75\%}$, while the large-particle fast-inhalation test induced a response in 7-9 out of 11 subjects for the FEV_1 and $FEF_{25-75\%}$. Interestingly, the large-particle tests, i.e. with a slow and fast inhalation, induced in 8-10 out of 11 subjects also a response in the small airways.

We had hypothesized that the four different adenosine tests would elicit the site of airway inflammation based on different response patterns, i.e. a response in the large airways only, in the small airways only, in the large and small airways, or no response in the large and small airways (Figure 1). However, none of the subjects showed an isolated response of the large airways (Table 2b). An isolated small airway response was present in 2 out of 11 subjects to both large-particle tests, and also in one of these subjects in response to the small-particle slow-inhalation test. A response of both the large and small airways was present in the other subjects, i.e. 9 out of 10 subjects to the small-particle slow-inhalation test and 8 out of 11 subjects in response to the large-particle fast-inhalation test.

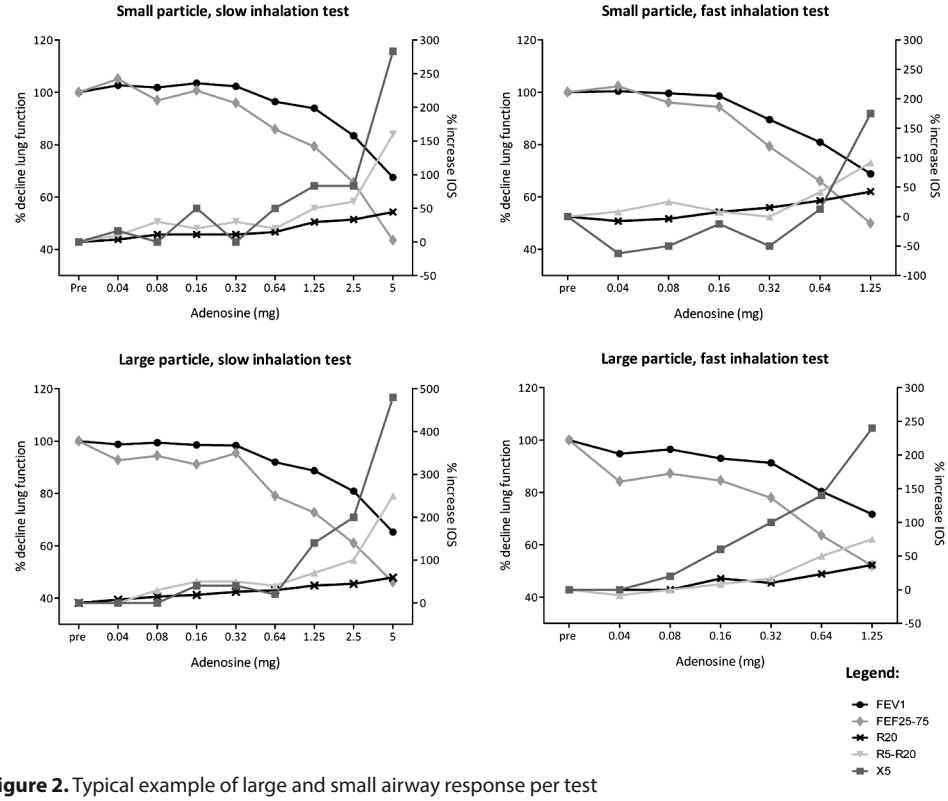

Figure 2. Typical example of large and small airway response per test

Differences in PD_{20} and PD_{40} threshold values between the four adenosine tests

No significant differences were found between the four tests for the $PD_{20}FEV_1$ (p=0.12), $P_{20}FEF_{25-75\%}$ (p=0.37), PD_{40}R5-R20 (p=0.60) and PD_{40}X5 (p=0.46). Pairwise comparison showed a few differences between the tests (Table 3, Figure 3), e.g. the small-particle slow-inhalation test induced a higher $PD_{20}FEF_{25-75\%}$ and $PD_{20}FEV_1$, indicating less severe bronchial hyperresponsiveness, than the small-particle fast-inhalation test. In addition, the small-particle fast-inhalation test induced a significantly lower $PD_{20}FEV_1$, indicating more severe bronchial hyperresponsiveness, than both large-particle tests. The large-particle slow-inhalation test induced a lower PD_{40}R5-R20 than the small-particle and large-particle fast-inhalation tests with p-values that approached statistical significance, i.e. p=0.06 and p=0.07 respectively.

We had hypothesized that small particles or particles inhaled with a low flow rate would induce an increased response of the small airways. However, analyses using a linear mixed effect model revealed no significant effect of particle size on the threshold value of the large and small airway parameters (Table 4). Inspiratory flow rate had a significant effect on the response in R5-R20, i.e. a slow inhalation induced a lower PD_{40}R5-R20. Provocative doses and maximal responses at the last given dose are shown for each subject in Supplementary table E2.

Table 2a. Responses per test

Percentage of subjects showing a response per test		Small particles, slow inhalation (n=10)	Small particles, fast inhalation (n=10)	Large particles, slow inhalation (n=11)	Large particles, fast inhalation (n=11)
Large airways	20% decrease in FEV_1	8	10	9	7
	40% increase in R20	2	2	1	3
Small airways	20% decrease in $FEF_{25-75\%}$	9	10	10	9
	40% increase in R5-R20	8	7	9	9
	40% increase in X5	8	7	9	8

Table 2b. Response patterns

Subjects showing a response in the large and/or small airways	Small particles, slow inhalation (n=10)	Small particles, fast inhalation (n=10)	Large particles, slow inhalation (n=11)	Large particles, fast inhalation (n=11)
Only a response in the large* airways	0	0	0	0
Only a response in the small** airways	1	0	2	2
Response in the large* and small** airways	9	10	9	8
No response in the large* or small** airways	0	0	0	1

* large airway response based on ≥20% decrease in FEV_1

** small airway response based on ≥20% decrease in $FEF_{25-75\%}$, or ≥40% increase in R5-R20 or X5

Borg score

There was no significant difference in the absolute change in Borg scores between the four adenosine tests (p=0.74). The median change in Borg score was similar for each test with a median change of 3.0 (range 1-9 for all four tests).

Baseline predictors of large or small airway response

A few significant associations were found between the baseline lung function parameters, e.g. FEV_1 %predicted, FEV_1/FVC and $FEF_{25-75\%}$ %predicted, and the threshold values of the large and small airway response (Supplementary table E3). Backward regression analysis showed that FEV_1 and FEV_1/FVC were not independently associated with the response to the adenosine provocation tests (Supplementary table E4). $FEF_{25-75\%}$ %predicted was a predictor for the $PD_{20}FEV_1$, $PD_{20}FEF_{25-75\%}$ and $PD_{40}X5$ of the large-particle fast-inhalation test, and not for the small-particle slow-inhalation test.

Table 3. Threshold values of adenosine challenge with the four provocation tests

	Small particles, slow inhalation	Small particles, fast inhalation	Large particles, slow inhalation	Large particles, fast inhalation	p-value#
Large airways					
$PD_{20}FEV_1$ (mg)*	2.95 (1.36-12.16)	2.49 (0.46-14.81)‖	4.62 (0.60-18.51)†	3.40 (1.07-40.00)†	0.116
Small airways					
$PD_{20}FEF_{25-75\%}$ (mg)*	1.29 (0.83-4.88)	1.30 (0.24-3.89)‖	3.15 (0.48-8.63)	1.72 (0.54-12.16)	0.373
PD_{40}R5-R20 (mg)*	0.83 (0.08-1.57)	0.68 (0.29-6.59)‡	0.15 (0.09-0.80)	0.98 (0.19-11.67)‡	0.603
PD_{40}X5 (mg)*	0.74 (0.15-3.01)	0.82 (0.26-13.66)	2.59 (0.60-13.61)	0.84 (0.11-13.50)	0.461

* values were log2 transformed. Median (IQ-range) #p-value of Friedman test. Median (IQrange) of PD_{40}R20 are not shown, because of a too small sample size

Pairwise comparison of the four adenosine tests, results of linear mixed effect model.

‖ significant different from small-particle slow-inhalation test ($p<0.05$)

† significant different from small-particle fast-inhalation test ($p<0.05$)

‡ different from large-particle slow-inhalation test ($p<0.1$)

$PD_{20}FEV_1$: adenosine dose causing a 20% fall in FEV_1, $PD_{20}FEF_{25-75\%}$: adenosine dose causing a 20% fall in $FEF_{25-75\%}$, PD_{40}R5-R20: adenosine dose causing a 40% increase in R5-R20, PD_{40}X5: adenosine dose causing a 40% increase in X5.

Table 4. Effect of adenosine particle size and inhalation flow rate on the response.

		Estimate	95% CI	P-value
$PD_{20}FEV_1$ (mg)	Particle size (small)	-0.52	-1.28;.25	0.174
	Inhalation (slow)	0.64	-.11;1.39	0.092
$PD_{20}FEF_{25-75\%}$ (mg)	Particle size (small)	-0.33	-1.20;.53	0.438
	Inhalation (slow)	0.52	-.32;1.37	0.217
PD_{40} R5-R20 (mg)	Particle size (small)	0.44	-1.09;1.97	0.563
	Inhalation (slow)	-1.61	-3.11;-.10	0.037
PD_{40} X5 (mg)	Particle size (small)	-0.16	-1.42;1.09	0.794
	Inhalation (slow)	0.18	-1.06;1.41	0.773

Estimate is calculated using a linear mixed effect model

$PD_{20}FEV_1$: adenosine dose causing a 20% fall in FEV_1, $PD_{20}FEF_{25-75\%}$: adenosine dose causing a 20% fall in $FEF_{25-75\%}$, PD_{40}R5-R20: adenosine dose causing a 40% increase in R5-R20, PD_{40}X5: adenosine dose causing a 40% increase in X5.

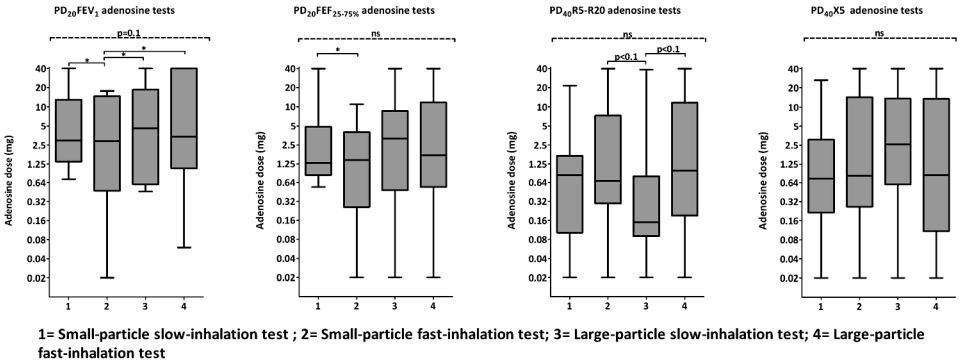

Figure 3. Box plots (25-75 percentile with range) of threshold values with differences between the four provocation tests. * p<0.05

$PD_{20}FEV_1$: adenosine dose causing a 20% fall in FEV_1, $PD_{20}FEF_{25-75\%}$: adenosine dose causing a 20% fall in $FEF_{25-75\%}$, PD_{40}R5-R20: adenosine dose causing a 40% increase in R5-R20, PD_{40}X5: adenosine dose causing a 40% increase in X5.

DISCUSSION

This study shows that the adenosine dry powder challenge can provoke large and small airway constrictive responses in subjects with asthma. However, in contrast to our hypothesis we could not demonstrate that small-particle slow-inhalation tests provoke a more pronounced response in the small airways than the three other combinations. Moreover, all four dry powder adenosine tests, even the one with large particles and a fast inhalation, provoked a response in the small airways. These findings are compelling and show that we need to reappraise the underlying mechanisms of bronchial hyperresponsiveness.

This is the first study showing that a provocation test with a dry powder agent is able to induce a significant response in the large and small airways in subjects with asthma. The provocation test with dry powder adenosine is an improvement to the small- and large-particle nebulized AMP test used in the study of Cohen and colleagues with respect to the response rate, since the small-particle dry powder adenosine tests induced a 20% fall in FEV_1 in 8-10 out of 10 subjects, while only 60% of the subjects responded to the nebulized small-particle AMP test used by Cohen and colleagues (6). The use of an inhaler with an air classifier dispersion system is also totally new and enabled administration of a consistent particle size over the total dose range. Furthermore, the duration of the test was substantially reduced since its use of a dry powder agent allowed inhalation of the provocative dose with one single inspiration instead of tidal breathing. The latter advantage also applies to mannitol provocation, however, small airway responses after mannitol provocation have not been studied yet.

We expected to observe a pronounced small and large airway response with the small-particle slow-inhalation test and a predominantly large airway response with the other combinations (Figure 1). However, almost every subject in our study demonstrated a response in both the large and small airways, independently of particle size and inhalation flow rate. We observed a minor effect of flow rate on the bronchial response, i.e. a low flow rate was related only to a lower R5-R20 threshold, using a linear mixed effect model. How can we explain such unexpected results? First, it may be that we did not achieve the expected selective deposition of large particles in the large airways and, substantially higher deposition of small particles in the small airways. As we did not perform an imaging study with radio-labeled adenosine particles, we relied on the findings of Usmani and colleagues showing the differential deposition patterns of salbutamol with different particle sizes and flow rates (1). We realize that the aerodynamic properties of adenosine may differ from salbutamol. The dry powder adenosine was not monodisperse even though adenosine had a relatively narrow size distribution. Possibly the use of monodisperse particles and particles with a smaller diameter for the finest aerosols would have been more discriminating. A second explanation may be that the lung function tests used in this study have limited specificity to discriminate between responses in the large and small airways. It has already been suggested that $FEF_{25-75\%}$ values not only reflect small airway dysfunction but also partly reflect large airway dysfunction (17). With regard to IOS, we could not perform analyses with the R20, because this parameter demonstrated a response in only 26% of all tests, probably reflecting the poor ability of the cartilaginous central airways to narrow. A final explanation may be that deposition of adenosine in the large airways not only leads to obstruction in the large airways but also in the small airways. The small airways may also respond to inflammatory mediators transported distally via superficial capillary vessels, or to stimulation of sensory nerves with excitation of cholinergic reflex pathways. A neural mechanism has not extensively been investigated in human bronchial hyperresponsiveness yet, but this may be worthwhile in perspective of our findings (18-20).

It is questionable whether we can develop a test that identifies potential responders and non-responders to small-particle treatment. Based on our results it is tempting to state that every asthmatic subject should be treated with small-particle anti-inflammatory and bronchodilator treatment given that almost every subject in our study demonstrated bronchoconstriction in the small airway function tests. On the other hand, because our study had a limited sample size, we cannot draw firm conclusions. Furthermore, regarding the treatment with ICS the situation is complicated, as the effect of small-particle ICS on the responsiveness of the small airways to a provocative agent has not yet been investigated. However, there is supportive evidence, since a close relationship has been demonstrated between PC_{20} AMP, assessed with FEV_1 and large-particle AMP, and airway inflammation in the large airways (21). The challenge with small-particle AMP in the study of Cohen and colleagues had promising results, however, we feel that a formal study analyzing the deposition of adenosine is needed before designing a study similar to that of Cohen and colleagues.

An important strength of this study is the use of the same inhaler in all four tests, in combination with a controlled inspiratory manoeuvre. By minimizing the factors that could affect the deposition of adenosine, we have been able to attribute our observations during the four tests to particle size and flow rate. A limitation of the study is that the provocations were stopped based on the fall in FEV_1, a large airway parameter. The increase in R5-R20, a small airway parameter, varied between 40 to 500% for the last given dose. We chose to stop the test at a 20% fall in FEV_1 for safety reasons, as we had limited experience with dry powder adenosine, as well as with specifically provoking the small airways. We used threshold values of a 20% fall in $FEF_{25-75\%}$ and 40% increase in IOS parameters in our analyses. These are arbitrary cut-off values based on previous studies, using a threshold of 40% increase in resistance measured with the forced oscillation technique. We anticipate that a better insight in the small airway response can be obtained if provocation continues to a predefined dose that is similar for all subjects. This enables a fair comparison of the tests and parameters between the subjects.

In summary, this study shows that a dry powder adenosine challenge is an appropriate test to induce bronchial hyperresponsiveness and can be readily used. All four dry powder adenosine tests, even the large-particle fast-inhalation test provoked a response in the small airways. In our opinion, the next phase in the investigation of the small airways should be to elucidate the exact sites of adenosine deposition and bronchoconstrictor response, e.g by using imaging techniques.

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SUPPLEMENT

Targeting the small airways with dry powder adenosine: A challenging concept

METHODS:

Questionnaires, lung function, airway resistance and ventilation heterogeneity

Subjects were characterized with questionnaires and lung function measurements. Patients filled in the Asthma Control Questionnaire (ACQ) and Bronchial Hyperresponsiveness Questionnaire (BHQ) (1,2). Spirometry and body plethysmography were performed according to guidelines after withholding bronchodilators (3,4). Reference values were obtained by Quanjer *et al* (5). IOS was measured using IOS masterscreen (E. Jaeger, Wurzburg, Germany) according to current available recommendations (6). Additionally, subjects performed an MBNW test (Exhalyzer, EcoMedics) obtaining Sacin and Scond. Sacin and Scond are expressed as value per 1L tidal volume. A tidal volume of 1L was encouraged but not strictly controlled. The MBNW measurement was completed according to the ERS/ATS consensus (7).

AMP provocation test

At the first visit, subjects performed an AMP provocation according to the tidal breathing method (Jaeger APS Pro system with the Medic-Aid Sidestream) (8,9). The AMP was nebulized with doubling concentrations ranging from 0.04 to 320 mg/mL. One IOS measurement, obtaining R20, R5-R20, X5 were performed 30 s after inhalation, followed by a forced vital capacity (FVC) manoeuvre, obtaining $FEF_{25-75\%}$, FEV_1 and FVC, 90s after inhalation. All provocation tests were scheduled during the same part of the day with an interval of at least 2 and maximally 14 days.

Dry powder adenosine particle size and inhalation flow rate

The adenosine dry powder provocation tests were performed with a spray-dried adenosine formulation delivered by an investigational test inhaler designed for this study. The development of the dry powder adenosine provocation test has been described in detail elsewhere (10,11). Dry powder adenosine was prepared with two different particle sizes, i.e. large-particle adenosine with an MMAD of 6.0 µm and a geometric SD of 1.8 and small-particle adenosine with an MMAD of 2.7 µm and a geometric SD of 1.5. The adenosine dry powder provocation test was performed with doubling adenosine doses ranging from 0.04 to 20 mg. Each dose was provided in a single blister, except the 20 mg dose, which was provided in two blisters of 10 mg. After full expiration, subjects inhaled the adenosine with one deep inspiration with the required flow rate, and then held their breath for 10s. The blisters were directly checked upon dose release and if required, a second inspiration was performed to ensure administration of the full dose. The test inhaler has an air classifier dispersion system similar to the Novolizer® and Twincer™ (12,13). Performance testing results and specifications of the inhaler in combination with dry powder adenosine are described by Lexmond and colleagues (11). Adenosine provocation tests were performed with a slow inhalation of 30-40 L/min or a fast inhalation of 60-70 L/min (14). The inhalation flow rate was measured and shown on a computer screen during each inhalation of adenosine in order to give visual feedback and target the desired flow rates. Peak and mean (min-max) flow rates were recorded for every inhalation manoeuvre. Before starting the tests, subjects practiced the inhalation manoeuvre until a proper inhalation was performed.

Borg score

Dyspnea during the provocations tests was rated with the Borg scale from 0 (no breathlessness) to 10 (maximal breathlessness) (15). Borg scores were filled in by the subjects after the IOS measurement of each provocation step.

Small and large airway response

The adenosine dose causing a 20% fall (PD_{20}) or 40% increase (PD_{40}) was calculated with interpolation using the log-transformed doses. If a 20% fall or 40% increase in the spirometric or IOS parameters respectively was reached after the first dose (0.04 mg) a PD of 0.02 was noted. If a 20% fall or 40% increase was not reached after the highest dose (20 mg), $PD_{20}$and PD_{40} were calculated by extrapolation with a maximum of 40 mg. If the test was stopped due to a 20% fall in FEV_1, extrapolation of PD_{20}or PD_{40} was only allowed if the calculated provocative dose was not higher than twice the last given dose, otherwise no PD_{20}or PD_{40} was assigned. $PD_{20}$and PD_{40} values were not based on outliers and were verified visually. FEV_1 and R20 were considered as large airway parameters, $FEF_{25-75\%}$, R5-R20 and X5 as small airway parameters (16).

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Supplementary table E1.

A. Peak and mean flow rates attained per test

	Peak inspiratory flow rate		Mean inspiratory fow rate	
	(L/min)	(min to max)	(L/min)	(min to max)
Small particle, slow inhalation	39.8	(35.1-46.4)	33.0	(28.9-40.4)
Small particle, fast inhalation	64.5	(60.3-72.1)	49.6	(41.3-53.3)
Large particle, slow inhalation	40.1	(35.1-46.1)	32.9	(29.1-38.6)
Large particle, fast inhalation	62.8	(57.0-70.9)	48.6	(44.2-52.3)

B. Number of incomplete adenosine dry powder releases after one inhalation per adenosine test

	Number of provocation steps	2nd inhalation required	3th inhalation required
Small particles, slow inhalation	82	9	0
Small particles, fast inhalation	73	6	0
Large particles, slow inhalation	94	17	5
Large particles, fast inhalation	90	1	0

Supplementary table E2. Threshold dose ($PD_{20}FEV_1$, $PD_{20}FEF_{25-75\%}$, $PD_{40}R20$, $PD_{40}R5\text{-}R20$, $PD_{40}X5$) and percentage change response at last given dose of FEV_1 $FEF_{25-75\%,}$ R20, R5-R20, X5 compared to baseline (max change)

Subject:	1		2		3		4		5		6		7		8		9		10		11	
	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)
Small-particle slow-inhalation test																						
$PD_{20}FEV_1$	2.90	(32.5)	9.46	(20.1)	22.12	(19.6)	1.42	(28.0)	0.72	(30.9)	na		9.96	(20.0)	2.99	(34.6)	2.66	(43.2)	1.21	(21.1)	40#	(2.2)
$PD_{40}R20$	3.79	(44.4)	1.02	(46.3)		(4.7)		(15.9)		(-13.1)	na			(-22.5)	6.86*	(30.3)		(20.0)		(18.2)		(-5.3)
$PD_{20}FEF_{25-75\%}$	1.16	(56.5)	1.08	(38.0)	5.35	(32.9)	0.70	(50.1)	0.54	(44.7)	na		4.74	(35.9)	1.43	(43.4)	1.87	(59.8)	0.88	(46.2)	40#	(11.7)
$PD_{40}R5\text{-}R20$	1.00	(160.0)	2.93	(43.6)	0.75	(131.0)	0.24	(180.0)	1.28*	(38.9)	na		0.02	(190.0)	0.13	(172.7)	0.02	(530.8)	0.91	(50.0)	33.3*	(37.5)
$PD_{40}X5$	0.56	(283.3)	0.28	(50.0)	0.02	(345.5)	0.70	(200.0)	0.79	(122.2)	na		3.97	(110.0)	2.75	(212.5)	0.02	(1600.0)	1.38*	(33.3)	26.4*	(33.3)
Small-particle fast-inhalation test																						
$PD_{20}FEV_1$	0.67	(31.2)	15.51	(21.4)	17.86	(21.0)	1.45	(33.9)	0.53	(25.8)	na		7.82	(23.4)	4.29	(23.5)	0.02	(23.8)	0.30	(20.9)	14.58	(28.6)
$PD_{40}R20$	1.13	(42.3)		(12.7)		(-2.2)		(15.9)		(-22.0)	na			(0.0)		(17.4)		(29.7)	0.26	(48.6)		(21.4)
$PD_{20}FEF_{25-75\%}$	0.31	(50.2)	2.06	(44.6)	5.63	(39.1)	0.82	(47.3)	0.36	(46.2)	na		3.43	(41.1)	2.48	(43.3)	0.02	(44.9)	0.11	(48.0)	11.01	(47.5)
$PD_{40}R5\text{-}R20$	0.62	(91.7)	13.72	(85.1)	0.61	(143.3)	0.74	(250.0)	0.20	(122.2)	na		4.02	(75.0)	5.16*	(38.1)	0.02	(145.5)	0.33*	(38.9)	40#	(-7.1)
$PD_{40}X5$	0.72	(175.0)	22.97*	(36.4)	0.62	(284.2)	0.93	(340.0)	0.27	(176.2)	na		11.49*	(36.4)	4.03	(75.0)	0.02	(133.3)	0.26	(58.8)	40#	(0.0)
Large-particle slow-inhalation test																						
$PD_{20}FEV_1$	2.60	(34.8)	40#	(14.7)	18.51	(20.6)	4.62	(22.0)	0.60	(21.8)	10.88	(30.5)	13.16	(27.8)	4.05	(23.6)	0.46	(23.3)	0.48	(28.6)	40#	(4.8)
$PD_{40}R20$	1.20	(59.3)		(-27.1)		(2.4)		(4.9)		(6.12)		(10.6)	31.02*	(29.7)		(19.1)		(20.5)		(12.5)		(11.1)
$PD_{20}FEF_{25-75\%}$	0.61	(54.0)	6.63	(30.8)	3.87	(35.9)	3.15	(40.7)	0.48	(35.3)	11.1	(37.9)	8.63	(47.9)	2.12	(41.5)	0.02	(46.4)	0.37	(45.6)	40#	(10.2)
$PD_{40}R5\text{-}R20$	0.11	(250.0)	38.37*	(19.6)	2.84	(125.0)	0.09	(137.5)	0.80*	(34.9)	0.60	(233.3)	0.05	(342.9)	0.10	(127.3)	0.02	(253.9)	0.38	(137.5)	0.15	(42.9)
$PD_{40}X5$	0.72	(480.0)	40#	(17.6)	3.13	(177.8)	0.60	(42.9)	0.64	(40.0)	13.61	(90.0)	10.82	(91.7)	2.59	(166.7)	0.02	(230.0)	0.34	(145.0)	40#	(0.0)
Large-particle fast-inhalation test																						
$PD_{20}FEV_1$	0.66	(28.3)	40#	(8.4)	10.56	(27.1)	3.40	(23.8)	2.11	(25.8)	40#	(5.3)	28.8*	(16.2)	2.30	(21.1)	0.06	(23.5)	1.07	(22.2)	40#	(5.7
$PD_{40}R20$	1.48*	(36.7)		(12.0)		(-10.9)	2.03	(59.0)		(-13.6)		(-3.8)		(2.6)	1.24	(40.5)		(8.7)	0.35	(43.8)		(10.3)
$PD_{20}FEF_{25-75\%}$	0.26	(48.1)	7.73	(22.4)	6.48	(33.1)	1.72	(44.0)	1.59	(41.6)	40#	(11.8)	11.83	(29.4)	0.71	(39.3)	0.02	(40.3)	0.54	(44.6)	30.11*	(13.7)
$PD_{40}R5\text{-}R20$	0.52	(75.0)	0.16	(97.3)	6.32	(51.2)	0.19	(445.5)	0.98	(56.7)	40#	(18.8)	11.67	(110.0)	1.14	(88.2)	0.02	(134.8)	0.19	(271.4)	40#	(22.2)
$PD_{40}X5$	0.11	(240.0)	0.19	(107.7)	1.51	(154.6)	0.25	(320.0)	2.57*	(37.0)	40#	(27.3)	13.5	(77.8)	0.84	(83.3)	0.02	(191.7)	0.02	(644.4)	40#	(-14.3)

Values are shown as threshold dose (percentage change compared to baseline for last given dose)

* threshold was not reached and PD was extrapolated

thereshold was not reached, a PD of 40 mg was assigned

$PD_{20}FEV_1$*: adenosine dose causing a 20% fall in* FEV_1, $PD_{20}FEF_{25-75\%}$*: adenosine dose causing a 20% fall in* $FEF_{25-75\%}$, PD_{40}*R5-R20: adenosine dose causing a 40% increase in R5-R20,* PD_{40}*X5: adenosine dose causing a 40% increase in X5.*

Supplementary table E3 Correlation of baseline characteristics with the response for the small-particle slow-inhalation test and large-particle fast-inhalation test.

	$PD_{20}FEV_1$		$PD_{20}FEF_{25-75\%}$		$PD_{40}R5\text{-}R20$		$PD_{40}X5$	
Baseline parameters:	Small particles, slow inhalation	Large particles, fast inhalation	Small particles, slow inhalation	Large particles, fast inhalation	Small particles, slow inhalation	Large particles, fast inhalation	Small particles, slow inhalation	Large particles, fast inhalation
Correlation coefficient:								
Age (years)	.006	-.130	.197	-.032	.377	.267	-.093	.028
ICS dose (µg)§	.038	-.024	.133	-.033	-.309	.040	.019	.014
FEV_1 (%pred)	.661*	.495‡	.527‡	.527‡	.347‡	.424‡	.091	.384
FEV_1/FVC (%)	.309	.826**	.018	.855**	.164	.446‡	.547‡	.676*
$FEF_{25-75\%}$ (%pred)	.721*	.881***	.418	.900***	.182	.610*	.389	.676*
RV (%pred)	-.079	-.284	-.030	-.236	-.529‡	-.005	.383	-.174
R20 (kPa/L/s)	.036	.290	.018	.210	-.171	-.164	-.451‡	.046
R5-R20 (kPa/L/s)	-.116	.032	-.401	-.082	.058	-.411	-.409	-2.17
X5 (kPa/L/s)	.310	-.143	.584‡	-.137	-.216	.215	.335	.007
AX (kPa/L)	-.176	.166	-.511‡	.123	.262	-.227	-.451‡	-.009
Sacin (L^{-1})	-.167	-.031	-.250	-.042	.433	-.231	.192	-.378
Scond (L^{-1})	-.217	-.227	-.017	-.152	-.033	.407	.234	.244
Difference:								
Gender (f/m)	1.0	0.16‡	0.91	0.16‡	0.35	0.41	0.04*	0.16‡
ICS use (yes/no)	0.22	0.75	0.22	0.75	0.86	0.53	0.16‡	0.75

§ beclomethason equivalent

***p<0.001, **p<0.01, *p<0.05, ‡p<0.2

Univariate correlations calculated with the Spearman's rank correlation coefficient. Differences are calculated with the Mann Whitney U test (added below). All parameters with a p-value<0.2 are included in the backward linear regression model.

AX: reactance area, $FEF_{25-75\%}$: forced expiratory flow between 25% and 75% of FVC, FEV_1 : forced expiratory volume in 1 second, FVC: forced vital capacity, ICS: inhaled corticosteroids, R5-R20: difference between the resistance of the respiratory system at 5 Hz and 20 Hz, R20: resistance of the respiratory system at 20 Hz, RV: residual volume, PC_{20}: provocative concentration causing a 20% fall in FEV_1, Sacin: ventilation heterogeneity of the acinar lung zone, Scond, ventilation heterogeneity of the conductive lung zone, X5: reactance at 5 Hz.

Supplementary table E4. Baseline predictors of small and large airway response on adenosine challenge

Dependent variable	Adenosine test	Baseline predictor	B	CI 95%	P-value
$PD_{20}FEV_1$ (mg)	Small particles, slow inhalation	FEV_1 (%pred)	0.102	-.010;.214	0.069
	Large particles, fast inhalation	$FEF_{25-75\%}$ (%pred)	0.096	.035;.158	0.006
$PD_{20}FEF_{25-75\%}$ (mg)	Small particles, slow inhalation	-			
	Large particles, fast inhalation	$FEF_{25-75\%}$ (%pred)	0.105	.040;.171	0.005
PD_{40}R5-R20 (mg)	Small particles, slow inhalation	-			
	Large particles, fast inhalation	-			
PD_{40}X5 (mg)	Small particles, slow inhalation	Gender (f/m)	4.45	1.19;7.71	0.015
		AX (kPa/L/s)	-1.42	-3.10;.26	0.086
	Large particles, fast inhalation	$FEF_{25-75\%}$ (%pred)	0.128	.004;.251	0.044

Values based on backward linear regression analysis. Associations with a p-value <0.2 were included in the multivariate regression analysis (**Supplementary table E3**).

$PD_{20}FEV_1$: adenosine dose causing a 20% fall in FEV_1, $PD_{20}FEF_{25-75\%}$: adenosine dose causing a 20% fall in $FEF_{25-75\%}$, PD_{40}R5-R20: adenosine dose causing a 40% increase in R5-R20, PD_{40}X5: adenosine dose causing a 40% increase in X5

CHAPTER 8

Eosinophilic inflammation in subjects with mild-to-moderate asthma with and without obesity: Disparity between sputum and biopsies

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To the editor,

In the September 15, 2013, issue of the *Journal,* Desai and colleagues showed that, in severe asthma, sputum eosinophils do not differ between obese and nonobese patients, yet obese patients have higher sputum IL-5 levels and eosinophil numbers in the bronchial submucosa (1). These findings contrast with two widely held beliefs; first, that sputum cellular profiles reflect airway inflammation; and second, that the obese asthma phenotype, identified by the cluster analysis by Haldar and colleagues (2), is characterized by high symptom perception but not eosinophilic inflammation.

The findings by Desai and colleagues (1) were obtained from two groups of subjects with asthma, one investigated with sputum and airway wall biopsies, and the other with sputum and blood differential cell counts. The investigation was restricted to severe asthma. We wondered whether these findings could be replicated in patients with mild-to-moderate asthma, in whom cell counts had been obtained from all three compartments (i.e., airway wall biopsies, sputum and blood). To this end, we investigated a large cohort (3-5) of 147 patients with predominantly mild-to-moderate asthma, as indicated by 56% of the patients requiring treatment step 1, 10% step 2, 12% step 3, and 21% step 4, according to Global Initiative for Asthma guidelines (6). Patients in steps 2-4 were receiving a median dose of inhaled corticosteroids of 800 µg/day beclomethasone equivalent. No patient was being treated according to Global Initiative for Asthma step 5 (oral corticosteroids or omalizumab), resulting in exclusion of severe asthma. Obesity (body mass index [BMI] > 30 kg/m^2) was present in 32 patients (28%). Patients had a mean prebronchodilator FEV_1 of 89% predicted (interquartile range 79-102%), a median Asthma Control Questionnaire score of 0.57 (interquartile range 0.29-1.3), and all had bronchial hyperresponsiveness to histamine or Adenosine 5′-monophosphate.

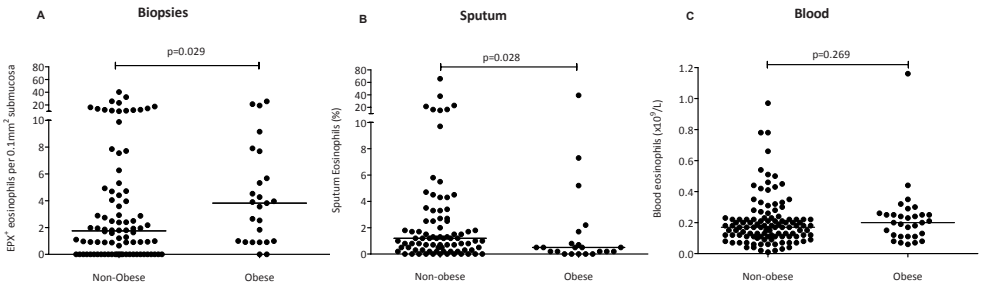

Figure 1

Eosinophilic inflammation, reflected by (**A**) number of eosinophilic peroxidase$^+$ eosinophils in the bronchial submucosa, (**B**) percentage of sputum eosinophils and (**C**) number of blood eosinophils in nonobese (body mass index [BMI] < 30 kg/m^2) and obese (BMI ≥ 30 kg/m^2) patients with asthma. Differences between groups were tested with the Mann-Whitney *U* test one-sided for biopsies and two-sided for sputum and blood. *Horizontal lines* represent median values. Each *circle* represents one patient.

We have previously reported differences in the relationship between clinical control and eosinophilia from biopsy compared with sputum (4). Now, we have investigated the difference between obese and nonobese patients in eosinophil counts from biopsies, sputum and blood and whether eosinophils in sputum, blood, and biopsies are correlated.

In our cohort, obese patients had significantly higher numbers of submucosal eosinophils and lower sputum eosinophil percentages than nonobese patients (Figure 1A and 1B; Table E1). Blood eosinophil numbers were comparable (Figure 1C). Using conventional criteria, sputum or blood eosinophilia was rarely found in the group of obese subjects, despite the higher number of submucosal eosinophils. In obese subjects with bronchial biopsy data, sputum eosinophilia (≥3%) was present in 3 out of 18 patients, and blood eosinophilia (≥300 cells/μL) in 3 out of 14 patients. These findings suggest a differential signal of airway wall biopsies, sputum, and blood in obese patients with asthma.

Table 1. Characteristics of subjects with asthma with and without submucosal eosinophils

	Subjects with submucosal eosinophils (n=84) [EPX^+ per 0.1 mm^2 > 0.00]		Subjects without submucosal eosinophils (n=29) [EPX^+ per 0.1 mm^2 = 0.00]		P value
Age (years)	49	(11)	46	(15)	0.243
Male, n (%)*	49	(58)	11	(38)	0.058
BMI, kg/m^2	28	(5.1)	26	(3.3)	0.047
Smoking, current/ex/never (%)	17/25/42		7/15/7		0.040
Pack-years (years)†	0.0	(0.0-7.7)	4.3	(0.1-13.1)	0.013
ICS use, n yes (%)*	38	(45)	12	(41)	0.718
ICS dose, μg/day†	800	(400-1000)	800	(425-1750)	0.516
Atopy present, n (%)*	62	(77)	17	(59)	0.066
Pre-BD FEV_1 (% pred)	87	(17)	93	(18)	0.176
Pre-BD FEV_1/FVC (%)	69	(11)	71	(11)	0.359
Post-BD FEV_1 (% pred)	97	(16)	100	(19)	0.365
Post-BD FEV_1/FVC (%)	72	(11)	75	(16)	0.391
PC_{20} AMP (mg/ml)†	50	(7.2-640)	244	(22.9-640)	0.136
Sputum neutrophils (%)*	52	(36-70)	61	(46-76)	0.189
Sputum eosinophils (%)*	1.0	(0.2-2.5)	0.2	(0.0-1.2)	0.035
Blood eosinophils ($x10^9/L$)†	0.18	(0.12-0.27)	0.15	(0.09-0.22)	0.117

Data are presented as mean (SD) or *number (percentage proportion) or †median (interquartile range) . Differences between groups are tested with the student's T-test, Mann-Whitney *U* test or Chi-square test, as appropriate.

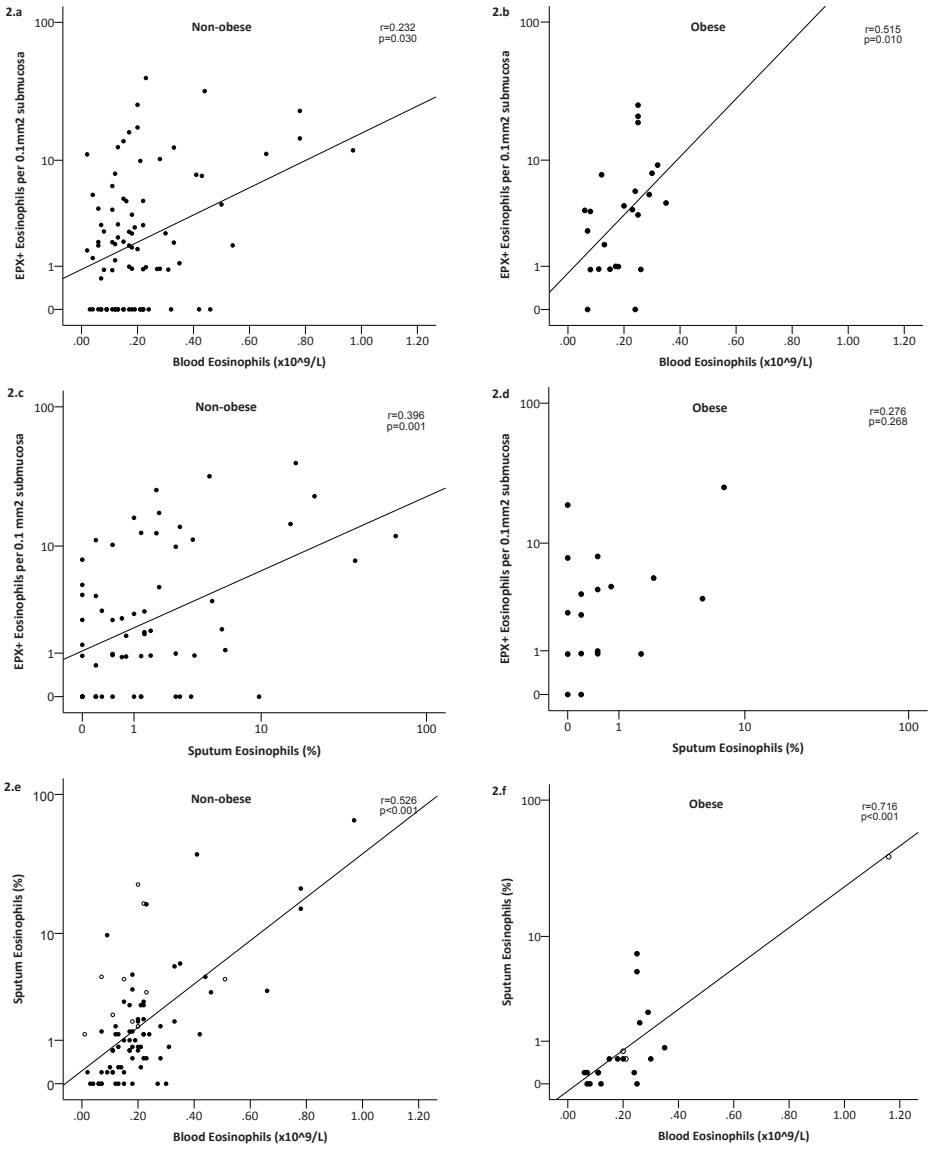

Figure 2

Correlations are shown for the number of blood eosinophils with the number of eosinophilic peroxidase$^+$ eosinophils in the bronchial submucosa in (**2a**) nonobese and (**2b**) obese patients with asthma, the percentage of sputum eosinophils with the number of eosinophilic peroxidase$^+$ eosinophils in the bronchial submucosa in (**2c**) nonobese and (**2d**) obese patients with asthma, and the number of blood eosinophils with the percentage of sputum eosinophils in (**2e**) nonobese and (**2f**) obese patients with asthma. Spearman's rank correlation coefficients (r) are shown in the *upper right corner* of the graph. *Open circles* represent patients without bronchial biopsy data available (E and F).

Note on F: If the outlying data point (sputum eosinophils >10%) was excluded, the correlation coefficient was 0.670 (p=0.001).

In nonobese subjects, we found significant, but weak, positive associations for numbers of submucosal eosinophils and blood eosinophils ($r = 0.232$, $P = 0.030$; Figure 2A) between number of submucosal eosinophils and percentage of sputum eosinophils ($r = 0.396$, $p = 0.001$; Figure 2C), and a stronger positive association between number of blood eosinophils and percentage of sputum eosinophils ($r = 0.526$, $p < 0.001$; Figure 2E). In obese subjects, there were significant positive associations between numbers of submucosal eosinophils and blood eosinophils ($r = 0.515$, $p = 0.010$; Figure 2B) and between blood eosinophils and sputum eosinophils ($r = 0.716$, $p < 0.001$; Figure 2F). However, we found no significant association between the number of submucosal eosinophils and the percentage of sputum eosinophils in obese subjects ($r = 0.276$, $p = 0.268$; Figure 2D). The latter, although potentially due to the small number of observations ($n = 18$), is consistent with our observation that the number of eosinophils in biopsies was increased in obese compared to nonobese patients with asthma, whereas the converse was found for sputum eosinophil percentages.

We wondered whether patients with and without submucosal eosinophils would differ in BMI or other clinical characteristics. Comparing patients with asthma with and without submucosal eosinophils (>0.00 and 0.00 eosinophilic peroxidase$^+$ eosinophils respectively), we found that the presence of submucosal eosinophils was associated with a higher BMI, never smoking, and fewer pack-years smoking (Table1). When adjusting for parameters with a P value less than 0.1 in a multivariate regression analysis on data of the complete cohort, a higher BMI was still positively associated with a higher number of eosinophilic peroxidase$^+$ eosinophils ($b = 0.095$, $p = 0.009$; Table E2). Thus, the association of obesity with higher bronchial eosinophil numbers in asthma appears to be independent of sex, smoking, pack-years, and atopy.

In conclusion, we confirmed in our cohort the finding of Desai and colleagues, that submucosal eosinophil numbers are higher in obese compared to nonobese subjects with asthma. Thus, the observation of Desai and colleagues is not restricted to patients with severe asthma, but also applies to those with mild-to-moderate asthma. However, in contrast to the results of Desai and colleagues, we observed significantly *lower* sputum eosinophils in obese patients with asthma than in nonobese patients with asthma (Figure 1B). This is compatible with the earlier reported obese noneosinophilic phenotype that was based on sputum (2).

A possible explanation for the discrepancy between our results and those of Desai and colleagues may be that we investigated a group with predominantly mild-to-moderate asthma group, whereas Desai and colleagues investigated patients with severe asthma. It is possible that patients with severe asthma have more inflammation and therefore higher sputum eosinophils, which would also explain the finding of elevated sputum IL-5 by Desai and colleagues. However, a previous study of Van Veen and colleagues showed an inverse association between sputum eosinophils and BMI in obese patients with severe asthma (7).

The absence of an association between the number of submucosal eosinophils and sputum eosinophil percentage in obese subjects, and the low prevalence of blood and sputum eosinophilia in this group, further supports our findings that sputum and blood may not reflect airway wall inflammation in patients with asthma with obesity.

So far, research of patients with asthma with obesity has predominantly used sputum, blood or exhaled nitric oxide instead of biopsies to investigate eosinophilic inflammation. The study by Desai and colleagues and our own study together show increased submucosal eosinophil numbers in obese subjects with asthma, irrespective of the severity of asthma. The discrepancy between inflammatory cell findings in airway wall biopsies and sputum in obese and nonobese subjects with asthma raises questions of whether sputum reflects airway wall inflammation in a reliable way. Furthermore, the underlying biological mechanisms are as yet unresolved. We therefore recommend to include airway wall biopsies in future studies seeking to clarify mechanisms of relationships between obesity in asthma and symptoms or steroid response.

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SUPPLEMENT

Eosinophilic inflammation in mild-to-moderate asthmatics with and without obesity: Disparity between sputum and biopsies

Table E1. Characteristics of non-obese and obese patients with asthma

	Non-obese (BMI<30 kg/m^2) (n=115)		Obese (BMI≥30 kg/m^2) (n=32)		p-value
Age (years)	47	(13)	51	(11)	0.124
Male, n (%)*	64	(56)	10	(31)	0.015
BMI, kg/m^2	25.4	(2.6)	35.0	(4.3)	<0.001
Smoking, % current/ex/never	31/26/43		31/16/53		0.413
Pack-years (years)†	0.4	(0.0-10.1)	0.0	(0.0-10.6)	0.525
ICS use, n yes (%)*‡	46	(40)	18	(56)	0.101
ICS dose, µg/day†	800	(400-1000)	1000	(475-2000)	0.103
Atopy present, n (%)*	83	(72)	17	(53)	0.075
Pre-BD FEV1 (% pred)	89	(17)	91	(19)	0.431
Pre-BD FEV1/FVC (%)	69	(11)	72	(10)	0.247
Post-BD FEV1 (% pred)	98	(16)	100	(18)	0.560
Post-BD FEV1/FVC (%)	73	(12)	74	(10)	0.629
ACQ (score) [n= 86 versus 29]	0.76	(0.0-2.9)	0.83	(0.0-2.6)	0.463
PC_{20} AMP (mg/ml)†	63.2	(8.9-640)	37.8	(8.0-640)	0.330
Sputum neutrophils (%)*	55	(33-70)	52	(37-72)	0.925
Sputum eosinophils (%)*§ [n= 83 versus 22]	1.20	(0.30-2.7)	0.50	(0.15-1.03)	0.028
EPX+ eosinophils per 0.1mm^2† [n= 88 versus 25]	1.75	(0.00-4.71)	3.82	(0.95-6.68)	0.029
Blood eosinophils (x10^9/L)† [n= 113 versus 31]	0.17	(0.11-0.23)	0.20	(0.11-0.25)	0.269

Data are presented as mean (SD) or *number (percentage proportion) or † median (interquartile range) . ‡ No oral steroid use. Differences between groups are tested with the student's T-test, Mann-Whitney *U* test or Chi-square test as appropriate. † Difference between groups is tested one-sided.

Table E2. Results of a multivariate linear regression analyzing the association between submucosal eosinophils (EPX+ eosinophils) and BMI, adjusting for sex, smoking habits, pack-years and atopy

	B	SE	p-value
BMI (kg/m^2)	0.095	0.036	0.009
Sex (f/m)	1.048	0.336	0.002
Smoking (never/current)	-0.655	0.528	0.218
Smoking (never/ex)	-0.754	0.401	0.063
Pack-years (years)	-0.020	0.016	0.212
Atopy present (n/y)	0.791	0.366	0.033

R-square 0.21

Distribution of EPX$^+$ eosinophils was normalized by Ln-transformation. Residuals were normally distributed.

CHAPTER 9

Summary and general discussion

SUMMARY

This thesis focuses on the relation between small airway dysfunction and asthma symptoms as well as clinical features in patients with asthma. We investigated the current literature about this relationship and explored the association of small airway dysfunction with respiratory symptoms, asthma control, the response to environmental stimuli and bronchial hyperresponsiveness (BHR) in our own study populations. Next, we started with the development of new tools to assess small airway dysfunction in patients with asthma. The main findings of the performed studies are summarized below.

In **chapter 2** we performed a formal literature search to find articles analyzing the relation between small airway dysfunction and clinical signs or symptoms of asthma. We selected 80 original research articles for this systematic review. We observed an association between small airway dysfunction and worse control of asthma, higher occurrence of exacerbations, presence of nocturnal asthma, more severe BHR, presence of exercise-induced asthma, and the late-phase allergic response. Interestingly, these associations were observed in patients with severe asthma, as well as in patients with mild asthma. We also discovered that exposure to fine-particle air pollution is related to worse asthma control accompanied by a fall in large and small airway function. In addition, treatment with fine-particle inhaled corticosteroids (ICS) was related to an improved quality of life and asthma control. We concluded based on this literature review, that small airway dysfunction in patients with asthma appears to be clinically relevant.

We chose to investigate our own asthma populations and used a two-way approach. First, we investigated whether small airway dysfunction was related to the clinical expression of asthma in a study population of 58 patients with mild-to-moderate asthma, who were extensively characterized with respect to large and small airway function (chapter 3). Secondly, we analyzed this relationship in a very large asthma population of 3,155 patients with a simple characterization (chapter 4).

In **chapter 3** we focused on the association of large and small airway dysfunction with asthma symptoms and BHR. We found that symptoms of nocturnal asthma, exercise-related symptoms, and symptoms of BHR as obtained with questionnaires were not significantly associated with large or small airway dysfunction. Only higher symptom scores on shortness of breath and wheezing were significantly associated with higher R5-R20 and AX values, variables that represent small airway dysfunction. The small airway variables $FEF_{25-75\%}$ and R5-R20 were also related to more severe BHR to methacholine, independently of the large airway variables FEV_1 and R20. The increase in dyspnea during the methacholine provocation was also strongly and independently correlated with the fall in large and small airway variables FEV_1 and X5. These findings show that the small airways are involved in the clinical expression of asthma, as reflected by BHR severity. In general, small and large airway dysfunction poorly correlate with asthma symptoms, although the methacholine-induced increase in dyspnea strongly correlated with the fall in small airway. The poor correlation of symptoms with small airway dysfunction contrasted with our expectations

based on the observations reported in chapter 2 of this thesis. On the other hand, our findings are compatible with previous studies observing a poor relation between asthma symptoms and large airway dysfunction, reflected by the FEV_1.

In **chapter 4** we investigated whether asthma control and the clinical response to environmental stimuli associate with large and/or small airway dysfunction in a large primary care-derived population. We selected 3,155 patients with a doctors' diagnosis of asthma, who were characterized with the asthma control questionnaire (ACQ), a tick-list including several stimuli that are able to induce respiratory symptoms, and spirometry before and after a bronchodilator. Patients with uncontrolled asthma had significantly lower pre-bronchodilator FEV_1 and $FEF_{25-75\%}$ values and a higher degree of reversibility than the group with controlled asthma. Separate multivariate regression models showed that a higher ACQ score was associated with both lower FEV_1 and $FEF_{25-75\%}$ values. Different environmental stimuli eliciting a respiratory response were associated with FEV_1 or $FEF_{25-75\%}$ values. Responses to fog and exercise were related to lower FEV_1 values, while responses to animals with lower $FEF_{25-75\%}$ values. These results show that small and large airway dysfunction are both related to the clinical expression of asthma in a large cohort of primary care patients as reflected by worse asthma control and the response to environmental stimuli. These results also suggest that environmental stimuli may have differential effects on the large and small airways.

Together, the results of chapters 3 and 4 show that small airway dysfunction is poorly correlated with asthma symptoms and control. This may suggest that small airway dysfunction drives these clinical features only mildly. Alternatively, conventional questionnaires on symptoms and control of asthma may be insufficiently sensitive to measure small airway dysfunction. Although this interpretation may seem far-fetched, it should be noted that these questionnaires have been developed using only large airway parameters. For this reason, it is of interest to develop a specific small-airway questionnaire.

Chapter 5 describes the first step in the development of a questionnaire to assess small airway dysfunction. A specific questionnaire may help to identify asthma patients with small airway dysfunction and to enable an early start of targeted therapy to improve symptoms. To this end, we selected asthma patients with and without small airway dysfunction based on both low $FEF_{50\%}$ and high R5-R20 values. First, patients were individually interviewed about symptoms, signs, habits and health related issues, subsequently patients discussed these items in small focus groups. All items potentially differentiating between patients with and without small airway dysfunction were selected. For example, patients with small airway dysfunction reported more exercise-induced symptoms and more symptoms after exposure to cats and birds. In total 63 items were selected for the first preliminary small airway dysfunction tool (SADT). The next step will be to test and validate all items in a large asthma population and retain the most relevant items to create a short and simple small airway dysfunction tool.

Recent studies have shown that treatment with small particle ICS have clinical benefits. However, we are currently not able to specifically diagnose asthma patients with small airway dysfunction, those who will probably benefit most from the inhalation of small particle medication. A few years ago researchers of our group tried to identify patients with small airway dysfunction using a tidal breathing provocation test with small and large particles adenosine '5-monophosphate (AMP). However, this conventional nebulized AMP test has several disadvantages. For instance, the particle size and nebuliser output is inconsistent over the AMP concentration range, and the maximum concentration AMP is restricted to approximately 320 mg/mL leaving a considerable percentage of asthma subjects not responding to AMP. To overcome the disadvantages, we developed a new provocation test with dry powder adenosine enabling a narrow particle size and higher dose steps to 80 mg adenosine. The aim of the study, described in **chapter 6**, was to compare the new dry powder adenosine test with the conventional nebulized AMP test. Five subjects with asthma performed both challenge tests. All subjects reached a 20% fall in FEV_1 with the new dry powder adenosine test, compared to four out of five subjects with the conventional AMP test. The subject without a response to the AMP test reached a PD_{20}after inhaling 80 mg of adenosine with the new dry powder adenosine test. This dry powder adenosine dose was higher than the last step of the conventional AMP test, i.e. 80 mg versus ±28 mg (320 mg/mL AMP). The new dry powder adenosine test was well tolerated by all subjects and much easier and quicker to carry out than the conventional AMP test. The new dry powder adenosine provocation test appeared to be feasible to assess BHR in subjects with asthma.

After the above described feasibility of the dry powder adenosine test, we tried to identify patients with small airway dysfunction using this dry powder adenosine provocation test in **chapter 7**. We aimed to challenge the small and large airways selectively using small- and large-particle adenosine, inhaled with a fast or slow flow. We hypothesized that the small-particle slow-inhalation provocation test gives a higher deposition in the small airways and thus induces a higher response in the small airways than a test with large-particle adenosine and/or a high flow rate. Eleven subjects with asthma performed the adenosine provocation tests with both small (2.7 µm) and large (6.0 µm) particles, and inhaled once with a low flow rate (30 L/min) and once with a high flow rate (60 L/min). We found that a challenge with adenosine dry powder is suitable to induce a large and small airway response. In contrast to our hypothesis, we could not demonstrate that the small-particle slow-inhalation test provokes a higher response in the small airways than the other three combinations. All provocation tests induced a response of the small airways, even the large-particle test with a high flow rate, which probably achieves nearly no deposition in the small airways. These findings are compelling and may have profound implications for our understanding of the underlying mechanisms of BHR, as the current thought is that a stimulus only elicits bronchoconstriction at the place of deposition.

In response to a study of Desai and colleagues, presence of eosinophilic inflammation in obese patients with asthma is discussed in **chapter 8** (1). The study of Desai and colleagues had shown that sputum IL-5 and bronchial submucosal eosinophils were elevated in obese patients, whereas the eosinophil percentage did not differ between obese and nonobese patients with severe asthma. These findings are in contrast with the obese asthma phenotype as reported in the cluster analysis of Haldar and colleagues, which characterized the obese asthma phenotype as a cluster of patients with a high symptom expression, but without eosinophilic airway inflammation (2). In order to replicate the findings of Desai and colleagues, we analyzed data from 147 patients with mild-to-moderate asthma in whom cell counts had been obtained from airway wall biopsies, as well as sputum and blood. We found that obese patients with asthma had significantly higher numbers of submucosal eosinophils and lower sputum eosinophil percentages than nonobese patients. Blood eosinophil numbers did not differ significantly between obese and nonobese patients with asthma. These findings confirm the results of Desai and colleagues and partially extend their findings showing a differential signal from cells in airway wall biopsies, sputum, and blood in obese patients with asthma.

GENERAL DISCUSSION

Clinical expression of the small airways in asthma

This thesis showed that small airway dysfunction contributes to the clinical expression of asthma, as expressed by asthma symptoms, asthma control, bronchial hyperresponsiveness (BHR) and the response to environmental stimuli. These observations together with data from our literature review seem to provide conclusive evidence about the contribution of small airway dysfunction to the clinical expression of asthma. However, there are a few important limitations that need to be considered before drawing a definite conclusion.

Study design

Studies reviewed in chapter 2, and presented in chapters 3 and 4, had a cross-sectional design showing correlations between small airway dysfunction and clinical features of asthma. A positive or negative correlation in a cross-sectional study does not imply causation, indicating that presence of clinical features cannot be attributed with certainty to small airway dysfunction. Longitudinal studies investigating the relation between small airway dysfunction and symptoms are lacking. As a result, we have no definitive information about the long-term course and variability of small airway function.

Study population

A point of discussion is the selection of the study populations. Several studies presented in chapter 2 investigated a subgroup of patients with asthma e.g. severe asthma or nocturnal asthma (3-5). This may lead to the false idea that small airway dysfunction is only present in selective subgroups of asthma patients. Furthermore, findings of these studies are thus limited to these selective subgroups and disregard the majority of the asthma patients (6,7). Therefore, it is recommended to perform population studies with representative study populations (6). We investigated in chapters 3 and 4 a heterogeneous study population of subjects with mild to severe asthma. The advantage of such a heterogeneous study population is that it reflects a real life situation and conclusions can be translated to a broad spectrum of asthma patients. However, the heterogeneity of our small sample study population investigated in chapter 3, can also be seen as a drawback, because associations may for example differ in obese versus nonobese subjects, smokers versus never smokers, subjects with versus without established BHR. In contrast, in chapter 4 we investigated data from 3,155 non-selected subjects with asthma, but the clinical characterization of these subjects was limited. In conclusion, small sample size studies may discover associations in selective subgroups of asthma patients, which cannot be generalized to the total asthma population. In addition, small sample size studies may lack enough statistical power to discover associations that are present in the total asthma population. Consequently, we recommend to perform large population studies as this may cover the total spectrum of small airway dysfunction present in real life.

Tools to assess clinical features in the perspective of small airway dysfunction

In this thesis we used different tools to investigate the clinical expression of asthma. To assess asthma symptoms we used 5 questions of the asthma control questionnaire (ACQ) and 7 questions of the bronchial hyperresponsiveness questionnaire (BHQ) and detected a significant relation with higher scores on these questionnaires with small airway dysfunction for only two questions. Both BHQ-wheezing and BHQ-shortness of breath were related to the small airway parameters R5-R20 and AX. The total score of the ACQ was used to quantify the level of asthma control and correlated significantly, but weakly, with the small airway parameter $FEF_{25-75\%}$. These findings suggest that small airway dysfunction, as measured with spirometry, body plethysmography, IOS and alveolar exhaled nitric oxide, poorly correlate with asthma symptoms and asthma control. This is in line with the poor correlation between large airway dysfunction and patients' perceived symptoms as described by several studies (8,9). However, it is also possible that these questionnaires were not sufficiently sensitive to detect small airway dysfunction, as both questionnaires were validated in the past with large and not small airway parameters (10,11). This probably reduces the chance to find relationships of these clinical variables with small airway parameters.

In the study presented in chapter 3, we observed a moderate association between small airway dysfunction and the severity of bronchial hyperresponsiveness to methacholine. In addition, we found that the slopes of the large airway parameter FEV_1 and the small airway parameter X5 both were strongly and independently related to the methacholine-induced increase in dyspnea. These findings suggest that BHR and symptoms elicited by BHR at least partly originate from the small airways. In the review presented in chapter 2, we described several studies showing that the small airways are involved in BHR to methacholine, exercise-induced bronchoconstriction and the response to allergens (12-14). Currently, the severity of the response to a stimulus is usually defined by the fall in large airway function, like the provocative concentration of methacholine inducing a 20% fall in FEV_1 (PC_{20}). Therefore, it would be interesting to develop new cut-off values for the response of the small airways, like the 40% increase in R5-R20 used in chapter 7.

In chapter 4 we assessed the respiratory responses to environmental stimuli with a tick-list. Patients reported if they had either chest-tightness, breathlessness or wheezing with exposure to stimuli like animals, (house)dust, or grasses. We found that a positive, questionnaire based, respiratory response to animals was related to small airway dysfunction, while positive responses to fog and exercise were related to large airway dysfunction. A positive response is considered to reflect BHR, however it is questionable if the used tick-list is an appropriate tool. For example the type of response to exercise was not specified and a positive response was only based on patient reported symptoms. Future studies are required including skin-prick tests or allergen challenges, to confirm the link between environmental stimuli and large as well as small airway dysfunction as described in the current study.

Tests to assess small airway dysfunction

An important limitation of all studies is the lack of a gold standard to assess small airway dysfunction. We used several different variables that are assumed to reflect small airway dysfunction. The drawbacks of small airway dysfunction tests will be discussed in more detail below.

In summary, we have to interpret associations between small airway dysfunction and clinical features of asthma carefully. New studies with a longitudinal design and investigating large representative study populations are required to provide more robust evidence whether small airway dysfunction contributes to clinical features of asthma. It is important to acknowledge that variables of clinical expression have been validated with large airway parameters only. Therefore, we have to consider new approaches to study small airway dysfunction like developing a specific small airway dysfunction questionnaire.

Challenging the small airways in asthma

Findings

One of the aims of this thesis was to develop a new bronchial provocation test that could identify patients with and without small airway dysfunction. To this end, we tried to challenge the large and small airways selectively with large- and small-particle adenosine inhaled with a high or a low flow rate. We investigated the response of the small and large airways and hypothesized that a small-particle slow-inhalation provocation test would give a higher deposition in the small airways and thus would induce a higher response in the small airways than a provocation test with large particles and/or with a fast inhalation (chapter 7). In contrast, to our hypothesis we observed no difference between the four dry powder adenosine tests. Furthermore, we found that all tests induced a small airway response, even the test with large particles. These intriguing findings may provide a new insight in the mechanisms of bronchial hyperresponsiveness (BHR) of the small airways. Implications of our observations are discussed below.

Interpretation and implication

Challenging the small airways with dry powder adenosine is a new concept. We observed that dry powder adenosine was able to induce bronchoconstriction of the large as well as the small airways. Since not only the large but also the small airways likely contribute to the severity and clinical expression of asthma, this is an important finding. The dry powder adenosine provocation test has many advantages above the conventional AMP test and was tolerated by all subjects with asthma (15,16). For these reasons, the new dry powder adenosine test may be suitable for further development for implementation in clinical practice. Before introduction, further studies are required investigating the response in healthy subjects and determining the sensitivity and specificity of the adenosine test compared to the conventional AMP or methacholine challenge.

In contrast to our hypothesis, we found no difference in the small airway response between the four adenosine tests with large or small particles and inhaled with high or low flow rate. A possible explanation is that we did not achieve a selective deposition in the large or small airways. Table 1 shows the deposition of monodisperse albuterol for the different particle sizes and flow rates as used by Usmani and colleagues (17). Based on these findings the deposition of dry powder

Table 1. Calculated deposition

Particle size (µm)	Inhalation flow rate (L/min)	Small airways (%)	Large airways (%)	Oropharynx (%)	Total deposition (%)*
Dry powder adenosine					
2.7	31	17	34	30	81
2.7	67	12	34	40	86
6.0	31	11	35	42	88
6.0	67	6	19	64	89
Albuterol by Usmani					
3.0	31	17	34	31	82
3.0	67	12	39	37	88
6.0	31	11	35	43	89
6.0	67	4	18	67	89

* remainder is exhaled

Adenosine was calculated for the four tests. We found that the dry powder adenosine deposition was comparable with albuterol in the different compartments. Although deposition in the small airways with the small-particle slow-inhalation test is higher with a factor of 2.8 than the large-particle fast-inhalation test, it is questionable if this difference is large enough to induce a differential response in the small airways. Additionally, the deposition in the small airways was very small compared to the deposition in the large airways and oropharynx, with a maximum deposition of 17% with the small-particle slow-inhalation test. Comparing particles with a smaller particle size than 1.5 µm and a large particle size between 8-10 µm and with a monodisperse distribution, would probably give better discrimination.

The observation that all provocation tests were able to induce a small airways response, even the large-particle fast-inhalation test, was surprising. We expected that the large-particle fast-inhalation provocation test would lead to deposition mainly in the large airways and oropharynx, and only minimally in the small airways. The total area of the small airway is enormous compared to the large airways, which makes it unlikely that the estimated deposition of 6% is sufficiently high to induce a small airway response. These findings suggest that a provocation not only leads to a local response at the spot of deposition but also to airway responses deeper in the lungs. To state this in a different manner, our observations suggest that deposition of adenosine in the small airways is not obligatory to induce a small airway response. We may speculate about underlying mechanisms in this respect. One potential mechanism may be the transportation of adenosine, or mediators released by adenosine, to the small airways via the capillary network that is present just beneath the basement membrane (18,19). Another potential mechanism is via the neural pathway, as adenosine probably also induces bronchoconstriction via activation of sensory nerve pathways that subsequently stimulate the local axon reflexes and central nervous system reflex pathways (20,21). Activation of the central nervous system via adenosine deposition in the large airways may lead to activation of efferent vagal nerves that cause contraction of smooth muscle

cells elsewhere, e.g. in the small airways. The existence of this central bronchial reflex and role in BHR has not yet extensively been investigated in humans (22,23). Of interest in this respect is the investigation of the nasobronchial reflex, to unravel the relation between allergic rhinitis and asthma (24). Several studies suggest that nasal stimulation can also induce a bronchial response (25). However, it remains difficult to dissect the neural pathway from other possible mechanisms and despite accumulating evidence, the role of this nasobronchial reflex is also still debated.

Tests to assess small airway dysfunction in asthma

The small airways are relatively inaccessible and therefore difficult to investigate. Transbronchial biopsies have been used to directly measure inflammation of the small airways, but application of this invasive technique is limited in daily practice (26). In recent years several tests have been put forward to assess small airway dysfunction (27). Functional techniques are frequently used to assess small airway dysfunction, like spirometry, impulse oscillometry (IOS) and multiple breath nitrogen washout (MBNW). These techniques are only able to provide indirect information of the small airways. Unfortunately, there are no established cut-off values of these tests available. As a result, there is a recurrent discussion whether tests are sufficiently sensitive and specific to reflect small airway dysfunction. Parameters, advantages and disadvantages of small airway dysfunction tests are presented in chapter 1, table 1. Tests that have been used in the studies presented in this thesis will be discussed below

The dilemma of FEV_1 versus $FEF_{25-75\%}$

The mid-expiratory flow rate of the forced vital capacity, i.e. $FEF_{50\%}$ or $FEF_{25-75\%}$, is thought to reflect small airway function, while the FEV_1 is thought to reflect large airway function. In this thesis we made comparisons between the $FEF_{25-75\%}$ and FEV_1 values in order to compare small and large airway function. In chapter 4 we showed that the FEV_1 and $FEF_{25-75\%}$ were very closely correlated with ρ=0.717, a p-value of 0.001. Unfortunately, this correlation was too strong to determine the independent contribution of these parameters to asthma control. This raises the question whether the $FEF_{25-75\%}$ and FEV_1 reflect airway dysfunction in a different area of the tracheobronchial tree. Interestingly, the correlation graph of the $FEF_{25-75\%}$ and FEV_1 shows not a straight line, but a curved line with more variability in the range of higher FEV_1 and $FEF_{25-75\%}$ values (figure 1). This finding suggests that both parameters provide a distinct signal, especially when FEV_1 is >85% of predicted. Interestingly, the $FEF_{25-75\%}$ has also been put forward as a sensitive tool to detect early stage small airway dysfunction in asthma, when the FEV_1 still is in the normal range (28,29). Since the small and large airways are connected in series, it is likely that severe air flow limitation in the small airways affects the $FEF_{25-75\%}$ as well as the FEV_1 and that severe air flow limitation in the large airways affects the FEV_1 as well as the $FEF_{25-75\%}$. Together, these findings show that lower $FEF_{25-75\%}$ and FEV_1 values probably reflect different areas of airway obstruction in the lungs, especially with an FEV_1 value in the normal range. However, when obstruction occurs in a partly overlapping area both $FEF_{25-75\%}$ and FEV_1 values will probably be reduced.

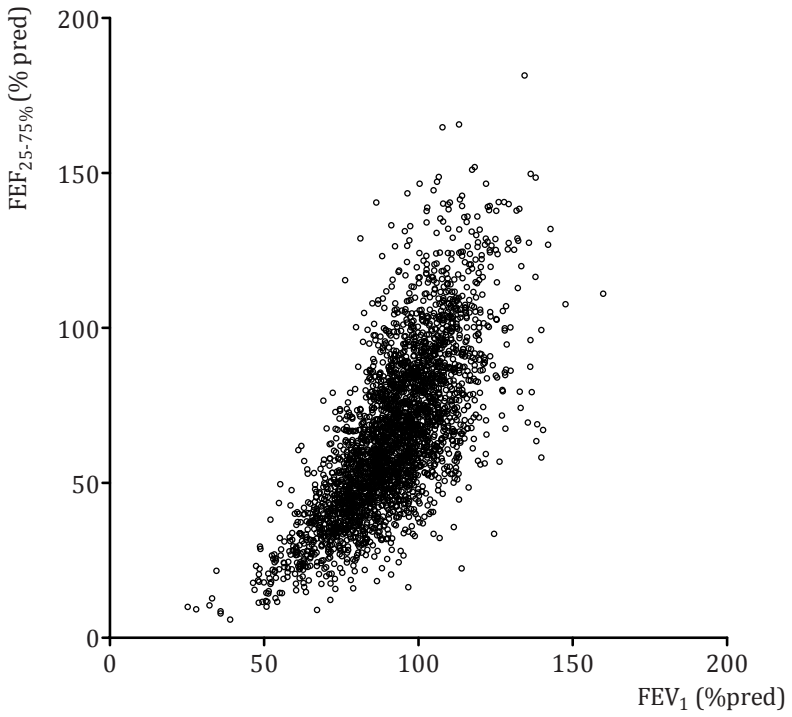

Figure 3. Correlation plot of the FEV_1 %predicted with the $FEF_{25-75\%}$ %predicted (r=0.717, p<0.001). Pearson correlation coefficient with pre-bronchodilator values.

IOS parameters during bronchial challenging

The division of large and small airway parameters for the IOS is based on a theoretical model that high-frequency impulses around 20 Hz only pass through the first generations and low-frequency impulses of 5 Hz pass further until the end of the bronchial tree. Small airway resistance is reflected by the frequency dependence of the resistance and is usually calculated by subtracting large airway resistance (R20) from the total airway resistance (R5), i.e. R5-R20 (30). It is assumed that at 5 Hz the imaginary reactance (X5) reflects also small airway dysfunction. A limitation of this model is that it is not known at which level of the bronchial tree the R5 and R20 are being measured and whether small airway parameters like R5-R20 and X5 are influenced by large airway dysfunction, or by other differences in airway geometry. We observed hardly any increase in R20 during the provocation tests with methacholine or dry powder adenosine, even when the FEV_1 fell ≥ 20% compared to baseline. These findings are in line with the study of Segal and colleagues observing a minimal change in R20 during the methacholine provocation (31). In addition, Lee and colleagues found no association between the exercise induced response in FEV_1 and the R20, while the R5-R20 and reactance at 5 Hz (X5) did associate (32). An explanation for this minimal change in R20 in response to BHR may be the little possibility of the first generations of the bronchial tree to narrow given the fact that they are surrounded by cartilage. It is also possible that airway narrowing during provocation prevents the high frequency impulses of 20 Hz to pass

to deeper parts of the bronchial tree, leading to a more central measurement of R20. Goldman and colleagues described a similar phenomenon after bronchodilator use (33). They observed no change in R20 after inhalation of a bronchodilator and proposed that the little change in R20 does not imply that the large airways do not dilate after bronchodilator but that dilation allows 20 Hz impulses to pass further into the bronchial tree. Nevertheless, the findings suggest that the R20 is not a sensitive parameter to assess changes of the large airways. This poses also questions about the validity of the calculated R5-R20 variable as marker of small airway resistance during provocation. In chapter 3, we found that the R5-R20 was not associated with the change in dyspnea during a methacholine provocation test independently of the FEV_1 while the X5 was significantly and independently associated. These findings seem to confirm that the R20 is not a sensitive parameter to detect changes of the large airways during bronchial provocation, leading to the suggestion that also the derivative R5-R20 as small airway parameter may be restricted.

MBNW technique

The MBNW technique has only recently become clinically available and is increasingly used to assess small airway dysfunction. We performed MBNW measurement in the study presented in chapter 7 and found no significant association between the Sacin and Scond, obtained with MBNW, and the response of the FEV_1or $FEF_{25-75\%}$ to small-particle slow-inhalation or the large-particle fast-inhalation adenosine test in our subjects. Studies using the MBNW technique showed conflicting results whether small airway ventilation heterogeneity is correlated with worse asthma control or more severe BHR (34-37). For example, Farah and colleagues investigated 105 asthma patients and found that an increase in Sacin and Scond, parameters of the MBNW, were independently related to worse asthma control (34). In contrast, Gonem and colleagues could not detect an association between Sacin or Scond and asthma control in 74 patients with asthma (35). The discrepancy between both studies can probably be explained by the fact that MBNW testing was performed after 400 µg salbutamol in the study of Gonem and colleagues, while patients had to withhold bronchodilators before MBNW testing in the study of Farah and colleagues. We cannot conclude that the MBNW technique is an optimal test, however there is suggestive evidence that MBNW technique is sensitive to detect small airway dysfunction. A few studies showed that MBNW is more sensitive than spirometry to detect associations between small airway dysfunction and clinical features, like asthma control and BHR. In addition, a recent study showed that the MBNW was also more sensitive than the FOT to detect abnormalities in asymptomatic smokers (38). Therefore, it would be worthwhile to include this technique together with a resistance measurement in further research on small airway dysfunction.

We conclude that there is an important unmet need for a gold standard to assess small airway dysfunction in the research field of clinical asthma. All tests have their specific shortcomings and many small airway parameters are probably also influenced by large airway dysfunction. As a result, without a gold standard, it is difficult to draw definitive conclusions about the exact role of the small airways.

Future perspectives

The development of a *gold standard* to assess small airway dysfunction would be a big advance in the research field of small airway dysfunction. Ideally, we would like to assess sensitivity, specificity and minimally clinically important change for each test. However, since we have no reference to represent small airways dysfunction, we are not able to provide these specifications. It is questionable if we are able to find one test as standard small airway dysfunction in the near future, because current available tests measure several different aspects of small airway dysfunction, like flow, resistance, inflammation and air trapping. Furthermore, the majority of the tests provide only indirect information about small airway dysfunction. Direct information about the small airways can be obtained by small airway biopsies, or imaging techniques, like imaging of deposition with radiolabelling or the new optical coherence tomography (OCT). Future studies should consider evaluating combinations of indirect non-invasive tests like IOS and MBNW and direct techniques. For example, a strong relationship between small airway inflammation, obtained by transbronchial biopsies, and peripheral airway resistance assessed with IOS, may bring us further in the validation of small airway dysfunction tests. Another approach for future research is to find a combination of tests that are complimentary in detecting different aspects of small airway dysfunction.

In addition to the development of new techniques to assess small airway dysfunction, it would also be of interest to develop new simple *questionnaires* to assess small airway dysfunction. In chapter 5 we presented the first step in the development of such a questionnaire, i.e. SADT. This preliminary list of 63 items has to be reduced and validated before it can be applied to recognize small airway dysfunction in daily clinical care. All items should be tested cross-sectionally and longitudinal in a population consisting of asthma patients with and without small airway dysfunction and of healthy controls.

Future research about clinical features of asthma and small airway involvement should also address the normal variability in small airway dysfunction over time. *Longitudinal data* may reveal whether small airway dysfunction varies over time or relates to further lung function decline. Longitudinal data may also help to detect subjects at risk for small airway dysfunction. It has been suggested that small airway dysfunction is more frequently present in asthma patients who smoke, are older, or have severe asthma (39). Performing a longitudinal cluster analysis in a large asthma population, that is extensively characterized with different small airway dysfunction tests, may discover whether there exists a distinct small airway dysfunction phenotype. In addition it can provide a combination of tests that differentiates this phenotype.

Studies reviewed in chapter 2 demonstrated that exposure to fine-particle *air pollution* was associated with worse asthma control accompanied by a fall in large and small airway function. Nowadays, there is increasing interest in air pollution and several studies have shown an association between exposure to air pollution and decline in lung function (40,41). Current research about air pollution mainly focuses on the spirometric parameters FEV_1 FVC and PEF and does not pay attention to small airway parameters (42). However, it is likely that fine-particle air

pollution will also have an effect on the small airways. Moreover, De Jong and colleagues recently showed that occupational exposure, i.e. vapors, gasses, dust and fumes, was related to lower $FEF_{25-75\%}$ values in subjects without large airway obstruction, i.e. $FEV_1 \geq 80\%$ and $FEV_1/FVC \geq 70\%$ (43). It would be interesting to gather the knowledge of both fields and investigate the effect of fine-particle and coarse-particle air pollution on the large and small airways, because if research focuses on the large airways only an effect on the small airways may be overlooked.

The findings about small airway response to a *dry powder adenosine challenge* presented in chapter 7 ask for further studies. Repeating the study of Cohen and colleagues, with application of the dry powder adenosine test to predict responders and non-responders to treatment with large and small-particle inhaled corticosteroids would be of interest, yet a deposition study with radio-labeled adenosine should precede such a study (44). Images of adenosine deposition in the large and small airways can determine if our assumptions are correct. In addition, we would like to determine the exact site of bronchoconstriction in response to the adenosine challenge. Unfortunately, currently available tools are not able to detect the site of bronchoconstriction. A first attempt can be made with expiration high-resolution computed tomography imaging (HRCT) performed before and after a large and small particle provocation test. Comparing the patterns of air trapping on HRCT may provide new insights in the site of the airway response to the large- and small particle provocation tests.
Based on the findings that all adenosine provocation tests were able to induce a small airway response, we speculated that local deposition of adenosine in the small airways may not be necessary to induce a response of the small airways. By inference, it would be interesting to investigate if a deposition of a bronchodilator in the small airways is needed to induce bronchodilation of the small airways or that deposition in the large airways is sufficient. Interestingly, Usmani and colleagues already showed that the greatest improvement in $FEF_{25-75\%}$ was achieved with the largest particle size of 6 μm (17). Therefore, it would be of interest to investigate the large and small airway response to large and small particle β2-sympathicomimetics.

Finally, this thesis showed the importance of the small airways in several clinical features of asthma. Still many questions remain unanswered and new studies as described above are required to get more in-depth insight in the role and relevance of the small airways in asthma. Hopefully future research will provide new findings and disentangle the independent functions and interrelations of the large and small airways in clinical features of asthma and BHR. This will ultimately benefit patients' well-being in case we can develop novel treatments or treatment combinations that target the large and small airways optimally.

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ABBREVIATIONS

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ACT: Asthma Control Test
ACQ: Asthma Control Questionnaire
AMP: Adenosine 5'-monophosphate
AQLQ: Asthma-related Quality of Life Questionnaire
ATS: American Thoracic Society
AX: Reactance area
BAL: Bronchoalveolar lavage
BDI: Baseline Dyspnea Index
BDP: Beclomethasone dipropionate
BHR: Bronchial hyperresponsiveness
BHQ: Bronchial Hyperresponsiveness Questionnaire
BMI: Body mass index
CaIv: Alveolar concentration of eNO
CC: Closing capacity
CCQ = Clinical COPD Questionnaire
CFC: Chlorofluorcarbon
CT scan: Computed tomography scan
CV: Closing volume
COPD: Chronic obstructive pulmonary disease
dN2: Slope of phase III of SBNT
DPI: Dry powder inhaler
EHV: Eucapnic hyperventilation test
$FEF_{25-75\%:}$ Forced expiratory flow at 25% to 75% of the FVC
$FEF_{50\%}$: Forced expiratory flow at 50% of the FVC
FEV_1: Forced expiratory volume in one second
FOT: Forced oscillation technique
FRC: Functional residual capacity
Fres: Resonant frequency of reactance
FVC: Forced vital capacity
GSD: Geometric standard deviation
HDM: House dust mite
He: Helium
HFA: Hydrofluoralkane
HRCT: High resolution computed tomography
ICS: Inhaled corticosteroids

IOS: Impulse oscillometry
JNO: Bronchial flux of eNO
LLN = Lower limit of normal
MBNW: Multiple breath nitrogen washout test
MCT: Methacholine provocation test
NA: Nocturnal asthma
NNA: Non-nocturnal asthma
NCT= Clinical trial registry number
NO of eNO: Exhaled nitric oxide
Nv: Number per volume
PC_{20}: Provocative concentration causing a 20% fall in FEV_1
PEEP: Positive end-expiratory pressure
PEF: Peak expiratory flow
PM: Particulate matter
PM2.5: Particulate matter <2.5 μm in diameter
PM10: Particulate matter <10 μm in diameter
pMDI: Pressurized metered dose inhalers
PD20: Provocative dose causing a 20% fall in FEV_1 or $FEF_{25-75\%}$
PD_{40}: Provocative dose causing a 40% increase in R20, R5-R20 or X5
R5: Resistance of the respiratory system at 5 Hertz
R20: Resistance of the respiratory system at 20 Hertz
R5-R20: Difference between R5 and R20
Rsr: Reactance of the respiratory system
Rp: Peripheral lung resistance
RV: Residual volume
Sacin: Ventilation heterogeneity generated in the acinar lung zone
SAD: Small airway dysfunction
SADT: Small Airway Dysfunction Tool
SBNT: Single breath nitrogen test
Scond: Ventilation heterogeneity generated in the conductive lung zone
SF6: Sulfur hexafluoride
Sgaw: Specific airway conductance
SGRQ: St. George Respiratory Questionnaire
SVC: Slow vital capacity
TLC: Total lung capacity
VC: Vital capacity
X5: Reactance of the respiratory system at 5 Hertz

NEDERLANDSE SAMENVATTING

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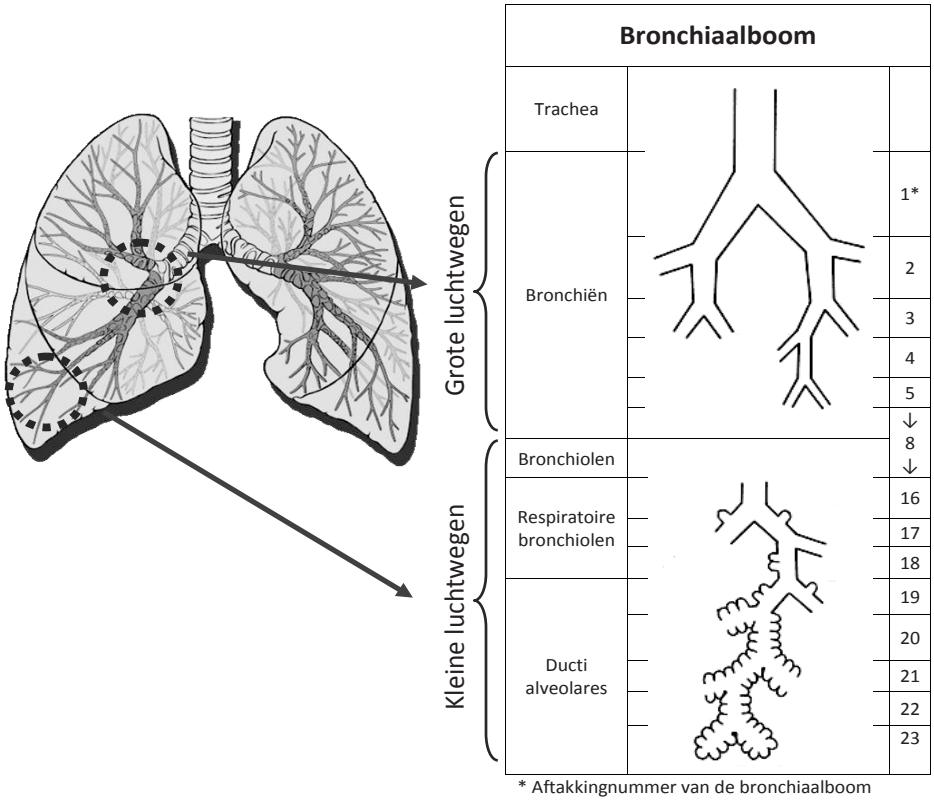
Astma is een chronische luchtwegaandoening die gekenmerkt wordt door ontsteking en een variabele luchtwegobstructie. Astmapatiënten hebben last van kortademigheid, piepen, hoesten en nachtelijke luchtwegklachten. Het ontstekingsproces van de luchtwegen kan zowel de grote als de kleine luchtwegen aantasten. De verdeling tussen grote en kleine luchtwegen is gewoonlijk gebaseerd op de interne diameter van de luchtweg, namelijk groter of kleiner dan 2 mm, wat overeenkomt met ongeveer de 8e aftakking van de bronchiaalboom (Figuur 1) (1,2). Er is lange tijd gedacht dat astma voornamelijk een ziekte was van de grote luchtwegen en dat de kleine luchtwegen niet belangrijk zouden zijn (3). Sinds een aantal jaren is er echter meer aandacht voor de kleine luchtwegen. Verschillende studies hebben aangetoond dat er in astma ook afwijkingen aan de kleine luchtwegen voorkomen (4). Bovendien lijken afwijkingen van de kleine luchtwegen samen te hangen met klinische verschijnselen, zoals plotselinge toename van luchtwegklachten (exacerbaties) en nachtelijke luchtwegklachten (5,6).



Figuur 1: Verdeling van de bronchiaal boom in grote en kleine luchtwegen (figuur naar Weibel(2))

Dit proefschrift onderzoekt de relatie tussen kleine luchtwegafwijkingen en symptomen ten gevolge van astma. We hebben een literatuurstudie gedaan naar deze relatie en vervolgens hebben we bij onze eigen astmapatiënten onderzocht of kleine luchtwegafwijkingen gerelateerd zijn aan astmasymptomen, astmacontrole, prikkels die luchtwegklachten veroorzaken en aan hyperreactiviteit van de luchtwegen. We zijn ook gestart met het ontwikkelen van nieuwe testen waarmee we afwijkingen aan de kleine luchtwegen kunnen vaststellen bij patiënten met astma.

De relatie tussen kleine luchtwegfunctie en klinische verschijnselen van astma

Om de relatie tussen de kleine luchtwegen en klinische verschijnselen van astma te onderzoeken zijn we gestart met een literatuurstudie. *Hoofdstuk 2* bevat een overzicht van alle artikelen die een relatie beschrijven tussen kleine luchtwegafwijkingen en klinische verschijnselen of symptomen. Na een systematische zoekstrategie zijn in totaal 80 artikelen geselecteerd. We vonden dat verminderde kleine luchtwegfunctie bij astma verband houdt met een slechtere controle van astma, frequenter optreden van exacerbaties, nachtelijke astmaklachten, inspanningsastma, ernstiger hyperreactiviteit van de luchtwegen en een late reactie op allergenen. Verder vonden we dat luchtvervuiling met deeltjes kleiner dan 2,5 µm in doorsnede gerelateerd is aan een slechtere astmacontrole en slechtere functie van de grote en kleine luchtwegen. Ook vonden we dat behandeling met kleine deeltjes inhalatie steroïden gerelateerd is aan een verbeterde kwaliteit van leven en astmacontrole. Samen laten deze artikelen zien dat de kleine luchtwegen voor astmapatiënten van belang zijn.

Het bewijs van de studies beschreven in hoofdstuk 2 is echter beperkt, aangezien een groot aantal van deze studies een klein aantal proefpersonen bestudeerde en in eerste instantie niet was opgezet om de relatie tussen de kleine luchtwegen en klinische symptomen te onderzoeken. Om deze redenen hebben we dit verband ook onderzocht in onze eigen studiepopulaties met twee verschillende benaderingen. In de eerste plaats hebben we de relatie tussen kleine luchtwegfunctie en klinische symptomen onderzocht in een populatie van 58 astmapatiënten waarvan de kleine luchtwegfunctie met verschillende testen was bepaald (hoofdstuk 3). In de tweede plaats hebben we een grote studiepopulatie onderzocht van 3.155 astmapatiënten. Van deze patiënten was de kleine luchtwegfunctie slechts in beperkte mate getest (hoofdstuk 4).

In ons onderzoek, beschreven in *hoofdstuk 3*, vonden we een relatie tussen verminderde functie van de kleine luchtwegen en symptomen van piepen en kortademigheid in de populatie van 58 milde tot ernstige astmapatiënten. We vonden geen andere verbanden tussen grote en kleine luchtwegfunctie en luchtwegklachten, nachtelijke klachten, inspanningsgerelateerde klachten, of klachten van bronchiale hyperreactiviteit. Bronchiale hyperreactiviteit is de overdreven reactie van luchtwegen om te vernauwen op aspecifieke prikkels, zoals bijvoorbeeld mist. De aanwezigheid van bronchiale hyperreactiviteit is een belangrijk kenmerk van astma en wordt gewoonlijk getest met een provocatietest. In ons onderzoek was de ernst van de bronchiale hyperreactiviteit, gemeten met een provocatietest, significant geassocieerd met een meer verstoorde kleine luchtwegfunctie. Bovendien was de toename in benauwdheid gedurende de test onafhankelijk geassocieerd met de afname in grote en kleine luchtwegfunctie.

In *hoofdstuk 4* gebruikten we gegevens van 3.155 astmapatiënten die bekend waren bij de huisarts. We vonden dat een slechtere astmacontrole significant gecorreleerd is met een verminderde grote en kleine luchtwegfunctie. Deze relaties waren echter niet sterk. Daarnaast analyseerden we of prikkels die luchtwegklachten veroorzaakten, gerelateerd zijn aan de grote en kleine luchtwegen. We toonden aan dat reacties op inspanning en mist geassocieerd zijn aan een slechtere grote luchtwegfunctie, terwijl de reactie op (huis)dieren gerelateerd is aan een slechtere kleine luchtwegfunctie.

De resultaten van hoofdstuk 3 en 4 laten zien samen dat een verminderde kleine luchtwegfunctie bijdraagt aan klinische verschijnselen van astma, zoals luchtwegklachten, astmacontrole, bronchiale hyperreactiviteit en ook het ontstaan van luchtwegklachten na blootstelling aan (huis) dieren. Het verband tussen kleine luchtwegfunctie en astmasymptomen en astmacontrole is niet sterk. Dit kan komen doordat de vragenlijsten die we hebben gebruikt niet gevoelig genoeg zijn om dit verband vast te stellen. De huidige vragenlijsten zijn namelijk ontwikkeld met gebruik van grote luchtweg parameters.

Een nieuwe kleine luchtweg vragenlijst

Hoofdstuk 5 beschrijft de eerste stap in de ontwikkeling van een nieuwe vragenlijst om specifiek afwijkingen van de kleine luchtwegen vast te stellen bij astmapatiënten. Hiervoor hebben we patiënten met en zonder kleine luchtwegafwijkingen individueel geïnterviewd over klachten, gewoontes, beperkingen en andere gezondheidsgerelateerde onderwerpen. Vervolgens werden de items bediscussieerd in kleine focusgroepen. Daarna hebben we 63 items geselecteerd voor de eerste versie van de nieuwe vragenlijst. Deze items zullen nog worden getest en gevalideerd in een grote astmapopulatie om tot de uiteindelijke vragenlijst te komen.

Een nieuwe provocatie test met adenosinepoeder

In *hoofdstuk 6* laten we de eerste testresultaten zien van een nieuwe provocatietest voor hyperreactiviteit uitgevoerd met adenosinepoeder. Deze provocatietest is een vernieuwing van de bestaande provocatietest die werkt met verneveling van adenosineoplossing (7). Voor dit onderzoek hebben 5 vrijwilligers met astma de reguliere provocatietest uitgevoerd met adenosineverneveling en de nieuwe provocatietest met adenosinepoeder. De nieuwe provocatietest met adenosinepoeder leidde tot luchtwegvernauwing bij alle vijf vrijwilligers. De test was goed uitvoerbaar en werd goed verdragen door alle vrijwilligers. Samengevat werd duidelijk dat de nieuwe droge poeder test met adenosine geschikt is om bronchiale hyperreactiviteit aan te tonen.

Vervolgens zijn we een nieuw onderzoek gestart met adenosinepoeder met als doel om astmapatiënten met kleine luchtwegvernauwing te kunnen identificeren op basis van de reactie op de provocatietest wat beschreven staat in *hoofdstuk 7*. Hiervoor probeerden we de kleine en grote luchtwegen selectief te prikkelen met behulp van kleine en grote deeltjes adenosine en met een snelle en langzame inhalatie van deze deeltjes. Op basis van eerder onderzoek naar

de depositie van verschillende deeltjes groottes verwachtten we dat langzaam geïnhaleerde kleine deeltjes dieper in de luchtwegen komen en meer neerslaan in de kleine luchtwegen dan de provocatietesten met grote deeltjes of snel geïnhaleerde kleine deeltjes (8). Daarom was onze hypothese dat de test met langzaam geïnhaleerde kleine deeltjes een verhoogde reactie zou veroorzaken in de kleine luchtwegen in vergelijking met de andere testen. Op deze manier hoopten we te kunnen differentiëren tussen mensen met en zonder kleine luchtwegvernauwing. Deze hypothese hebben we getoetst door elf patiënten met astma de verschillende adenosineprovocatietesten uit te laten voeren met kleine (2.7 µm) en grote deeltjes (6.0 µm) eenmaal met een langzame inhalatie (30 L/min) en eenmaal met een snelle (60 L/min) inhalatie. We toonden aan dat de provocatietesten in staat waren om een vernauwing van de grote en kleine luchtwegen te induceren. In tegenstelling tot onze hypothese vonden we echter niet dat de test met langzaam geïnhaleerde kleine deeltjes leidde tot een verhoogde respons in de kleine luchtwegen in vergelijking met de andere testen. Integendeel, alle adenosineprovocatietesten veroorzaakten een respons in de kleine luchtwegen. Zelfs de test met snel geïnhaleerde grote deeltjes was in staat om een respons in de kleine luchtwegen te induceren, terwijl we niet verwachten dat de adenosinedeeltjes de kleine luchtwegen bereiken. Deze resultaten stellen onze huidige visie over de onderliggende mechanismen van bronchiale hyperreactiviteit en van inhalatie met grote en kleine deeltjes ter discussie.

Luchtwegontsteking bij astma patiënten met overgewicht

In *hoofdstuk 8* bespreken we de aanwezigheid van luchtwegontsteking in astmapatiënten met obesitas. Met spreekt van obesitas als de BMI (Body Mass Index) groter is dan 30 kg/m^2. Luchtwegontsteking bij astmapatiënten kenmerkt zich door de betrokkenheid van zogenaamde eosinofiele granulocyten, een type witte bloedcellen. Een eerder onderzoek toonde aan dat astmapatiënten met obesitas, hoewel ze veel klachten ervaren, geen 'eosinofiele' ontsteking hebben (9). Deze bevindingen zijn gedaan in bloed en sputum (slijm uit de luchtwegen). Een recent onderzoek vindt echter dat er wel sprake is van eosinofiele ontsteking in patiënten met obesitas en ernstig astma. Er is zelfs sprake van verhoogde eosinofiele ontsteking in de obese patiënten met ernstig astma vergeleken met de niet-obese patiënten met ernstig astma. Dit onderzoek bestudeerde sputum en kleine stukjes weefsel uit de luchtwegen (luchtwegbiopten) in twee verschillende groepen van respectievelijk 131 en 45 patiënten met ernstig astma (10). Wij hebben geprobeerd of we deze bevindingen konden bevestigen in een groep van 147 patiënten met mild tot matig ernstig astma, waarvan zowel bloed, sputum als weefsel uit de luchtwegwand was geanalyseerd. We vonden dat astmapatiënten met obesitas een verhoogd aantal eosinofiele ontstekingscellen hebben in de luchtwegbiopten en een lager aantal eosinofiele ontstekingscellen in sputum vergeleken met de groep zonder obesitas. In bloed vonden we geen verschillen. Deze bevindingen bevestigen dat er bij astmapatiënten met obesitas sprake is van een eosinofiele ontsteking in de luchtwegen.

Conclusies en toekomstig onderzoek

De studies beschreven in dit proefschrift tonen aan dat afwijkingen in de kleine luchtwegen bijdragen aan de klinische expressie van astma. We vonden dat een slechtere kleine luchtwegfunctie gerelateerd is aan meer astmaklachten, slechtere astmacontrole, ernstigere bronchiale hyperreactiviteit en prikkels die luchtwegklachten veroorzaken. Dit betekent dat de kleine luchtwegen belangrijk zijn om verder te onderzoeken. We hebben een vragenlijst voor afwijkingen aan de kleine luchtwegen opgezet en deze zal in een vervolgonderzoek worden gevalideerd.

Verder hebben we een nieuwe provocatietest ontwikkeld met adenosine poeder. De resultaten laten zien dat deze test geschikt is om bronchiale hyperreactiviteit te meten en dat de test in staat is om een respons te veroorzaken in zowel de grote als de kleine luchtwegen. We vonden geen verschil tussen de verschillende testen (met kleine en grote deeltjes en langzame en snelle inhalatie) in reactie van de kleine luchtwegen. Om deze resultaten goed te kunnen begrijpen bevelen wij aan om in de toekomst de depositie van adenosine poeder in de grote en kleine luchtwegen goed in kaart te brengen.

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DANKWOORD

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