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Stenberg, Henning

2017

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Stenberg, H. (2017). *The asthmatic airway response – effects on physiology and biomarkers.* [Doctoral Thesis (compilation), Respiratory Medicine, Allergology, and Palliative Medicine]. Lund University: Faculty of Medicine.

Total number of authors:
1

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– effects on physiology and biomarkers

Henning Stenberg

Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
Department of Clinical Science, Lund BMC D12, 221 84 Lund	Date of issue September 29th, 2017
Author: Henning Stenberg	Sponsoring organization

Title and subtitle:
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Abstract

Asthma is a common airway inflammatory disease, associated with a variable degree of airway obstruction. It is a heterogeneous condition with large variations in severity and clinical presentation. Moreover, disease activity can vary from time to time. Taken together, these factors make the diagnosis, monitoring and treatment of asthma challenging. Peripheral airway inflammation has been shown to be of clinical importance in asthma, but the methods used to assess the airways in clinical practice today are not adequately designed to detect peripheral airway dysfunction. Exhaled breath temperature (EBT) has been suggested as a marker of airway inflammation, and club cell protein (CC16) has been suggested as a marker of airway epithelial dysfunction.

The aim of this thesis was to explore the different aspects of the asthmatic airway response, beyond the mechanisms that can be measured by the traditional physiology assessment, i.e. spirometry. To study the airway response, we used different types of airway provocations that are often used in asthma research and occasionally in clinical practice to confirm the diagnosis of asthma or assess airway inflammatory status.

Five different types of airway provocations were used to elicit airway responses in asthmatic subjects: exercise, eucapnic voluntary hyperventilation (EVH), methacholine challenge test (MCT), mannitol and inhaled allergen challenge. Responses to exercise, EVH and MCT were also tested in healthy controls for comparison. EBT was measured before and repeatedly after exercise, MCT, EVH and allergen challenge. Small airway physiology was measured with impulse oscillometry (IOS), inert gas washout (IGW) and body plethysmography before and repeatedly after the allergen challenge. Levels of CC16 were measured in several compartments before and after exercise and allergen challenge.

We found that EBT is increased after exercise, EVH, MCT and during the late phase of the allergic airway response. The increase in EBT correlated to the drop in FEV_1 after exercise and EVH, but not after the MCT, indicating an airway epithelial effect. EBT did not differ between asthmatic subjects and healthy controls before or after provocations, suggesting that an increase in EBT is a normal physiological response rather than a specific sign of airway inflammation. However, the duration of the increase in EBT was longer in asthmatic subjects with a more pronounced airway response after exercise, indicative of a sustaining effect of airway inflammation. CC16 is increased in plasma after exercise and during the early phase of the allergic response. It is also increased in urine after exercise but not after the allergen challenge, indicating differences in renal excretion after the two provocations. A late phase response after the allergen challenge was associated with a significant small airway dysfunction, consisting of both increased airway resistance and ventilation heterogeneity.

Our results have increased our understanding of effects on the airway physiology, the respiratory epithelium and small airway dysfunction by different types of stimuli that are known to elicit asthmatic airway responses. We have explored effects on physiology parameters and biomarkers that may be used in research and care of pulmonary diseases in the near future.

Key words: asthma, exhaled breath temperature, exercise, allergen challenge, small airways, airway provocations, spirometry, impulse oscillometry, inert gas washout, body plethysmography, club cell protein (CC16).

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English

ISSN and key title: 1652-8220, Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:122	ISBN 978-7619-505-5

Recipient's notes	Number of pages: 81	Price
	Security classification	

Signature   Date: August 21, 2017

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Front cover: a colorized electron microscope image of miscellaneous pollen magnified by approximately x500, by Dartmouth Electron Microscope Facility, Dartmouth College.

Till Hugo och Johanna

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Contents

List of papers

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

I. Svensson H[*], Nilsson D[*], Bjermer L, Tufvesson E: Exhaled breath temperature after exercise in asthmatics and healthy controls. *Respiration* 2012;84:283-290.

    [*]Shared authorship.

II. Tufvesson E, Svensson H, Ankerst J, Bjermer L: Increase of club cell (Clara) protein (CC16) in plasma and urine after exercise challenge in asthmatics and healthy controls, and correlations to exhaled breath temperature and exhaled nitric oxide. *Respiratory Medicine* 2013;107:1675-1681.

III. Svensson H, Bjermer L, Tufvesson E: Exhaled breath temperature in asthmatics and controls after eucapnic voluntary hyperventilation and a methacholine challenge test. *Respiration* 2014;87:149-157.

IV. Stenberg H, Diamant Z, Ankerst J, Bjermer L, Tufvesson E. Small airways involvement in the late allergic response in asthma. Submitted to *Clinical & Experimental Allergy* July 2017

V. Stenberg H, Wadelius E, Moitra S, Åberg I, Ankerst J, Diamant Z, Bjermer L, Tufvesson E. Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics. Submitted to *Biomarkers* August 2017

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Other papers

Additional papers, not included in the thesis:

Ekstedt S, Säfholm J, Stenberg H, Tufvesson E, Bjermer L, Kumlien Georén S, Cardell LO. Role of neutrophils in the allergic asthma response. Submitted to *Science Translational Medicine*.

Weitoft M, Rolandsson Enes S, Tykesson E, Roschupkina T, Tufvesson E, Stenberg H, Rydell-Törmänen K, Bjermer L, Westergren-Thorsson G. Circulating fibrocytes and possible recruitment factors in a human allergen provocation study. Manuscript in progress.

Abbreviations

ACT	Asthma control test
AHR	Airway hyper-responsiveness
AX	Area of integrated reactance between 5 Hz and resonant frequency
BAL	Broncho-alveolar lavage
CC16	Club cell 16 kDa protein
EAR	Early allergic response
EBT	Exhaled breath temperature
EIA	Exercise-induced asthma
EIB	Exercise-induced bronchoconstriction
EVH	Eucapnic voluntary hyperventilation
FDR	Frequency dependence of resistance
$F_E NO$	Fractional nitric oxide concentration in exhaled breath
FEV_1	Forced expiratory volume in 1 second
FOT	Forced oscillation technique
FRC	Functional residual capacity
F_{res}	Resonant frequency
FVC	Forced vital capacity
HDM	House dust mite
IC	Inspiratory capacity
ICS	Inhaled corticosteroids
IGW	Inert gas washout
IOS	Impulse oscillometry
LABA	Long-acting β_2-agonist

LAR	Late allergic response
LCI	Lung clearance index
MBW	Multiple breath washout
MCT	Methacholine challenge test
PD_X	Cumulative dose required to decrease FEV_1 by X%
RV	Residual volume
R5	Resistance at 5 Hz
R20	Resistance at 20 Hz
SABA	Short-acting β_2-agonist
S_{acin}	Ventilation heterogeneity of intra-acinar airways
S_{cond}	Ventilation heterogeneity of conducting airways
Sn_{III}	Concentration normalized slope of phase III
TLC	Total lung capacity
V_T	Tidal volume
X5	Reactance at 5 Hz

Introduction

The subjects of this thesis are asthma and airway provocations. Asthma is a common but heterogeneous disease of the airways, with large variations in clinical, physiological and histopathological features. The severity of asthmatic disease is also varied, to say the least, ranging from mild symptoms with minimal impact on daily life to uncontrolled inflammation with devastating long-term effects and even fatal attacks. Although asthma has been studied extensively for a long time, our knowledge on why some patients do not reach acceptable clinical control despite intensified treatment is limited. Furthermore, we do not know how to correctly phenotype our patients with asthma and how to individualize treatment according to their specific features and how to adjust it properly over time as the severity of the disease fluctuate or progress. Even the seemingly simple task of making the diagnosis of asthma is still a challenge in many situations. Symptoms and classical clinical features are not always present from day to day, and the diagnostic methods commonly used are not adequately designed to detect all aspects of asthmatic disease.

Airway provocations are used both in every day clinical practice and in pulmonary research. It is both a way to elicit a response to confirm a diagnosis suspected by the clinician, and to mimic asthmatic exacerbation for purposes of studying the response.

After establishing the diagnosis of asthma in a certain patient, it is still necessary to assess the airways on a regular basis in order to adjust treatment as the inflammatory activity fluctuates over time. There is consequently a need for easily available methods to monitor the asthmatic disease, ideally managed primarily by the patient with support from the health care provider when needed.

Asthma has previously been viewed as a disease of the large airways, possibly due to the fact that commonly used methods have only been able to assess the large airways. More recently, however, there has been numerous reports on small airway disease in asthma, and there is an ongoing discussion about its importance and how it influences what type of symptoms and what severity the disease will present. In the future, we will most likely evaluate not only large airway pathology but also the rest of the respiratory tract, to characterize our patients with asthma.

This thesis will address different means to measure asthmatic airway responses in relation to airway provocations. The studies presented here explore several different methods and compare the outcomes in asthmatic and non-asthmatic subjects, and also compare results derived from different methods. Some methods used are well established in clinical practice while others are recently developed and are just investigated on an experimental stage. The aim of this thesis is to aid in understanding the asthmatic airway responses, and to increase our knowledge on the methods we may use to diagnose and follow pulmonary diseases in the future.

Background

Asthma

The Global Initiative for Asthma (GINA) suggests the following definition of asthma:

> "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation."[1]

Asthma is estimated to affect approximately 300 million people across all age groups worldwide. It imposes a huge economic burden on health care systems and society in general. As can be understood from the definition stated above, the diagnosis and monitoring of asthma is not always easy, since symptoms and clinical findings may vary between two individuals that both have asthma and from day to day in one single individual. There are however some common features of asthma that can be utilized to improve our assessment. One such feature is the airway hyper-responsiveness (AHR), an increased tendency of the airway smooth muscle cells to react to different types of stimuli. Providing such stimuli, the airway provocations have been developed both as research tools to study the asthmatic airway response, and as a way to improve the diagnostic accuracy in the clinical setting. The diagnosis of asthma can be based on a typical medical history and a pulmonary function test confirming a variable expiratory airflow limitation (with or without the use of an airway provocation) or, in cases where testing is unable to identify the variability in airflow limitation but the medical history is typical for asthma and other diagnoses are unlikely, on a positive response to treatment.

Phenotypes and treatment

Not long ago, when therapeutic options were few, asthma was regarded as a single disease. However, with the introduction of corticosteroids and later targeted immunomodulatory treatment, the need of assessing the specific triggering factors,

pathophysiology and immunological type of disease in each individual has become apparent. In an attempt to categorize the many forms of asthma, a number of different phenotypes with a set of shared clinical or pathophysiological features are often used. Commonly reported phenotypes include early onset allergic asthma, non-allergic asthma, late onset asthma, exercise-induced asthma, aspirin exacerbated respiratory disease and obesity related asthma[2]. The most common phenotype is the early onset allergic asthma[3]. Several categorizations of phenotypes have been suggested over time, and today a frequently used classification is based on the molecular pattern of inflammatory mediator expression, i.e. the degree of a T-helper type 2 (T_H2) inflammation[4] (Fig. 1). More recently, the concept "endotypes" has been introduced as an alternative way to view the different aspects of asthma, being based on genetic variants and biomarkers in blood and taking into consideration the driving factor behind the disease. The work on endotypes is ongoing, and no endotype has yet been fully characterized. However, with new discoveries and studies on the effect of immunotherapy in different patients, many believe that we will see a gradual transition from the phenotypes towards the endotypes in asthma care and research.
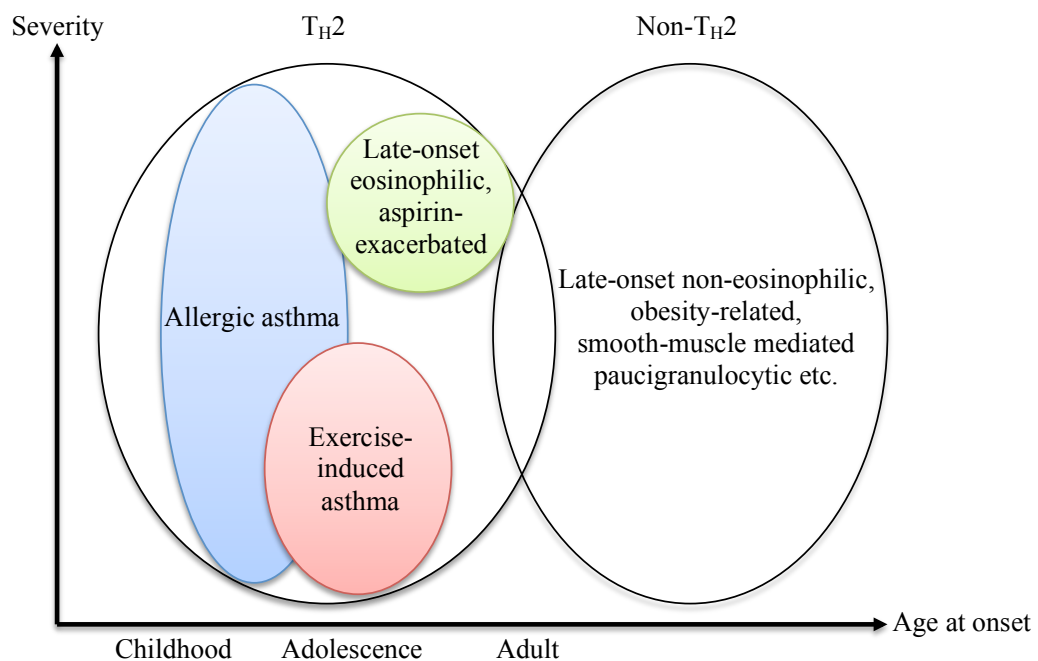

Figure 1. Asthma phenotypes.
Some of the commonly reported asthmatic phenotypes, divided into the two main categories of T_H2-driven and non-T_H2-driven. The circles also show the severity and age at onset that are usually seen. Adapted from Wenzel SE[5].

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Despite the development of advanced immunotherapy, the vast majority of patients with asthma are still treated with inhaled corticosteroids (ICS), the basis of asthma maintenance therapy. ICS has a wide-ranging inhibitory effect on several parts of the immune system, e.g. T-cells and eosinophils. The effect on neutrophils is however limited, and some patients do not respond to ICS. Taking other factors such as low adherence to treatment or comorbidities into account, the consequence is that only about 50% of patients with asthma reach an acceptable clinical control[6].

The other foundation of asthma treatment apart from ICS is the use of β_2-agonists. There are short-acting β_2-agonists (SABA) used as rescue medication, and long acting β_2-agonists (LABA) used as a symptom reliever over the course of the day. β_2-agonists exert their effect primarily on airway smooth muscle cells, but may also inhibit bronchoconstriction through effects on mast cells and sensory nerves, and possibly prevent plasma exudation into the airway lumen[7].

Allergic asthma and rhinitis

The genetic predisposition associated with a tendency to develop immunoglobulin (Ig) E antibodies when exposed to certain allergens, i.e. proteins that can cause an allergic reaction, is called atopy. Although there are many non-atopic phenotypes of asthma, there is a strong link between allergic sensitization and the development of asthma[8, 9]. Allergic asthma is often preceded and paralleled by allergic symptoms of the upper airways, i.e. allergic rhinitis[10]. In fact, allergic rhinitis and asthma are sometimes viewed as one and the same disease with the only difference being the anatomical distribution[11].

Not all patients with allergic rhinitis develop asthma though, and the cause of progression from upper to lower airway inflammation has been under investigation. Patients with concomitant asthma and allergic rhinitis have been shown to have involvement of peripheral airways, with increased peripheral resistance compared to those with allergic rhinitis only (with or without bronchial hyper-responsiveness to methacholine)[12]. The number of IgE-receptor positive alveolar mast cells is also increased in asthmatics when compared to those with allergic rhinitis[13]. This indicates that the tendency of developing asthma could be related to the degree of peripheral airway inflammation.

Exercise-induced asthma

A common feature of asthmatic disease is an increased responsiveness of the airways to exercise, often leading to symptoms of abnormal dyspnea, cough and

increased airway mucus production during and after exercise[14]. Bronchoconstriction as a response to exercise has also been documented in some individuals without any asthma or any evident symptoms during exercise[15], and might be associated with an increased risk of developing asthma later in life[16]. The terms exercise-induced asthma (EIA) and exercise-induced bronchoconstriction (EIB) are used to describe symptomatic and non-symptomatic bronchoconstriction, respectively, arising during or directly after exercise. Both EIA and EIB are found in increased prevalence among elite athletes, especially those practicing endurance sports[17]. The proposed explanation for this is that their airways are subject to repeated epithelial injury and repair processes due to frequent high-intensity hyperventilation[18].

The airways – structure and function

The portion of the respiratory system below the larynx is a complex, somewhat asymmetrical structure with a range of different types of airways, from the large cartilaginous trachea with an inner diameter of about 2.5 cm to the microscopic alveolar ducts containing the alveolar openings. Dichotomous branching gives rise to approximately 23 generations of airways. Two different systems of categorization are used to separate and name the different compartments of the lung. The first principle is the division of airways into central and peripheral (or large and small), where by convention the first 8 generations constitute the central part and have inner diameters of >2 mm and walls containing cartilage. The peripheral airways, composed of non-cartilaginous bronchioles, have inner diameters of <2 mm. The second principle is the division of airways into conducting and respiratory zones, based on where the gas exchange takes place. The border between these two compartments is located where the branching of the terminal bronchioles results in two (or more) respiratory bronchioles, around the 16th generation.

The airway tree is supplied with oxygenated blood from the tracheobronchial vasculature, which stems directly from the aorta and the carotid arteries, and runs along the bronchi and bronchioles approximately down to the level of the transition between conducting and respiratory airways. The tracheobronchial vasculature forms a capillary network that runs just below the epithelium, and sinuses that lies deeper and functions as a distensible capacitance system. A minor portion of the blood is carried back to the heart through the bronchial veins, while the largest remaining part flows to the pulmonary veins. Initially viewed as a source of oxygen and nutrition only, the tracheobronchial vasculature has later also been found to be of importance in delivering cells, mediators and hormones

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from the blood stream, removing inflammatory mediators and cells resident in the airway walls, affecting the distribution and clearance of inhaled particles and drugs, and, last but not least, conditioning the ventilated air by exchanging heat and water[19].

Peripheral airways

The peripheral airways represent about 95% of the total lung volume. However, due to the dramatic increase in total airway cross-sectional area for each generation of branching, only 10-20% of the total airway resistance to flow in healthy adult lungs originates from the peripheral airways[20]. Therefore, large changes in peripheral airway physiology can occur before they are detected as a reduction in flow by the standard lung function test of spirometry. In fact, the peripheral airway compartment has often been termed "the silent lung zone" due to its inaccessibility. Invasive methods, such as transbronchial biopsies obtained during bronchoscopy, can provide evidence of peripheral airway inflammation, but are simply not feasible in everyday clinical practice.

Several studies have shown links between a peripheral airway inflammation/dysfunction and impaired asthma control[21, 22], EIB[23], higher frequency of exacerbations[24, 25] and nocturnal symptoms[26, 27]. Andersson et al showed that inflammatory patterns in biopsies from central airways did not differ between asthmatic subjects and subjects with allergic rhinitis only, while those taken from peripheral airways contained a markedly increased number of IgE-receptor positive mast cells in asthmatic subjects[13]. Subjects with an increase in peripheral airway resistance also had an increase in airway hyper-responsiveness to methacholine in a study by Beretta et al[28].

Sensitization to so-called perennial (present at all seasons of the year) allergens, i.e. house dust mite (HDM) and animal allergens, seems to increase the risk of developing AHR[29] and asthma[30, 31], or presenting with wheeze in the event of a rhinovirus infection[32]. Sears et al showed in a longitudinal study of a birth cohort of 714 children that sensitization to HDM or cat dander, but not grass pollen, was an independent risk factor for developing asthma[33]. Apart from a potentially longer time of exposure during the year, an explanation for this link between sensitization to perennial allergens and asthma development might be attributable to the fact that perennial allergen particles are smaller in size and deposit more easily in the peripheral airways. While common pollen particles have the size of about 20-30 μm in diameter and approximately 99% of them are caught in the nasopharynx during breathing[34], a large proportion of the perennial allergen particles are <5 μm[35] and has a greater potential to deposit peripherally. As demonstrated by Zeidler et al, exposure to natural cat allergen results in a

pronounced peripheral reaction with increased amount of air trapping as measured by high-resolution computed tomography, even when changes in parameters reflecting large airway physiology are modest[36].

Despite several studies assessing the pathophysiology of small airways, no biomarker or physiological parameter has yet been accepted as the "golden standard" of small airway disease in asthma.

Airway provocations

Airway provocations are used to produce an airway response in a controlled environment. They can be used as a diagnostic tool and in research of pulmonary disease. There are a number of different airway provocations that are divided into two groups, the indirect and the direct tests, based on their mechanism of action (Fig. 2). While the indirect tests initiate processes that lead to a cellular response with release of inflammatory mediators, the direct tests target the smooth muscle cells directly, thus not requiring an inflammatory cellular presence/activity.

Since the indirect tests rely on a pre-established presence of inflammatory cells in the asthmatic airways, they are considered more specific than the direct tests[37]. Their sensitivity in clinical assessment of asthma is however lower.
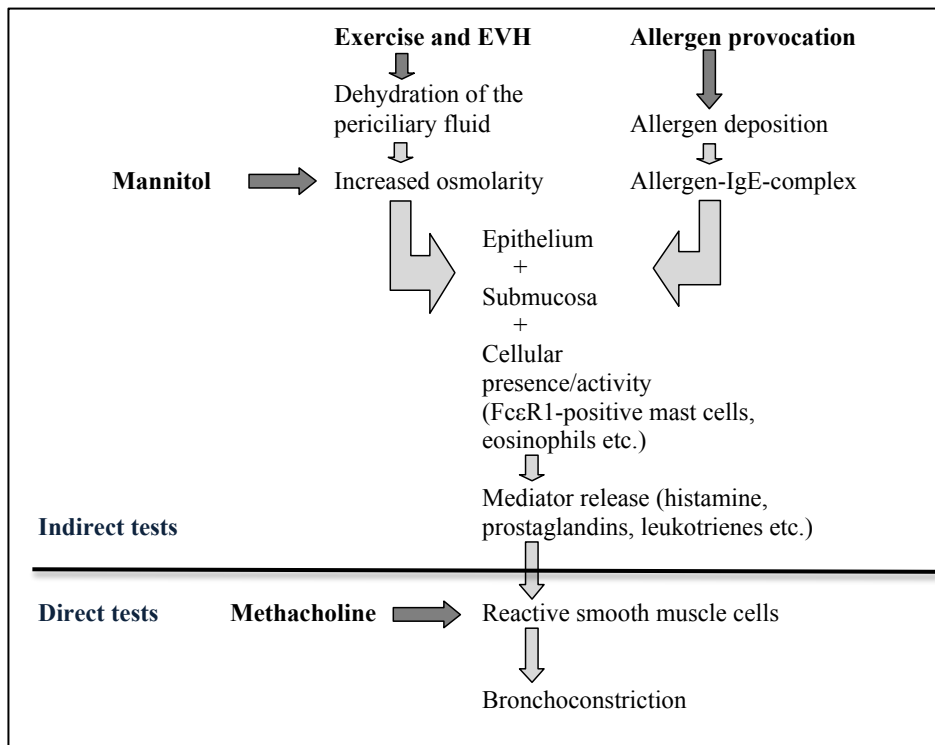
Figure 2. Airway provocations.
The current view on the mechanisms of the indirect and direct airway provocations presented in this thesis. There are also a few additional tests that are not described here (e.g. histamine, hypertonic saline and adenosine monophosphate). EVH = eucapnic voluntary hyperventilation. Adapted from O'Byrne et al[38].

Exercise challenge test

The standardized exercise challenge test is an indirect test, often used in research but also in clinical practice to confirm EIB in patients with exercise-induced respiratory symptoms. The current hypothesis is that exercise induces bronchoconstriction by evaporation of the airway surface fluid due to hyperventilation, causing increased osmolarity and a release of inflammatory mediators[39]. At a lower intensity of exercise, the air is conditioned to a certain temperature and humidity mainly by the most proximal generations of airways[40]. As the intensity of exercise (and ventilation) increases, the fluid volume of the central airways is insufficient to condition the air and smaller airways are recruited, thus becoming vulnerable to epithelial dehydration injury[41]. The repair process following that injury may lead to airway smooth muscle cells being exposed to plasma and cell mediators altering their structure and function[18].

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Eucapnic voluntary hyperventilation

The idea behind eucapnic voluntary hyperventilation (EVH) is to mimic the hyperventilation created by exercise, but without the actual need of exercising. By adding extra CO_2 to the inhaled gas, hyperventilation can be sustained for a prolonged period of time without the risk of hypocapnia. The supposed mechanism for inducing bronchoconstriction is thus the same as for the exercise challenge test. A lower humidity of the inhaled gas will lead to more dehydration of the epithelial lining fluid, and the magnitude of the response is proportional to the amount of water loss from the mucosal surface[42]. A vulnerability of peripheral airways to hyperventilation has been shown in experimental animal studies[43], and peripheral airway resistance and inflammatory markers were increased in dogs after repeated hyperventilation[44].

Mannitol challenge test

Mannitol is a sugar alcohol that can be inhaled as a dry powder to cause bronchoconstriction in predisposed individuals. The mechanism for this is believed to be a release of mediators in response to increased osmolarity of the airway surface liquid[45], similar to exercise-induced bronchoconstriction and bronchoconstriction due to EVH. Mast cells are believed to be mainly responsible as the source of mediator release, and inhibition of mast cells by disodium cromoglycate reduced the reaction to mannitol in a study by Brannan *et al*[46].

Although both the mannitol challenge test and the EVH induce bronchoconstriction in the same principal way as exercise, the question of whether either or both of these tests can replace an exercise challenge test in diagnosing exercise-induced bronchoconstriction is controversial[47, 48].

Methacholine challenge test

Methacholine is a non-selective muscarinic receptor agonist, used as a direct airway provocation upon inhalation. Three subtypes of muscarinic receptors have been identified in human airways: M1, M2 and M3[49]. Although the exact function of each of the muscarinic receptors is still not fully known, activation of the M1-receptor is believed to enhance cholinergic transmission while M2 acts as a negative feedback auto-regulator to inhibit the release of acetylcholine. M3 is located on smooth muscle cells and is believed to mediate bronchoconstriction. Muscarinic receptors are found at all levels in human lungs but the concentration is higher in central airways[50], and anti-cholinergic compounds seem to dilate large airways primarily while β_2-agonists has a relatively greater effect on

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peripheral airways[51]. Activation of the muscarinic receptors is also believed to cause vasodilation at all levels of the tracheobronchial circulation[52]. The methacholine challenge test (MCT) is frequently used to diagnose AHR, not only in research but also in clinical settings.

Inhaled allergen challenge

Allergen bronchoprovocation is used as a model of allergic asthma, mimicking both the acute asthmatic attack and some of the long-term chronic features of asthmatic disease. Inhalation of allergens leads to crosslinking of membrane bound IgE-antibodies on mast cells, releasing preformed inflammatory mediators such as histamine, leukotrienes and prostaglandins. The immediate bronchoconstriction is called the early allergic response (EAR). After the EAR has resolved (completely or partially), a second decline in lung function is seen in approximately 50% of subjects[53] after a delay of around four hours. This represents the inflammatory airway reaction of the adaptive immune system, and the phenomenon is called the late allergic response (LAR). It is still unclear if the occurrence of a LAR can be predicted in any way, and it seems that developing a LAR is not simply a question of disease severity. Although a large retrospective study on allergen provocations revealed that some subjects who had a LAR were more reactive to methacholine at baseline and had a larger response during the EAR, these results varied depending on the type of allergen used for the provocation[54]. During the LAR, activated Th2-cells release interleukin (IL)-4, IL-5, IL-13 and eotaxin[55], leading to eosinophil activation, a switch to IgE isotype by B cells and airway smooth muscle cell proliferation[56]. The LAR is thought to be clinically and pathophysiologically similar to the naturally occurring asthma, and the capacity to suppress the LAR experimentally has been used to evaluate the efficacy of novel asthma therapies[57].

As described before, the size of the inhaled allergen particles affects how the allergen is deposited in the airways, and deposition will differ between different types of pollen and other allergens[34, 58]. Repeated inhalations of the same allergen by the same subject but with different particle sizes may lead to varying types of airway responses[59]. Pollen particles are generally larger than for example cat or house dust mite allergens, and are therefore deposited mainly in the upper airways. For example, the mean size of birch pollen (*Betula)* particles is 25 μm[60], while a large proportion of cat allergen (*Fel d 1*) found in homes with a cat is smaller than 5 μm in diameter[61]. A well-documented phenomenon is an increased number of asthmatic exacerbations after thunderstorms occurring during pollen season, called "thunderstorm asthma". A possible explanation for this is that the pollen particles become electrically charged and are fragmented into smaller particles, which leads to a more peripheral deposition[62]. In our studies, allergens were delivered by a Spira Elektro 2 nebulizer, delivering an aerosol of lyophilized allergen extract with

an aerodynamic mass median diameter of approximately 1.6 μm[63]. Inhalation of a solution of allergen extract rather than intact particles ensured that the aerosol diameter was kept fairly consistent regardless of type of allergen. Had natural exposure to pollen or animal allergens been used instead, the deposition and resulting response could have differed.

Lung function tests

Spirometry

Spirometry is the standard method for assessing lung function. The most commonly reported parameters are forced expiratory volume in 1 second (FEV_1) and peak expiratory flow (PEF). Both being measures of flow, they are dependent on the resistance of the airways. It is widely accepted that FEV_1 does not reflect the resistance of the entire airway tree, but is rather determined by properties of those airways where resistance is highest. Since the resistance of central airways overshadows that of peripheral airways, changes in the latter may be missed by FEV_1 and PEF. Other parameters have been suggested to better reflect small airway physiology, including the forced expiratory flow between 25% and 75% of the expired volume (FEF_{25-75}) and the forced vital capacity (FVC). FEF_{25-75} has however been shown to be heavily affected by central airway obstruction and changes in lung volumes[64], and lacking correlations to indices of small airway inflammation found in biopsies from asthmatic subjects[65]. Besides, when distal airway obstruction is heterogeneous, increased flow through non-obstructed airways will conceal any change in spirometric flow parameters[66]. A decrease in FVC might however be an indirect sign of increased air trapping, a feature of small airway dysfunction. Studies evaluating treatment with extra-fine formulations of ICS compared to non-extra-fine compounds have shown a greater increase in FVC (but not in FEV_1 or PEF)[67, 68]. Although future research might clarify the role of FVC in assessing small airway dysfunction, the present view on spirometry is however that it is insensitive to subtle changes and not specific to small airway pathophysiology[69].

Body plethysmography

Since spirometry cannot estimate the volume of air that remains in the lungs after a maximal exhalation, i.e. the residual volume (RV), other methods are needed to complete the picture regarding total lung capacity (TLC) and the relative

contributions of each of the different lung volumes (Fig. 3). Body plethysmography is a widely available method used to acquire this information, along with measures of airway resistance. It is considered relatively easy to perform, has good reproducibility and is sensitive to early changes in lung volumes and airway resistance. One drawback is however that plethysmography cannot differ between resistance of small and large airways.
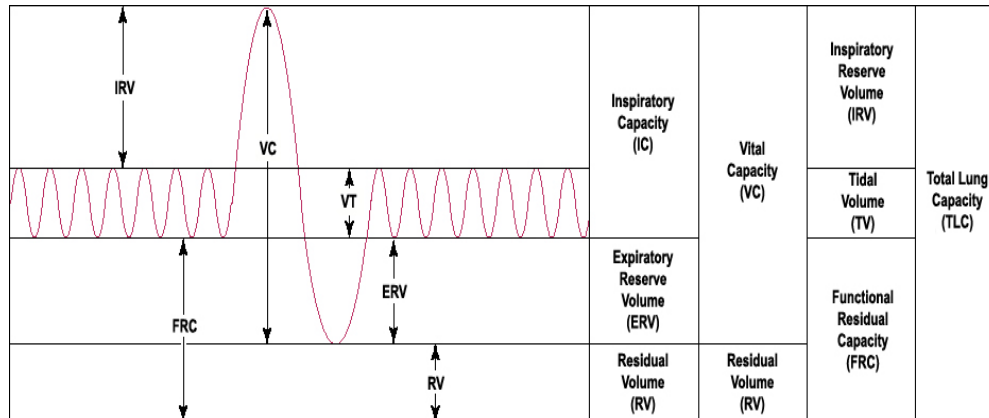
Figure 3. Lung volumes.
A graphic representation of the different lung volumes. The red line illustrates a few breaths of tidal breathing, followed by a full inhalation, a full exhalation and back to tidal breathing again. The different volumes and capacities (a capacity is the sum of several volumes) are denoted with abbreviations explained on the right side of the figure.

The theoretical background for plethysmographic measurements is based on Boyle's law, which states that when a constant mass of gas is compressed or decompressed under isothermal conditions, the volume of the gas will decrease or increase so that the product of volume and pressure at any given moment will always be constant. In practice, pressure is measured at the mouth of the subject and in a closed chamber where the subject is seated. During the measurement of thoracic gas volume, a shutter valve at the mouthpiece is temporarily closed, occluding the flow of air during breathing. As the subject inhales against the closed shutter valve, the thorax expands and the pressure at the mouth decreases while the volume of gas in the chamber (and outside of the subject's thorax) decreases and the pressure in the chamber increases. By measuring three factors (mouth pressure, chamber gas volume and chamber pressure), we can calculate the fourth factor in the equation, i.e. the thoracic gas volume. The airway resistance is measured while the subject is breathing tidally with the shutter valve open, by calculation of pressure and flow at the mouthpiece.

Body plethysmography is also useful for detecting hyperinflation, defined as an abnormally large persisting lung volume after full exhalation. This happens

because a preceding airway narrowing leads to earlier and more widespread closing of airways during an exhalation with dynamic compression. The resulting air trapping is seen as an increase in RV in relation to TLC. The increase in RV/TLC can be seen in asthmatic subjects before any abnormalities are found on a regular spirometry[70].

Carbon monoxide diffusion capacity

The estimation of lung volumes by gas dilution techniques was first developed more than 200 years ago. Different gases have been studied since, but modern standard methods use methane (CH_4) or helium to determine lung volumes, and carbon monoxide (CO) to measure the diffusion capacity of the lungs. CH_4 is not absorbed by the body, so with a known amount of CH_4 inhaled by the subject, the concentration of exhaled CH_4 will allow for calculation of the dilution and thereby the lung volumes. The absorption of CO from inhaled air is dependent on all aspects of gas transport, such as ventilation, the condition of the alveolar-capillary membrane, cardiac output and hemoglobin levels. One limitation of the gas dilution technique when it comes to measuring lung volumes is that only parts of the lung that communicate directly with the surrounding air will participate in the measurement, meaning that in for example severe obstructive disorders with air trapping, lung volumes will be underestimated. Therefore, results from gas dilution measurements and body plethysmography can differ when measured in the same patient.

Impulse oscillometry system

The forced oscillation technique (FOT) is a method used to measure respiratory mechanics by applying oscillations of pressure at the mouth superimposed on tidal breathing. Its non-invasiveness and the fact that minimal cooperation is required makes FOT a promising lung function test in many settings, including the pediatric range and in other patients who have difficulties performing spirometry.

The impulse oscillometry system (IOS) is a commercially available device utilizing the FOT. IOS uses a tidal oscillation volume of approximately 10 ml with frequencies ranging from 5 Hz to 35 Hz. Low oscillation frequencies have been shown to transmit more distally in the lung while high frequencies are blocked in intermediate-sized airways, a finding that has led to the concept that IOS can be used to isolate the resistance of peripheral airways from airway resistance in general[71].

In contrast to spirometry, IOS does not require any deep inspirations or forced expirations, and is thus performed under close to physiologic conditions. This is important, seeing as airway caliber can change with breathing pattern in different fashions in healthy and asthmatic subjects. Deep inspirations can cause dilatation of constricted airways[72, 73, 74], although there is a greater tendency of asthmatic airways to immediately return to a narrowed state, possibly due to an altered mechanical behavior of smooth muscle cells[75].

IOS measures respiratory impedance, which consists of resistance and reactance. While resistance is simply the part of the pressure-flow relation in-phase with flow, reactance is a complex quantity that is related to the parts of the pressure oscillations that are out-of-phase with flow. Reactance is the sum of two components, elastance and inertance. Elastance can be viewed as the "back pressure" created after some of the flow has already occurred, i.e. elastance is *delayed* relative to flow. In contrast, inertance of the airways is the opposing force that builds up *before* flow takes place[76]. By convention, reactant forces are designated a positive sign when pressure changes precede flow changes (and vice versa), meaning that inert forces have a positive sign and elastic forces have a negative sign.

Commonly reported parameters of resistance include R5 and R20, meaning the resistance to oscillations at 5 Hz and 20 Hz, respectively. Reactance is usually reported as the reactance at 5 Hz (X5), the resonant frequency (F_{res}), which is the frequency where elastic and inert forces are equal, and the integrated area of low frequency reactance from X5 to F_{res} (AX) (Fig. 4).
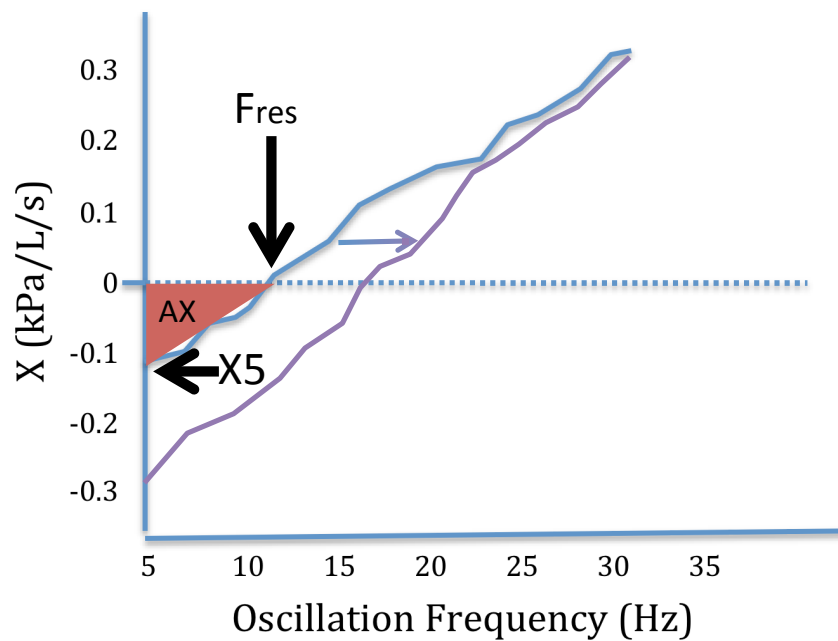
Figure 4. Airway reactance.
An example of airway reactance (X) at different oscillation frequencies. The reactance at 5 Hz (X5) is negative in sign, since the elastic forces are dominating at low frequencies. As the oscillation frequency increases, inert forces become increasingly important and the reactance becomes gradually more positive in sign. The frequency where elastic and inert forces are equal (and reactance is zero) is termed the resonant frequency (F_{res}). AX is the area of integrated reactance at frequencies between X5 and F_{res}. With increased airway stiffness, elastance increases (denoted by the arrow pointing from the blue to the purple line) with an increase in F_{res} and AX, and a decrease in X5 (towards more negative values).

In healthy lungs, resistance is fairly equal regardless of the oscillation frequency. In airway disease, however, a frequency dependence of resistance (FDR) is often visible, meaning that the resistance to low frequency oscillations is increased more than the resistance to high frequency oscillations. Since low frequency waves are transmitted more peripherally, FDR is often interpreted as a peripheral airway resistance. Another possible explanation is however also a resistance that is heterogeneously distributed among parallel airways of the same generation. When air flows into the lungs, expansion occurs through different parallel pathways simultaneously. The time required to expand the volume of every pathway, the time constant, is a product of resistance and compliance. In asthmatic disease, some of the airways will display increased resistance, and this is usually unevenly distributed[77]. With heterogeneous ventilation, an increase in breathing frequency will lead to an apparent overall decrease in resistance and compliance since pathways with time constants that exceed the breathing frequency will no longer participate in volume changes. Consequently, heterogeneity among parallel

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pathways as well as along a single pathway longitudinally, will result in a frequency dependent of resistance[71]. Models of airway resistance have shown that heterogeneous narrowing with near closing of some of the large airways raises resistance more than the same degree of narrowing distributed more homogenously[78, 79]. With airway closure the peripheral airways become less accessible by forced oscillations, and if closure is heterogeneous flow is directed into the airway walls leading to an increase in elastance, i.e. reactance becomes more negative in the low frequency range.

Inert gas washout

The inert gas washout (IGW) technique is designed to detect ventilation heterogeneity, and was first tested over 70 years ago[80]. The technique is based on the principles of gas transport and mixing in the lung. In the conducting airways, flow velocity is high and gas transport is mainly a result of convection. As the airways divide, giving rise to increasing total airway cross-sectional diameter, flow decreases rapidly and at the level of transition from conducting to respiratory airways, diffusion becomes increasingly important as a driving factor in gas transport. At the level where convection and diffusion are equal in force (corresponding approximately to the 17th generation for O_2 and similarly sized molecules in healthy airways) a "diffusion-convection front" is produced[81].

IGW utilizes analysis of exhaled gas composure, determining the concentration of an inert gas, i.e. a gas that is not taken up into the bloodstream. Nitrogen (N_2) was first used as the inert marker gas and later other molecules of different sizes were introduced, such as helium and sulphur hexafluoride. N_2 is present in ambient air at a concentration of approximately 78%, so in order to analyze how N_2 is exhaled from the lungs, the subject must "wash out" the ambient N_2 by inhaling 100% O_2. When inhalation of 100% O_2 is followed by an exhalation, the concentration of exhaled N_2 will follow a certain pattern with three (and sometimes a fourth) different phases[82]. The first phase represents the dead space, where no gas mixing occurs and the concentration of N_2 is close to zero, i.e. the subject is just "breathing back" the inhaled O_2. The second phase arises from gas mixing with a rapidly increasing concentration of N_2 as air from more peripheral airways arrives at the mouthpiece where the gas is analyzed. The third phase (sometimes called the alveolar phase) is where the concentration of exhaled N_2 reaches a plateau, representing the air from the most peripheral lung units. This plateau would be flat if ventilation was entirely even. There is however always some degree of uneven ventilation, also in completely healthy lungs, with regional differences due to gravity accounting for about a third of the heterogeneity and anatomical asymmetries within peripheral lung units for the rest[83]. As described above, airway obstruction in asthma is unevenly distributed and will lead to increased

ventilation heterogeneity with a steeper slope of the third phase (Fig. 5). The degree of ventilation heterogeneity in asthma has been shown to predict disease control and the response to changes in ICS treatment[84].
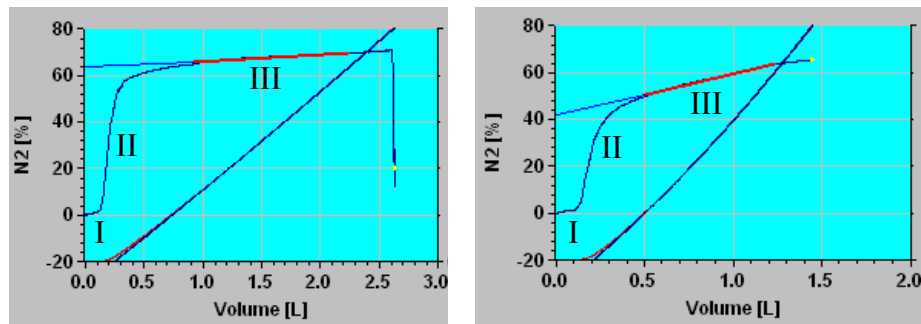
Figure 5. Slopes of expired N₂.
Expired N₂ concentrations during a single exhalation in a subject with moderate allergic asthma. The measurement is preceded by inhalation of 100% O₂, meaning that the first portion of exhaled air contains O₂ only. Three distinct phases are visible: phase I, representing the dead space; phase II, being the bronchial (or transitional) phase and phase III, termed the alveolar phase. The measurement depicted to the left was performed at baseline, while the measurement to the right was performed immediately after an inhaled allergen challenge. The red line indicates the slope that is extrapolated from phase III.

The analysis of exhaled inert gas concentrations can also be used over several consecutive breaths, producing more information about the different portions of the small airways. This is called the multiple breath washout (MBW). The drawback compared to the single-breath technique is that the MBW is more time consuming, usually taking a few minutes to complete. The subject inhales 100% O_2 during the entire measurement, and N_2 is gradually washed out until only $1/40^{th}$ of the starting concentration remains in the lungs (Fig. 6). Similar to the IOS, the MBW is performed at tidal breathing and thereby avoids any potential effect on smooth muscle tone by maneuvers like deep inspiration or forced expiration. The moment when the total volume of expired air during a measurement equals the functional residual capacity of the subject, one turnover is said to have occurred. The number of turnovers required to complete the test is called the lung clearance index (LCI). LCI is an overall measure of ventilation heterogeneity, and provides no detailed information of where the heterogeneity arises.
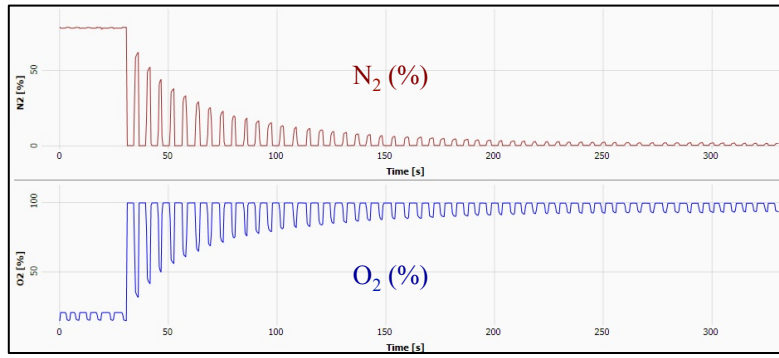
Figure 6. Multiple breath washout.
A complete multiple breath washout (MBW) measurement, with concentrations of exhaled N_2 and O_2 for each breath. The N_2 concentration is depicted at the top and the O_2 concentration at the bottom. The maneuver is continued until the expired N_2 concentration reaches 1/40th of its starting concentration.

After normalizing for dilution of the marker gas to allow for comparison between breaths during different parts of the measurement, the phase III slopes of each breath can be analyzed to dissect the ventilation defects of conducting airways from those of intra-acinar airways. The concentration normalized phase III slope is termed Sn_{III}. Ventilation heterogeneity of the conducting airways will lead to a progressive increase in Sn_{III} as the measurement proceeds, because of different time constants for sequential filling and emptying of parallel lung units with a dissimilar degree of obstruction. The slope of each Sn_{III} plotted between 1.5 and 6 turnovers (ΔSn_{III}) is called S_{cond} (Fig. 7).
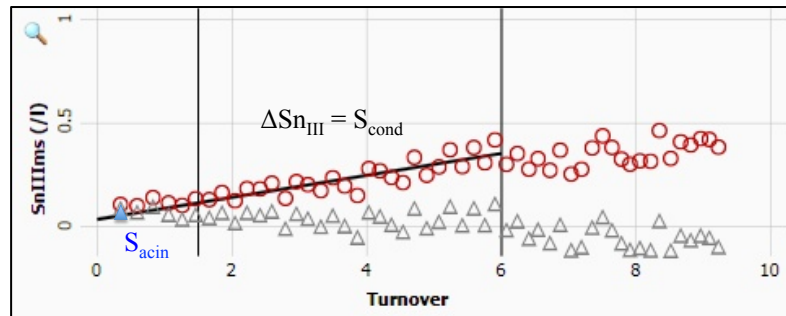
Figure 7. S_{acin} and S_{cond}.
The slopes of phase III are plotted for each breath. The x-axis denotes the number of turnovers. The phase III slope normalized for concentration (Sn_{III}) rises gradually when there is ventilation heterogeneity of small conducting airways. S_{acin} is the Sn_{III} of the first breath, while S_{cond} is the increase in Sn_{III} between 1.5 and 6 turnovers.

In contrast, the contribution of ventilation heterogeneity arising close to the diffusion-convection front to the Sn_{III} will increase initially to quickly reach a plateau with its maximum usually at around 1.5 turnovers[85], and the Sn_{III} of the

first breath is termed S_{acin} (strictly speaking, S_{acin} is the Sn_{III} of the first breath minus the convection-dependent heterogeneity, which is determined based on the ΔSn_{III} during the measurement).

Although the model of S_{cond} and S_{acin} measured with MBW is a theoretical one, and conclusions are complex regarding specific details of the locations of pathologic events giving rise to abnormal ventilation heterogeneity, several studies have shown that the peripheral conducting airways is the major site of dysfunction in the event of an increased S_{cond} among patients with mild asthma[86] and normal subjects after bronchoprovocation[87, 88]. Correlations between S_{cond} and FOT parameters of the lower frequency range (5 Hz) further strengthens the concept of pathophysiological changes of peripheral conducting airways contributing to an increase in S_{cond}, at least in healthy subjects[89]. In subjects with obstruction of the airways, however, the location of the diffusion-convection front could vary as flow velocity is changed, and the levels of airway generations represented by S_{cond} and S_{acin}, respectively, may be altered.

Biomarkers of airway pathology

Exhaled breath temperature

Exhaled breath temperature (EBT) was initially conceived as a potential marker of airway inflammation, based on the idea that the increases in blood flow in conducting airways that are found in different disease states would be followed by increased heating of the ventilated air. The airway mucosa of asthmatics is vascularized to a higher degree compared to healthy controls, with correlations among asthmatic subjects between the number of vessels and disease severity[90, 91], and there is increased activity of several vasodilating mediators, such as bradykinin, histamine and nitric oxide (NO)[92]. Several studies have shown an increased EBT in asthma[93, 94], especially when uncontrolled[95] and during exacerbations in children with asthma[96, 97].

Later it was also realized that remodeling processes of some respiratory diseases, with destruction of vasculature and reduced contact surface area, could lead to a downward shift of EBT[98]. In subjects with chronic obstructive pulmonary disease (COPD), EBT has been found to be lower during stable disease but increase during exacerbations[99]. It has therefore been suggested that EBT should be used to follow fluctuating changes in each individual, rather than as a diagnostic method to separate healthy airways from those affected by inflammatory processes.

Correlations between bronchial blood flow and exhaled breath temperature has been shown previously[100], and EBT has been shown to correlate to both exhaled nitric oxide and number of sputum eosinophils in asthmatic children[101, 102]. EBT was shown to increase after an exercise challenge test in asthmatic children and correlated to the decrease in FEV_1 after exercise, but only in subjects without ICS treatment[103].

Two different approaches to measuring EBT have been suggested: the rate of temperature increase ($\Delta e°T$) and the plateau of the exhaled air temperature during an exhalation (PLET). Pifferi *et al* found that, in children, the PLET but not the $\Delta e°T$ could be used to distinguish asthmatics from healthy controls, and hypothesized that the reason is that PLET better reflects peripheral airways[104]. Popov et al designed a device (X-Halo) that measures the plateau temperature, similar to PLET, but after a few minutes of tidal breathing instead of one single exhalation[93]. The X-Halo was used to investigate how 8 weeks of treatment with an extra-fine formulation of ICS changed EBT, among other markers of airway or systemic inflammation. EBT decreased significantly only among the "top responders" of improved peripheral airway function, supporting the notion of a link between peripheral airway inflammation and EBT[105].

Hamill *et al* tried to compare EBT measurements with clinical decisions to alter treatment in children with asthma, and with assessments of asthma control, without any evident associations[106]. The study was however cross sectional and did not describe the serial measurements that EBT could potentially be used for. They also described the issue that some children were unable to complete the measurements, which could take up to 8 minutes. A new device has since been designed that can complete the measurement within 10 breaths[107].

Measurements of EBT in elderly subjects revealed several factors influencing the temperature, including age, sex, BMI, ambient temperature and exposure to pollution from traffic[108]. However, only a small number of subjects had any respiratory disease (asthma or COPD). The reliability of EBT measurements has been discussed, and other factors apart from those mentioned above has been suggested as necessary to take into consideration, i.e. recent food intake, use of ICS and certain other medications, infections, co-morbidities and lung volumes[109, 110, 111]. To summarize, the debate on the role of EBT is ongoing.

Club cells and CC16

Club cells (previously known as Clara cells) are non-ciliated epithelial cells with secretory granules, found predominantly in respiratory bronchioles, and in fewer numbers in terminal bronchioles and more proximal airways[112]. The major

secretory protein of these cells is the club cell 16 kDa protein (CC16), which is secreted apically into the airway lumen.

CC16 has been suggested as a protective mediator in the peripheral airway inflammatory response[113]. It is an anti-inflammatory agent and natural immunosuppressor, inhibiting fibroblast[114], monocyte and neutrophil chemotaxis and phagocytosis in vitro[115], as well as the activity of secretory and intracellular PLA2[116]. Reduced concentrations of CC16-positive cells have been found in the airways of asthmatics[117], and lower levels of CC16 are seen in serum of asthmatic patients compared to healthy controls, both in children[118] and in adults, with a further decrease in patients with a longer disease duration[119]. CC16 is therefore a potential diagnostic marker of asthma.

A polymorphism of the CC16 gene with an alteration at position 38 (A38G) has been shown to predispose to asthma in adults[120]. A 6.9-fold risk increase for those homozygous for the mutation and a 4.2-fold increase for those heterozygous was shown in asthmatic children[121], the mutation was also associated with decreased plasma levels of CC16[122]. Other studies have however found no such association[123, 124]. In a study by Sengler et al, there was no association with the presence of asthma, but in asthmatic patients homozygous for the mutation there was an increase in bronchial hyper-responsiveness to histamine and a greater fall in FEV_1 after exercise[125].

CC16 has also been suggested as a marker of airway epithelial damage, with increased permeability of the bronchoalveolar/blood barrier (BA/BB) and leakage of CC16 into the bloodstream[126]. The acute effects of exacerbations and airway irritants exposure on CC16 levels are however still undefined. CC16 was increased in plasma of asthmatic children during an exacerbation, but only in those that did not have a polymorphism of the CC16 gene (the same subjects also had a milder disease)[127]. Exposure to ambient ozone in cyclists caused an increase in serum levels of CC16[128], while exposure to nitrogen trichloride in indoor chlorinated swimming pools (without exercising) resulted in an increase in surfactant-associated proteins A and B after 2 hours, but not any increase in serum CC16[129]. Possible explanations for an absent CC16 response include that some exposures could cause effects exclusively on compartments where club cells are fewer in numbers (dependent on e.g. particle size), or that there is a pre-established reduction in the capacity of the club cells to produce, store and/or secrete CC16 (due to genetic factors or due to repeated chronic exposure).

A low-dose allergen inhalation challenge for seven consecutive days caused a decrease in CC16 levels in broncho-alveolar lavage (BAL) in asthmatic subjects[130]. A challenge with inhaled glutaraldehyde, a low molecular-weight chemical, produced a significant decrease in CC16 levels in serum and BAL of individuals with occupational asthma due to glutaraldehyde, 24 hours after

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provocation, but not in healthy controls or patients with non-occupational asthma[131].

F_ENO

NO is produced in the human lung, and can be found in exhaled breath of all individuals. NO has a complex role in both healthy and asthmatic airways, working as a vasodilator, bronchodilator, neurotransmitter and inflammatory mediator all at the same time. NO is also a free oxygen radical which can be cytotoxic at higher concentrations. NO is produced by conversion of L-arginine to L-citrulline, a reaction catalyzed by enzymes called nitric oxide synthases (NOS) found in a large number of cell types, including vascular endothelial cells, inflammatory cells, epithelial cells and airway nerve cells[132]. Three different types of NOS have been identified, the endothelial NOS (eNOS), the neuronal NOS (nNOS) and the inducible NOS (iNOS)[133]. The eNOS and the nNOS are constitutively producing lower amounts of NO[134]. iNOS, on the other hand, can produce high concentrations of NO in instances of airway inflammation and as a non-specific defense mechanism against pathogens. The iNOS is found in the respiratory epithelium in humans[135] and is up-regulated in asthmatics compared to healthy controls[136, 137]. High concentrations of NO measured in exhaled breath (fractional exhaled NO, F_ENO) has been found in asthmatics with an atopic phenotype, associated with eosinophilic inflammation[138]. Correlations have been found between F_ENO levels and eosinophil counts in BAL, biopsies and sputum[139, 140, 141].

F_ENO is sometimes used in the diagnosis of airway inflammation. According to ATS guidelines, it can also be used to predict responsiveness to steroid treatment and to monitor airway inflammation[142], and an ERS technical standard defined a cutpoint of F_ENO <25 ppb to indicate that eosinophilic airway inflammation is less likely, while F_ENO >50 ppb suggests that a positive response to steroid treatment can be expected. Values between 25 and 50 ppb should be "judged within the clinical context"[143]. A problem with using F_ENO as a diagnostic tool is that a large proportion of asthmatics do not have eosinophilic inflammation, and have normal F_ENO levels[144]. Also, other underlying conditions with eosinophilic inflammation, such as some patients with COPD[145], eosinophilic bronchitis[146] and allergic rhinitis[147], can also lead to increased F_ENO levels. F_ENO should therefore be used not exclusively, but rather as a complement to other assessments of airway pathology. Analysis of the exhaled NO at different flow rates can also be used to allow for more detailed assessment of NO production in bronchial versus alveolar compartments[148].

Aims

The overall aim of this thesis was to explore the pathophysiology and some potential biomarkers of the asthmatic airway response. This was accomplished by eliciting airway responses using several types of airway provocations in both asthmatic subjects and healthy controls.

Specific aims of papers I-V

- Paper I: to evaluate the use of EBT as a biomarker of an airway inflammatory response induced by exercise, both in asthmatic subjects and healthy controls with the purpose of comparing any pathophysiological response to a normal physiological response.

- Paper II: to measure the response in systemic levels of CC16 after an exercise challenge test in asthmatic subjects and healthy controls, and relate levels of CC16 to EBT and $F_E NO$ to detect any injury or other effects on the airway epithelium.

- Paper III: to explore the response in EBT after two other types of airway provocations, EVH and methacholine inhalation challenge, to further evaluate the use of temperature measurements as a marker of an airway inflammatory response.

- Paper IV: to investigate the pathophysiology of small airways during the late allergic reaction induced by an allergen inhalation challenge, and to examine the effect on EBT by an allergic asthmatic reaction.

- Paper V: to study the effect on CC16 levels after an allergen inhalation challenge, as a way to estimate epithelial injury caused by the allergic reaction and to evaluate CC16 as a biomarker of asthma.

Materials and methods

Study population

Subjects were recruited locally in the Skåne region in the south of Sweden. Recruitment was mainly carried out by advertising in public spaces, in the university area of Lund University and in the allergy outpatient clinic at the university hospital in Lund.

Some subjects participated in more than one of the studies presented in this thesis. The overlapping numbers of subjects are distributed as follows: I and II: two subjects, I and III: twenty-four subjects, I and IV/V: three subjects, II and III: two subjects, II and IV/V: four subjects, III and IV/V: three subjects. Papers IV and V are based on the same study and thus include the same subjects.

General criteria

Subjects were not allowed to perform any exercise on the same day as any testing. No subjects were current smokers. Previous smokers had smoked less than five pack-years and stopped smoking at least 12 months before participation. A maximal daily dose of ICS equivalent to 800 μg of budesonide (I-III) or 400 μg of budesonide (IV-V) was allowed, and all subjects had been stable in their asthma for at least 6 months with no changes in treatment and no exacerbations. Short-acting β_2-agonists were withheld for at least 8 h prior to any tests, and long-acting β_2-agonists for at least 48 h (I-II and IV-V) or 24 h (III). Subjects were instructed to refrain from any caffeine intake for at least 2 h (I-II) or 6 h (III-V) before any testing. If subjects had suffered from any respiratory tract infections at any time during the last two (I) or three (II-V) weeks they were excluded or had to postpone visits. Subjects with other pulmonary or cardiovascular diseases or any other conditions affecting their ability to perform pulmonary function testing were excluded. Patients treated with allergen-specific immunotherapy, muscarinic or leukotriene receptor antagonists, phosphodiesterase inhibitors, oral corticosteroids, anti-IgE treatment, beta-blockers, anti-hypertensive medications, diuretics and other medications with significant pulmonary or cardiovascular effects were

excluded. Anti-histamines were not allowed for at least five days prior to any tests (IV-V).

Study design

Paper I

Twenty asthmatic subjects and 21 healthy controls performed an exercise challenge, running on a treadmill for 8 minutes. Lung function assessed by spirometry, body temperatures and EBT were measured before and repeatedly for 1 h after the challenge.

Paper II

Twenty-two asthmatics and 18 healthy controls performed an exercise challenge test, similar to the challenge in paper I. Spirometry, levels of CC16 in plasma and urine, EBT and F_ENO were measured before the challenge and repeatedly for 1 h after the challenge.

Paper III

Twenty-six asthmatics and 29 healthy controls performed both an EVH and a MCT. Spirometry, body temperatures and EBT were measured before the challenges and repeatedly for 1 h after each of the challenges. After the measurement at 1 h, subjects inhaled salbutamol and the measurements were repeated again after 15 min.

Paper IV

Thirty-four subjects with mild to moderate allergic asthma performed an allergen inhalation challenge. Lung function was assessed by spirometry, IOS, body plethysmography, CO diffusion capacity and MBW before the challenge and repeatedly for 23 h post-allergen challenge. EBT was also measured.

Paper V

Based on the same challenge as described for paper IV, levels of CC16 in plasma and urine were measured before and repeatedly afterwards for up to 23 h. Bronchoscopy was performed in 21 of the subjects before the allergen challenge and in 19 subjects 24 h after the end of the challenge. CC16 was measured in BAL fluid and club cells and CC16 mRNA were analyzed in brush samples.

Lung function tests

Spirometry

Spirometry was performed to obtain at least three acceptable spirograms before any challenge test (I-V), using a MasterScope (Erich Jaeger GmbH, Würzburg, Germany). Measurements were performed according to ATS/ERS standards and the highest and the second highest FEV_1 had to be at least within 0.150 L[149]. The best value was chosen and defined as baseline. One technically acceptable spirogram was generally considered enough at each time during and after the different challenges.

During a spirometry, subjects are equipped with a nose clip to avoid leaking of air through the nose, and are comfortably seated. Breathing into a mouthpiece connected to the spirometer, subjects exhale as fast as they can after a full inhalation. The exhalation continues until a plateau is reached in the volume-time curve (at least 6 s). FEV_1 is the volume of air exhaled during the first second of the maneuver, while FVC is the volume exhaled during the entire measurement. Percent of predicted values were calculated based on the reference values of Crapo *et al*[150].

Body plethysmography

Body plethysmography was measured (IV) with a MasterScreen Body (Erich Jaeger GmbH, Würzburg, Germany). Measurements were performed according to ATS/ERS recommendations[151] and reference values of Quanjer *et al* were used for calculating percent of predicted values[152]. Plethysmographic measurements are preceded by stabilization of temperature and pressure with the subject placed in the box with the door closed. Equipped with a nose clip, the subject will then start to breathe tidally through a mouthpiece, where flow and pressure are measured. Lung volumes are then measured by closing the shutter valve (as described previously in this thesis). Finally, the subject performs a slow full exhalation, followed by a full inhalation and directly after that a forced expiratory maneuver. After an expiratory time of at least 6 s and after reaching a plateau in the volume-time curve, the measurement is finished. The entire process was always repeated until at least two technically acceptable measurements were achieved, both before and after the allergen challenge.

Carbon monoxide diffusion capacity

Single breath (SB) methane (CH_4) dilution carbon monoxide (CO) diffusion was measured (IV) with a MasterScreen PFT system (Erich Jaeger GmbH, Würzburg, Germany), according to manufacturer's instructions and the ATS/ERS standardizations[153] and using the reference values of Quanjer *et al*[152]. Subjects used a nose clip, and were instructed to breathe tidally through the mouthpiece, followed by a slow full exhalation and then a quick full inhalation. Guided by the software, subjects were then instructed to hold their breath for at least 6 s, followed by exhalation to RV again. The measurement was then finished. The software reports diffusing capacity of the lung for CO ($DLCO_{SB}$), DLCO divided by alveolar volume ($DLCO_{SB}/VA$), residual volume (RV_{SB}) and functional residual capacity measured by single breath (FRC_{SB}).

Impulse oscillometry

Airway impedance was measured (III-IV) by IOS, using a MasterScreen Impulse Oscillometry System (IOS) (Erich Jaeger GmbH, Würzburg, Germany)[154]. When IOS and spirometry measurements coincided, IOS was always measured first to avoid any effects of deep inspirations or forced exhalations on results. Subjects were seated during measurement, they were equipped with a nose clip and had the palms of their hands pressed against their cheeks for the entire procedure in order to avoid shunting of the oscillometric pressure impulses. After waiting a few seconds for tidal breathing to stabilize, measurement of airway resistance and reactance was recorded for at least 30 s. If there was any coughing, swallowing or any other artifacts, the recording was rejected and a new test session was started. The software calculates and reports mean values of R5, R20, X5, F_{res} and AX. We also analyzed the FDR, by calculating R5-R20.

Multiple breath washout

Multiple breath washout of N_2 was measured (IV) with an Exhalyzer D (Eco Medics, Duernten, Switzerland) in accordance with previously described recommendations[155]. Subjects were comfortably seated and equipped with a nose clip throughout the measurements. They were instructed to breath at a normal relaxed rate, guided by the software's built-in feedback program. After a few tidal breaths of room air, the measurement started by influx of 100% O_2 into the mouthpiece. Inhaled and exhaled air was continuously analyzed for concentrations of N_2, O_2 and CO_2. The subjects were guided to keep their breathing at a relaxed pace, and the measurement continued until an automatic stop occurred when the

concentration of exhaled marker gas (N_2) had reached $1/40^{th}$ of the starting concentration (corresponding to just below 2%). The software calculates and reports LCI, S_{cond} and S_{acin}. The latter two are multiplied by tidal volume (V_T) for each breath as correction for lung size and breathing pattern (generating $S_{cond}*V_T$ and $S_{acin}*V_T$).

ACT

Subjects with asthma completed an asthma control test (ACT) before participation (I, III-V). The ACT consists of 5 questions with options for answers ranging from 1-5 points, where higher points indicate a better asthma control[156]. The questionnaire asks subjects to assess their symptoms and disease control during the last four weeks. Twenty-five points being the optimal score, total scores of 21-24 are usually considered acceptable while a score of less than 20 is regarded as indicative of poor asthma control.

Biomarkers

Exhaled breath temperature

EBT was measured (I-IV) with a hand-held device called X-Halo (Delmedica Investments, Singapore). The X-Halo has an insulated chamber containing a thermal block with high thermal capacity, reducing the influence of ambient environment. The subject breathes at a relaxed tidal rate and volume, inhaling through the nose and exhaling through the mouthpiece of the device. The chamber has outflow openings that release excess air, while the inflow opening to the chamber is equipped with a reversion valve that closes when the subject stops exhaling. This means that the thermal energy of the air primarily from the last portion of the exhalation (i.e. air flowing from the more peripheral airways) accumulates in the chamber. The thermal block has a high precision thermal sensor, and the device detects and signals when the temperature of the thermal block has reached a plateau. The measurement usually takes 2-6 minutes to complete.

F_ENO

Exhaled NO concentration was assessed with a NIOX Flex (Aerocrine AB, Stockholm, Sweden) before and after exercise (II), and before MCT and EVH

(III). During the measurement, the subject inhales ambient air depleted of NO and exhales into a mouthpiece at a steady flow rate, guided and quality controlled by the device. The measurement is repeated four times at four different flow rates (50, 100, 200 and 300 ml/s). By plotting the output of NO against the exhalation flow rate (100–300 ml/s), the slope and intercept can be calculated, representing alveolar NO concentration (CANO) and bronchial flux of NO (J'awNO), respectively[148].

CC16

CC16 was measured in plasma and urine (II and V), and in BAL fluid collected during bronchoscopy (V). Plasma was extracted from blood that was collected in sodium heparin tubes and centrifuged at room temperature for 12 min at 3000 rcf to separate the plasma portion. Since CC16 is produced in small amounts in the prostate, urine samples from all male subjects were collected after discarding the first 100 ml each time. All samples were stored at -80°C until analyzed.

BAL was collected by infusion and re-aspiration of 50 ml of 0.9% phosphate buffered saline (PBS) repeated three times. The first aliquot was defined as the bronchial sample while the subsequent two aliquots were defined as alveolar samples.

CC16 concentrations in plasma, urine and BAL were measured by ELISA (Human Club Cell Protein ELISA kit, BioVendor, Modrice, Czech Republic). To compensate for variations in sampling and dilution, BAL levels of CC16 were divided by total protein concentration in BAL and CC16 levels in urine were divided by creatinine concentration.

Airway provocations

Exercise challenge test (I-II)

The exercise challenge test was performed running on a treadmill. Four ECG leads were used to measure the heart rate throughout the challenge. Subjects were not allowed to warm up beforehand. The test was divided into three phases (a-c) ranging over 8 minutes in total: a) during the first two minutes, speed and level of upward slope were set to increase the heart rate of the subject to 90% of calculated maximum capacity (defined as 220 bpm - age in years) before the end of the phase, b) the speed and level of upward slope was adjusted continuously to

maintain the subject's heart rate as stable as possible during the next four minutes, c) during the last two minutes, the speed and level of upward slope were increased further to reach maximum capacity before the end of the challenge. The test was considered positive if FEV_1 dropped $\geq 10\%$ after the test, compared to baseline.

Eucapnic voluntary hyperventilation (III)

Subjects hyperventilated for six minutes through a mouthpiece connected to a container administering dry hypercapnic air (5% CO_2) at room temperature via a dry air ventilation device (Aiolos Medical AB, Karlstad, Sweden). Subjects used a nose clip during the entire test. The flow was set to 26 l/min times the baseline value of FEV_1. Subjects were supervised at all times and were guided by a reservoir balloon attached to the ventilation device to ensure that the rate and depth of breathing remained adequate. The test was considered positive if FEV_1 dropped $\geq 10\%$ compared to baseline at any time within 30 minutes after the end of the hyperventilation phase.

Mannitol challenge test (IV-V)

A mannitol powder kit (Aridol, Pharmaxis, Frenchs Forest, Australia) containing a hand held dry powder inhaler and pre-filled mannitol capsules was used. Gradually increasing doses were administered in eight steps according to the manufacturer's instructions, delivering a maximal cumulative dose of 635 mg of mannitol. FEV_1 was measured 1 min after each inhalation and the challenge was considered positive and completed if, at any time, FEV_1 dropped $\geq 15\%$ compared to baseline.

Methacholine challenge test (III-V)

A tidal volume triggered equipment (Aerosol Provocation System, APS; Erich Jaeger GmbH, Würzburg, Germany) was used for the MCT. One inhalation of 9 mg/ml NaCl was performed first, as a negative control. FEV_1 measured after five minutes was not allowed to drop $\geq 5\%$ compared to FEV_1 measured before inhalation of NaCl, in which case the subject would be excluded from further testing. Methacholine was then inhaled repeatedly in five steps, with gradually increasing doses (50, 150, 300, 600 and 900 µg) to a maximal cumulative dose of 2000 µg. IOS was conducted 1.5 min after each inhalation and spirometry 2 min after each inhalation. A positive test was defined as a drop in FEV_1 of $\geq 20\%$ compared to baseline.

Allergen inhalation challenge (IV-V)

The allergen inhalation challenge was conducted as an incremental allergen dose challenge, meaning that increasing doses of allergen are inhaled at several steps with intervals of approximately 10 minutes. Prior to the first inhalation of allergen, FEV_1 is measured before and 5 minutes after inhalation of 0.9% NaCl. This is made to ensure that there is no general irritant effect on the airways; if FEV_1 drops ≥5% compared to baseline after inhalation of NaCl the subject is excluded from further testing due to both safety precautions and due to the fact that it will be difficult to draw any conclusions from any further reaction to the allergen inhalations.

An automatic inhalation-synchronized dosimeter (Spira Elektro 2, Respiratory Care Center, Hämeenlinna, Finland was used for inhalation of NaCl and allergen. The allergen extract (Aquagen, ALK-Abelló, Hørsholm, Denmark) was diluted to eight separate concentrations (250, 500, 1000, 2000, 4000, 8000, 16000 and 32000 Standardized Quality units (SQ-U)/ml). The starting dose was 1.2 SQ-U. FEV_1 was measured 5 minutes and 10 minutes after the inhalation. If FEV_1 had not dropped ≥10% from baseline, the next dose was increased four-fold. The lowest FEV_1 of those measured at 5 and 10 minutes after each inhalation was used to evaluate the next dose. If FEV_1 had dropped by 10-15% from baseline, the next dose of allergen was doubled, and if FEV_1 had dropped by 15-20%, the next inhalation was postponed to 30 min later, with FEV_1 being measured every 5 minutes. If FEV_1 remained stable at a 15-20% decline, the previous dose was repeated once more. Whenever FEV_1 dropped ≥20% compared to baseline the challenge was immediately considered complete. If this had not been achieved when the cumulative dose reached 20 000 SQ-U, the test was cancelled and the subject was excluded from further testing.

Statistics

A limited number of subjects were included in our studies, and normal distribution could not be assumed for all parameters. Therefore, non-parametric statistical methods were used throughout the studies presented in this thesis, except for CC16 levels in paper II that followed normal distribution and were analyzed by parametric tests and reported as mean (SEM). All other results are presented as median (IQR). The median and the interquartile range are robust parameters, not heavily affected by outliers or skewness of data, which is why we chose to use them in most of our reports. Group comparisons were made using the Mann-Whitney test and paired measurements were analyzed with Wilcoxon's signed

rank test. Correlations were analyzed with Spearman's correlation test. To allow for analysis of the large amount of data acquired by repeated measurements (paper IV), area under the curve (AUC) was calculated for data collected during the early and the late phase after the inhaled allergen challenge. A p value of <0.05 (two-tailed) was considered statistically significant, while p values <0.1 could be described as "tendencies towards" depending on the context. For some comparisons between groups, the p-value was reported in figures regardless of its value to let the reader draw his/her own conclusions.

Statistical analysis was performed using GraphPad Prism (versions 4.0, 5.0 and 7.0) and SPSS (versions 21.0 for Windows and 23.0 for Mac OS).

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Results

Exhaled breath temperature

Paper I

EBT was elevated in all subjects after the exercise challenge test, but the elevation was not statistically different in asthmatic subjects compared to the healthy controls. Since exercise-induced bronchoconstriction (EIB) is not seen in all asthmatics and can in fact be found in several controls that are otherwise regarded as healthy, we also compared all subjects with EIB to those without EIB, but without finding any difference in the increase in EBT. However, there was a difference between asthmatic subjects with EIB and asthmatic subjects without EIB, in that EBT remained elevated for a longer time in the former group. Also, the drop in FEV_1 correlated significantly with EBT measured 5 min post-challenge in all subjects. EBT seemed to be closely related to oral temperature, but not to other body temperatures (axillary and auricular) to the same extent. EBT remained significantly elevated compared to baseline for at least 60 minutes.

Paper II

The findings from the previous study, that EBT is elevated after exercise, could be repeated in this study, with the only difference being that the duration of the increase in EBT was 45 min instead of 60 min. There were still no differences between asthmatic subjects and healthy controls. The correlation between EBT and the drop in FEV_1, seen previously, was not significant in this study. There were no significant correlations between absolute levels of EBT and F_ENO before or after exercise, and no correlations between the changes in levels from baseline to after the challenge.

Paper III

EBT was significantly increased in all subjects after both MCT and EVH, and the duration of increase compared to baseline was 45 and 30 min, respectively. There were no significant differences between asthmatics and healthy controls, or between responders and non-responders to the two different airway challenges. A significant correlation between the drop in FEV_1 and EBT 5 min post-challenge was seen in the asthmatic subjects after EVH, but not after MCT.

EBT and oral temperature correlated significantly at several time points before and after the challenges, as well as the changes in EBT and oral temperature from baseline to post-challenge. No correlations were seen between EBT and the other measurements of body temperature (axillary or auricular).

Paper IV

EBT was significantly increased in all subjects 7 h after the end of the allergen challenge compared to baseline temperature (Fig. 8). The increase did not differ between the single and dual responders. There was no increase during the early phase (30 min post-challenge).
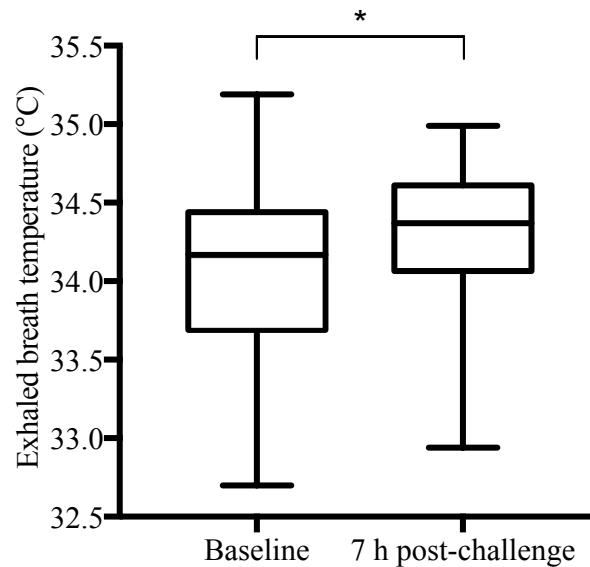
Figure 8. Exhaled breath temperature after inhaled allergen challenge.
Exhaled breath temperature in all subjects before and seven hours after the end of the inhaled allergen challenge. Seven hours post-challenge is regarded as the late phase of the allergic response in this thesis, although only approximately half of the subjects actually had a significant late allergic response by the traditional definition based on central airway obstruction.

CC16 and F_ENO

Paper II

CC16 was increased in plasma immediately after the exercise challenge in all subjects, and remained elevated for the entire study time of 60 min. There were no differences in plasma levels between asthmatics and healthy controls, or between

subjects with a positive response to the challenge and subjects with a negative response. Absolute levels of CC16 in plasma were significantly higher in male subjects compared to female subjects at all time points, both before and after the challenge. However, the relative increase was not different, i.e. the magnitude of the difference between female and male subjects remained constant throughout the test.

Urinary levels of CC16 increased in all subjects 30 min after the challenge. After 60 min, urine concentrations of CC16 had dropped again but were not fully restored to baseline levels. There were no differences in urinary levels between asthmatics and controls, between responders and non-responders, or between male and female subjects.

In addition, we could show significant correlations between the maximal increase in EBT and absolute levels of CC16 in plasma after the challenge. A reverse correlation was however seen between EBT at 60 minutes and the increase in CC16 levels in plasma from baseline to every individual time point.

Both alveolar concentrations and bronchial flux of NO decreased after exercise in all subjects. There were no differences between asthmatics and healthy controls at any time. The changes in CC16 and $F_E NO$ from baseline to post-challenge correlated significantly and inversely, meaning that subjects that had the greatest increase in CC16 also had the greatest decrease in $F_E NO$ levels.

Paper V

Immediately after the inhaled allergen challenge, plasma levels of CC16 were increased in all subjects. In contrast to our findings presented in paper II, this was not followed by an increase of CC16 in urine. Levels of mRNA remained constant, while BAL CC16 increased in a subgroup of subjects, i.e. those without a late allergic response, who also had a significantly lower level of CC16 in BAL at baseline. The number of CC16 positive cells in brush samples was significantly higher in the subgroup of subjects with a late allergic response after the challenge, but there was no significant change from baseline to post-challenge in single or dual responders.

Significant correlations were seen between levels of CC16 in plasma and in the alveolar portion of BAL, both expressed as the change in levels from baseline to post-challenge and as absolute values post-challenge. There was however no correlation at baseline.

There was also a significant correlation between plasma levels of CC16 at baseline and the PD_{15} of the mannitol challenge test, meaning that subjects with a greater airway hyper-responsiveness to mannitol had lower circulating levels of CC16. To assess any links to the peripheral airway response and allow for analysis also in

subjects with a PD_{15} >635 mg, we analyzed the slopes of R5-R20, X5, F_{res} and AX plotted against the cumulative inhaled dose of mannitol. The slopes of these parameters would reflect the development of a peripheral airway response, with more responsive small airways resulting in a higher (steeper) slope. The slopes were correlated with the baseline plasma levels of CC16, revealing significant associations between circulating levels of CC16 and the small airway reactance (Fig. 9).

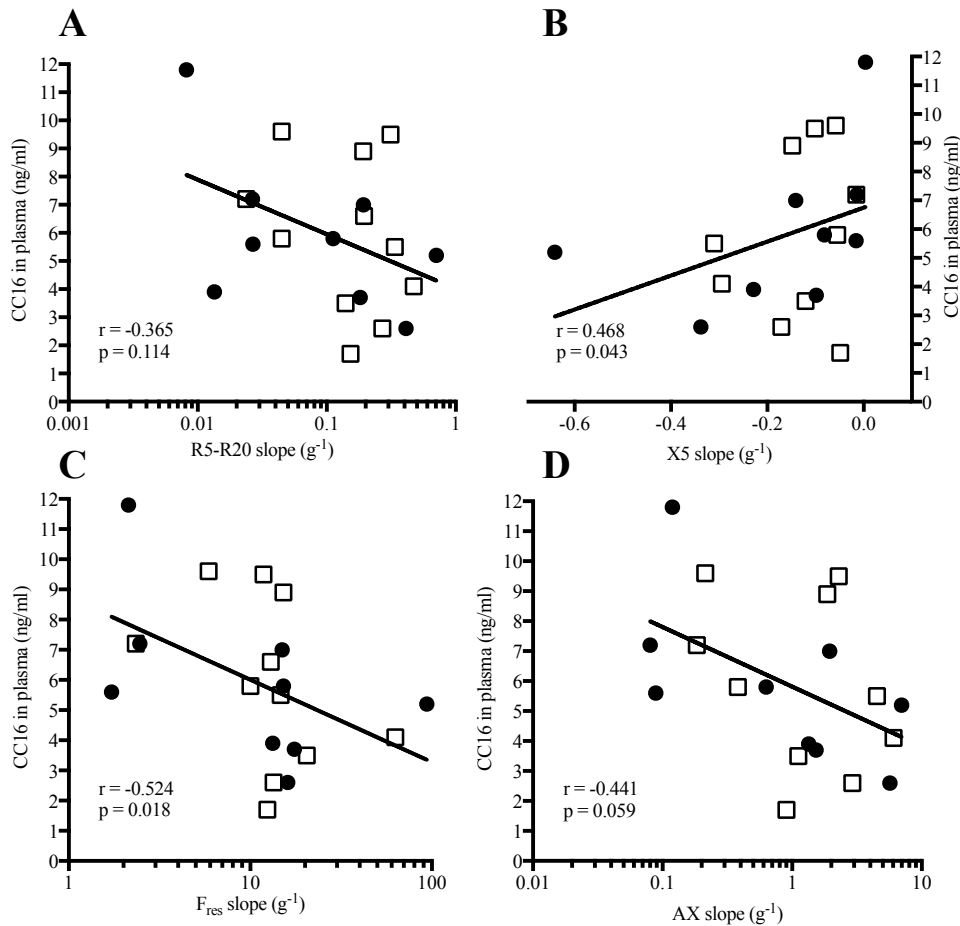**Figure 9. Slopes of airway resistance and reactance compared with CC16 levels in plasma.**
Plasma levels of CC16 at baseline were compared to the slopes of (A) R5-R20, (B) X5, (C) F_{res} and (D) AX. Slopes were calculated by plotting the outcome of IOS parameters against the cumulative dose of each inhalation step of mannitol. Spearman's rho and p-values are reported. □ = dual responders, ● = single responders.

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Small airway physiology

Paper III

IOS was measured before the MCT and during the challenge after each inhalation step. Increases in airway resistance after the challenge were seen in all subjects at both high and low frequencies. The frequency dependence of resistance was significantly higher in asthmatic subjects after the challenge. The MCT caused an increase in airway reactance in all subjects at the lower range of frequencies (X5 and AX). F_{res} was increased in controls only, while levels were not significantly different after the challenge, indicating that the asthmatic subjects had an established increase in airway reactance already at baseline. Airway resistance or reactance did not correlate with EBT.

Paper IV

After the inhaled allergen challenge, 15 subjects had both an early and a late allergic response and were defined as dual responders. Nineteen subjects were single responders, meaning they had an early allergic response only. The dual responders had significantly lower levels of FVC and X5, and significantly higher levels of RV/TLC, R5, F_{res}, LCI, S_{cond}, R_{tot}, R_{ex} and R_{in} during the late phase (4-8 h post-challenge).

Significant correlations were seen between several of the different parameters reflecting resistance, reactance, ventilation heterogeneity and airway obstruction. For example, R5-R20 and S_{acin} correlated significantly at baseline and at all times post-challenge, and LCI correlated to FEV_1 and FVC during the late phase.

Testing different approaches to defining the late allergic response, we found that there was a large but not complete overlap between central and peripheral airway responses during the late phase. Almost one fourth of the subjects had a central but not a peripheral response, and one fourth had a peripheral but not a central response. The latter group would be classified as single responders based on the traditional definition, but did display a dual airway response measured in peripheral airways (Fig. 10).
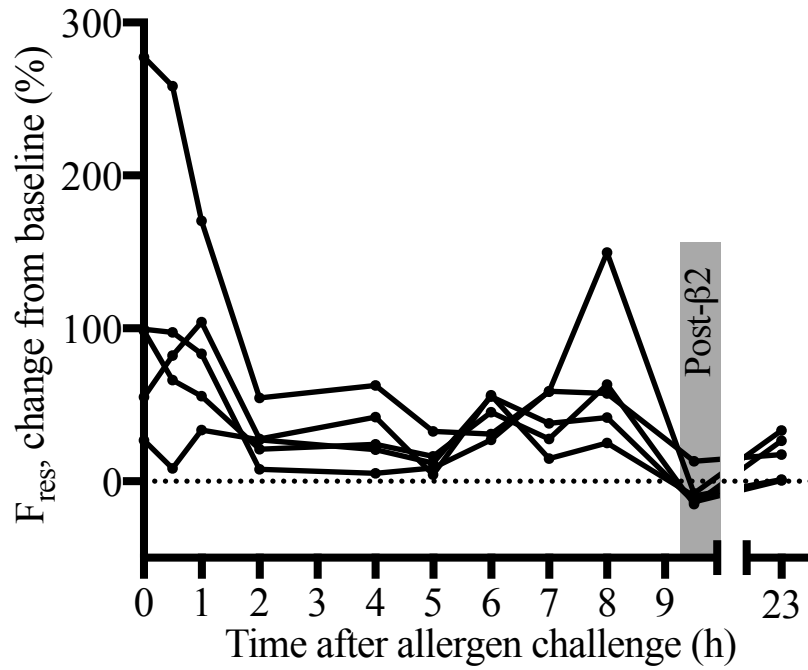
Figure 10. A peripheral dual response.
Illustrating the peripheral airway response in five subjects that were defined as single responders based on the traditional definition of a late allergic response, i.e. a drop in FEV_1. F_{res} = resonant frequency. Post-$\beta2$ = measurement performed 30 min after inhalation of salbutamol.

Discussion

EBT was measured after four different airway challenges with three principally different mechanisms of action. Our results point towards EBT being influenced mainly by a vascular effect, with or without any underlying inflammation. EBT was elevated after exercise, hyperventilation and methacholine, and during the LAR after an allergen challenge. No increase was seen during the EAR. The time point for measurement of EBT during the EAR of 30 min post-challenge was based on the maximal response seen after the previous airway provocations. The EAR is however more of an isolated bronchoconstriction, while the LAR is associated with an airway inflammatory response, leading to epithelial and endothelial damage, influx of inflammatory cells and hyperemia of the airway mucosa. Cardiac output would however not be affected, in contrast to exercise.

Relations between the increase in EBT and reduced expiratory flow could be due to a common cause, such as a release of inflammatory mediators leading to both bronchoconstriction and vasodilation. It could however also be that the reduction in flow leads to ventilated air residing in the airways for a prolonged time, increasing heat exchange. Mucosal swelling and plasma exudation could however have complex consequences with both a decrease in flow rates and an isolating effect between the airway lumen and the sub-epithelial capillary network, counteracting heat exchange.

EBT post-challenge correlated to the drop in FEV_1 after both exercise and EVH, but not after the MCT or the inhaled allergen challenge. This finding indicates that the elevation in EBT is not due to bronchoconstriction per se, but rather that the temperature increase and the bronchoconstriction have a common cause, i.e. hyperventilation with dehydration of the epithelial lining fluid. It would also indicate that the degree of hyperventilation and/or the pre-established vulnerability of the airways to a certain amount of hyperventilation would determine the resulting bronchoconstriction as well as the increase in tracheobronchial blood flow.

Morris *et al* showed that during exercise in 12 young healthy subjects, tracheobronchial blood flow was increased and correlated to both cardiac output and the minute ventilation, while vascular resistance was decreased, suggestive of vasodilation[157]. In the same study, both tracheobronchial blood flow and cardiac output returned to pre-exercise values within 10 minutes of rest. However, the

intensity of exercise was only low to moderate. We found that EBT remained elevated for 60 or 45 minutes (I and II, respectively) after high intensity exercise. Despite attempts to standardize the exercise challenge test, there are always variations between each individual test, most likely affecting the extent of the stimulus on the airways and causing results to differ somewhat from study to study. The difference in the duration of increase in tracheobronchial blood flow seen in previous studies compared to the duration of increase in EBT in our studies could however also be an indication that other factors than the tracheobronchial blood flow have significant effects on EBT. One such factor could be the pulmonary blood flow, and EBT could potentially be used to identify patients with peripheral airway inflammation, who possibly would benefit from extra-fine particle ICS. Further research will be needed to explore this concept.

Both α-adrenergic and β-adrenergic receptors are expressed on airway smooth muscle cells as well as airway vascular smooth muscle cells; the α-adrenergic receptors are more numerous on the vascular smooth muscle cells and vice versa[158, 159]. Brieva et al demonstrated that the airway blood flow was higher in subjects with mild asthma compared to healthy controls, and that an α_1-adrenergic receptor agonist could decrease the blood flow in asthmatics but not in the controls[160]. In contrast, a β-adrenergic receptor agonist increased the blood flow of controls but did not affect the blood flow in asthmatics. The authors suggest that the latter finding could be explained by a down-regulation of β_2-receptors on the vascular smooth muscle cells, something that could be restored by two weeks treatment with ICS[161]. The fact that airway blood flow is higher among asthmatics at baseline could also suggest that there already is vasodilation due to an uncontrolled airway inflammation and that there is limited capacity for further dilation in response to the β-agonist. It could also be secondary to an increased vascularization[162]. In our study (III), EBT was however increased after MCT in both asthmatics and healthy controls regardless of the response in FEV_1, which is consistent with methacholine increasing tracheobronchial blood flow[52]. Our asthmatics did however only have mild to moderate asthma, and were all clinically stable at the time of inclusion. If the study was repeated including subjects with severe or uncontrolled asthma, results may have differed with a higher EBT at baseline and no capacity for a further increase after challenges.

Similar to our findings, Couto et al found that the increase in EBT after a swimming training session was not dependent on asthma[163]. EBT was however only measured 5 min after exercise. Although the elevation of EBT seems to be a physiological response, it is possible that the increase in blood flow after exercise is allowed to persist for a longer time in those subjects whose airways are already primed with an increased vascularization due to long-standing airway inflammation. This would explain our findings of a longer duration of the elevation in EBT in the group of asthmatics with EIB.

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The close relationship between EBT and oral temperature raises questions of where in the respiratory tract that most of the heat exchange takes place, primarily influencing the temperature that we measure at the opening of the mouth. There is still controversy over this subject, with different studies claiming that the pulmonary arterial perfusion[164], the tracheobronchial vascular system[100] or even the upper airway above the glottis[165] represents the principal contribution. Most would probably agree though, that the site of heat exchange depends on the minute ventilation. This makes interpretation of EBT in relation to exercise complicated, since minute ventilation would change dramatically from rest before the challenge, to exercise and then back to rest with gradually decreasing ventilation rates as the subject recover.

The immediate increase in CC16 plasma levels after the inhaled allergen challenge supports the hypothesis that CC16 in plasma is a biomarker of airway epithelial dysfunction. CC16 is however always present in plasma, also in the absence of any airway disease. Furthermore, long-term chronic inflammation actually leads to lower circulating levels of CC16[118, 119], and the number of club cells is reduced in small airways of asthmatics[117]. This implies that an increase in CC16 plasma levels is not only a sign of epithelial dysfunction, but also dependent on other factors, such as the concentration in the epithelial lining fluid, with a greater concentration gradient driving the diffusion over the bronchoalveolar/blood barrier. We aimed to determine if acute inflammation, i.e. an allergic response, had any effect on pulmonary levels of CC16. There was no change in CC16 mRNA 24 h after the allergen challenge, but an increase in BAL levels of the single responders (with higher levels in BAL already at baseline in the dual responders). This could be explained by increased secretion and/or leakage from club cells, further boosting the diffusion over a dysfunctional epithelium and resulting in an increase in plasma CC16. The significantly higher proportion of club cells in brush samples from the dual responders compared to the single responders post-challenge is also interesting in this context, and we hypothesized that epithelial dysfunction could be associated with a disruption of cell-cell and/or cell-matrix junctions.

The correlations between CC16 and F_ENO (II) point towards an epithelial effect during the exercise challenge. A decrease in F_ENO after exercise has been found also in previous studies[166, 167], and is possibly due to the overall increase in ventilation with a washout effect and/or (in the case of a lower concentration of alveolar NO) a false improvement because of obstructed ventilation of respiratory units that are affected by inflammation to a higher degree. A higher rate of ventilation leads to more dehydration and epithelial involvement, explaining the correlations between the decrease in F_ENO and the increased CC16 levels in plasma.

In paper IV, we present results indicative of a peripheral airway involvement during the LAR. The occurrence of correlations between parameters measured with spirometry, IOS, body plethysmography and IGW vary from baseline to the different phases of the allergic response (EAR, LAR and 23 h post-challenge). At baseline, FEV_1, most of the IOS parameters and the R_{tot} correlate with each other while there are few correlations with indices of ventilation heterogeneity.

Apart from correlations that can be expected from a basic physiological perspective (such as FVC and RV/TLC), there were three pairs of parameters that correlated with each other at baseline and throughout all the different phases of the allergic response: R5-R20 and S_{acin}; R5-R20 and R_{tot}; and FEV_1 and R5. R5-R20 is an interesting parameter that has been interpreted in two different ways by different researchers. Some have stated that R5-R20 is an index of peripheral airway resistance[168], while others regard it as a parameter reflecting ventilation heterogeneity[169]. Based on our findings, we believe that R5-R20 is a parameter that reflects both peripheral airway resistance (based on the correlations with R_{tot}) and ventilation heterogeneity (because of correlations with S_{acin}). It is however possible that ventilation heterogeneity is the dominating, or even single, factor behind an increasing R5-R20, seeing as heterogeneous distribution of bronchoconstriction will lead to a higher total resistance compared to the same degree of bronchoconstriction distributed more equally[78, 79]. The correlations between FEV_1 and R5 have been seen in previous studies of both asthmatic children[170] and adults[171] before and after bronchodilation. It is explained by the fact that R5 represents the resistance of both distal and proximal airways, the latter of which is also reflected in FEV_1. Correlations between R5 and R_{tot} are also expected, and were seen both at baseline and 23 h post-challenge. The reason for the absence of correlations between R5 and R_{tot} during the EAR and the LAR could be that there is some degree of small airway closure during these phases, leading to an increase in R_{tot}[172] but a partial false improvement in R5 due to inaccessibility of oscillometric pressure impulses to non-ventilated units.

There are of course a few limitations to the studies presented in this thesis. Firstly, the groups are small, generally not allowing for any more advanced statistical analysis of causative relations and further subgroupings. Our findings can however be used as indications of what the methods described can be used for, and what some of the issues might be with interpreting the outcomes. Secondly, in the studies focusing on EBT (I-III) there was limited phenotyping of our subjects; participants could have different degrees of airway inflammation and the anatomical distribution may have differed. One could expect that our subjects constitute a mixture of diversities in their "physiotype" (central airway inflammation only vs. peripheral inflammation only or a combination of the two), inflammation status (low vs. high current inflammation status) and inflammatory phenotype (eosinophilic vs. non-eosinophilic). If one would, for example, select

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patients with signs of peripheral inflammation only, more differences between asthmatics and healthy subjects regarding EBT measurements could possibly have been found. However, in everyday clinical practice and especially in a primary care setting, we rarely know beforehand which type of airway inflammation our patients have, limiting the usefulness of a method that requires extensive phenotyping first. Our subjects were all relatively young and most asthmatic subjects had been diagnosed during childhood or adolescence. Furthermore, the majority of asthmatic subjects were atopic. These factors combined make the early-onset allergic asthma the expected leading phenotype in our asthmatic subjects.

Conclusions and future perspectives

There is increasing interest in the peripheral airway inflammation in asthma, but the difficulties associated with trying to assess them in clinical practice constitute a major obstacle. Future studies should address how and if sufficiently simple methods to detect small airway dysfunction can be used in e.g. a primary care or specialist outpatient clinical setting to improve asthma care. The methods presented in this thesis that have shown promise in detecting small airway dysfunction include IOS, IGW, body plethysmography and, from previous studies and in asthmatics with eosinophilic inflammation, F_ENO with estimation of alveolar NO.

IOS is a potentially useful test in the clinical setting, not only for its ability to detect peripheral airway dysfunction, but also because of its applicability in young children[173] and other patients that are not able to perform spirometry. There are also indications that IOS is more sensitive in detecting early signs of persistent asthma[174]. Further research will be needed to determine normal reference values and the utility of IOS in studies of long-term treatment effects. IOS and IGW, sharing many of the benefits of IOS, will however undoubtedly play an increasingly important role in our assessment of asthma and other pulmonary diseases in the future.

In our experience and based on findings reported in this thesis, EBT is not a method suitable for separating patients with asthma from those without. However, previous results have shown that EBT could play another role in the clinical setting, as a way to monitor the individual patient with asthma or other inflammatory airway diseases. Ideally, it would predict exacerbations and guide the patient and/or health care provider in adjusting the dose of ICS accordingly. Theoretically, EBT is influenced by peripheral airway inflammation, and our findings of sustained elevation of EBT in asthmatics with exercise-induced bronchoconstriction indicate that EBT could be used to phenotype patients with a pre-established diagnosis of asthma. Future studies should therefore further explore if a higher degree of peripheral airway inflammation is associated with an increase in EBT. Signs of peripheral airway inflammation could be an indication that the patient needs treatment with extra-fine particle ICS treatment and, based on previous reports[105], there is indication that this approach will normalize an elevated EBT.

Leakage of CC16 from the airway epithelial lining fluid into plasma has emerged as an interesting biomarker of epithelial damage, as demonstrated in this thesis. Previous studies have however shown that long-term chronic inflammation or toxic exposure can reduce CC16 levels, and circulating levels can be permanently decreased because of genetic variations. These factors combined make interpretation of a single measurement complex. High levels of plasma CC16 would however still indicate acute epithelial damage, especially if the baseline CC16 level is known, and there is a potential of using CC16 to confirm or rule out airway epithelial damage after airway challenges or exposures, even without assessing airway physiology. To follow CC16 plasma levels from year to year could perhaps be a way to monitor the progression of an airway disease, similar to the potential role of EBT measurements.

Epithelial dysfunction is believed to be of importance in asthma development, but knowledge about the effects of different inhaled particles, such as allergen, on the epithelium is limited. We have demonstrated that inhaled allergen induces an increase in plasma CC16, and we hypothesize that the reason for this is increased leakage due to an epithelial dysfunction. If future asthma research aims to find treatments that preserve the airway epithelial integrity, CC16 in plasma could be used as the outcome to evaluate the treatment effect.

The allergen challenge and the ability to experimentally reduce the LAR are often used in clinical trials that aim to find new treatments for asthma. We have demonstrated that the LAR is associated with a significant small airway dysfunction that is at risk of being overlooked if we would use only spirometry as the tool of assessing the response. Future studies could include an alternative method, such as IOS, IGW or body plethysmography, to make sure that dual responders are not incorrectly identified as single responders. This could potentially improve the quality of the clinical trials and increase chances of finding effective therapies targeting the small airways.

Our work may also aid in improving the clinical care of patients with asthma and other pulmonary diseases, as a potential small step towards the implementation of airway assessments that are easy to use, feasible at all ages and providing more of a comprehensive picture of the patient. This will possibly improve the diagnostic accuracy, the awareness of fluctuations in disease activity and the tailoring of appropriate individualized treatment.

In the studies presented in this thesis, we have explored several different methods of assessing the airway response to different stimuli. We have compared the responses of asthmatic and non-asthmatic subjects, and explored the outcomes of different methods assessing the asthmatic airway response. This has hopefully contributed to a deeper understanding of the asthmatic airway response and the ways in which we are trying to measure it.

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Populärvetenskaplig sammanfattning

Astma är ett mycket vanligt tillstånd i befolkningen. Astma drabbar luftvägarna, och innebär att det dels finns en varierande grad av ständigt pågående retning (inflammation) och dels att det kan finnas en tendens hos luftvägarna att reagera lättare på olika typer av påfrestningar. En sådan reaktion innebär att luftvägarna drar sig samman med hjälp av muskler som omsluter luftvägarna, och det blir då trångt i luftvägarna och svårt att andas för personen som drabbas.

Astma kan se väldigt olika ut hos olika personer, och det finns stora variationer i hur svår sjukdomen är. Hos en del ger den knappt märkbara besvär ett fåtal gånger i livet, medan den hos de allra svårast sjuka kan innebära dagliga symtom och begränsningar, och i ovanliga fall till och med luftvägsreaktioner som kan vara dödliga. Det är alltså av största vikt att man inte bara identifierar de personer som har astma, utan också försöker uppskatta hur svår astman är, för att kunna sätta in rätt sorts behandling till de som behöver det. Eftersom symtomen och graden av muskelsammandragning i luftvägarna varierar mycket från period till period kan det vara svårt att ställa diagnosen astma med de lungfunktionsundersökningar som vi har idag. Det finns därför ett behov av att utveckla nya mätmetoder som kan upptäcka astma även då lungfunktionen är normal, det vill säga även då det inte förekommer någon ökad muskelaktivitet i luftvägarna. För att undersöka personer med misstänkt astma och för att studera astma i forskningssammanhang används ofta så kallade luftvägsprovokationer. Detta innebär att man retar luftvägarna för att medvetet framkalla en reaktion. Det finns flera olika typer av luftvägsprovokationer, till exempel ansträngningstest samt inandning av olika retande ämnen.

Ett annat sätt att upptäcka astma kan vara att mäta inflammation i luftvägarna, som förmodligen är mer konstant närvarande. Vid inflammation är immunförsvaret, kroppens försvarssystem mot virus och bakterier, aktiverat. Oftast fyller inflammationen en roll i kampen mot de ämnen som kroppen vill få bort, men ibland förekommer inflammation utan att det utlösts av något ämne som är farligt. Kroppen har då av någon anledning feltolkat signaler och överreagerat. Ett sådant exempel är allergier, till exempel mot pollen eller kvalster. Med en långvarig inflammation riskerar de celler som ska bekämpa främmande ämnen att istället skada kroppens egna vävnader i det område där inflammationen är aktiverad. Det har hittills visat sig svårt att hitta en metod för att mäta inflammation som fungerar

hos alla patienter med astma, eftersom inflammationen kan ta sig olika uttryck hos olika personer. En optimal metod skulle alltså utnyttja en gemensam nämnare hos alla dessa personer. Den skulle kunna användas för att följa graden av inflammation hos en och samma person över tid, för att kunna utvärdera om behandlingen fungerar som den ska och kunna upptäcka eventuella försämringar i sjukdomen i god tid för att då kunna anpassa behandlingen på ett bra sätt.

I inflammation ingår bland annat att blodkärlen vidgas och blodflödet ökar till den del av kroppen där inflammationen uppstår, och därför har man tänkt att det förmodligen också innebär att personer med astma har ett högre blodflöde i kärlen som omger luftvägarna. Att direkt mäta blodflödet är svårt, men däremot kan man indirekt uppskatta detta genom att mäta temperaturen på den luft som värms upp av blodkärlen i lungorna, det vill säga utandningsluften. Metoden har testats ett fåtal gånger, och en del resultat tyder på att temperaturen är högre hos personer hos astma, men det behövs fler studier innan man kan säga säkert att temperaturmätning av utandningsluften kan användas inom sjukvården och av patienter med astma.

Tidigare studier av luftvägarna hos personer med astma har visat att sjukdomens svårighetsgrad och beteende beror på var i luftvägarna inflammationen finns. Hos de som har en inflammation som drabbar mer perifera luftvägar, det vill säga mindre luftvägar som finns längre ut i luftvägsträdet, så verkar astman ge mer symtom och vara mer svårbehandlad. Det finns alltså också ett behov av att identifiera vilka personer med astma som har en mer perifer inflammation. Även här saknas det bra välstuderade metoder som säkert kan användas i detta syfte.

Syftet med denna avhandling var att undersöka hur olika typer av luftvägsprovokationer påverkade temperaturen på utandningsluften och andra sätt att mäta luftvägarnas egenskaper hos personer med astma. Resultaten jämfördes med friska personer utan astma för att kunna se om reaktionerna skiljde sig åt.

Vi har visat att olika typer av luftvägsprovokationer leder till att temperaturen på utandningsluften höjs, oftast under cirka 30 till 60 minuter efteråt, vilket tyder på en reaktion med vidgade blodkärl och ökat blodflöde vilket kvarstår under en begränsad tid. Ett intressant fynd var att denna reaktion förekom i nästan lika stor utsträckning hos personer med astma, det vill säga de som har en inflammation i sina luftvägar, som hos helt friska personer. Detta betyder att ökad temperatur på utandningsluften efter någon form av retning av luftvägarna verkar vara en normal reaktion i kroppen, oberoende av om man har ett sjukdomstillstånd med inflammation i luftvägarna eller ej. Vi kunde dock också visa att reaktionen verkade kvarstå längre hos personer med astma, och att temperaturen ökade under en allergisk reaktion utan annan retning av luftvägarna, vilket tyder på att inflammation kan bidra till ännu större temperaturökning.

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Vi har också undersökt vad som händer vid luftvägsprovokationer genom att titta på ett protein som heter CC16 och som finns i luftvägarna. När luftvägarna skadas på något sätt tror man att CC16 kan läcka ut i blodet. Vi såg att nivån av CC16 i blodet mycket riktigt ökade efter både intensiv ansträngning och inandning av allergiframkallande ämnen, något som man tror kan skada luftvägarna både på kort och på längre sikt.

Dessutom har vi studerat andra metoder för att mäta luftvägsreaktionen i de små perifera luftvägarna. Av alla de personer som reagerar med en astmatisk reaktion av att andas in allergiframkallande ämnen så utvecklar cirka hälften även en så kallad sen-reaktion, som är en ny luftvägsreaktion som uppträder efter några timmar. Vi har visat att denna sen-reaktion är förknippad med en sjukdomsprocess i de små luftvägarna, och att det finns en risk att detta missas om man bara använder vanliga etablerade luftvägsundersökningar. Vi har därför föreslagit att definitionen av en sen-reaktion i framtiden ska ändras så att den även innefattar metoder som upptäcker reaktioner i små luftvägar.

Sammanfattningsvis har vi dels demonstrerat hur flera metoder kan användas för att upptäcka och mäta den astmatiska luftvägsreaktionen, och dels bidragit till förståelsen för hur luftvägarna reagerar vid olika typer av retningar. Förhoppningen är att detta ska bidra till att astma och eventuellt även andra luftvägssjukdomar i framtiden blir lättare att identifiera, klassificera och behandla.

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Acknowledgements

Many people have helped me along the way towards finishing this thesis. I would like to thank some of them especially.

My supervisor *Ellen Tufvesson*. Thank you for believing in me from the very start and for your continuing support and patience. You have always been a true mentor and the perfect guide to all aspects of the world of science.

My co-supervisor *Leif Bjermer*. Your creativity, passion and endless curiosity are an inspiration to every researcher!

Jonas Olsson, who has worked with me on each and every project, through some long days, early mornings and late nights. Thank you for your company, for always having the patient's perspective first and for never surrendering neither your professionalism, nor your kindheartedness.

Ida Åberg, your sincerity, thoughtfulness and your dedication to maintaining the highest level of both quality and safety in research have been invaluable to me. You also have an impressive ability to organize the seemingly chaotic!

Thank you *Zuzana Diamant*, for your never-ending support and for teaching me not only about allergen challenges, but also about the world of research outside of Sweden and a few things about life as well.

Many thanks to the staff at the research clinic: *Louise Qvist, Anna Sikesjö, Tina Olsson, Johan Olsson, Mette Solum, Kerstin Hansson, Jaro Ankerst* and *Alf Tunsäter*. I couldn't possibly count the number of times that I have asked you for help, but I know for certain that each time you have assisted me in the most kind and professional way imaginable.

My research group members during different times: *Linnea Jarenbäck, Julie Weidner, Subhabrata Moitra, Sofia Mogren* and *Cecilia Hagman*. Thank you for creating such a nice and friendly atmosphere, and for great company through long workdays and during numerous events outside of work. Thanks to all the people at BMC, for entertaining discussions about science as well as other things. Many thanks to *Jonas Einarsson* and the rest of the pulmonologists in Lund for inspiring me to take an interest in pulmonary medicine, even though I finally decided to practice it outside of the hospital. *Eva Körner Muhrbeck,* thank you for solving any issue with the utmost efficiency.

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Exhaled Breath Temperature Increases after Exercise in Asthmatics and Controls

Henning Svensson   David Nilsson   Leif Bjermer   Ellen Tufvesson

Respiratory Medicine and Allergology, Department of Clinical Sciences, Lund University, Lund, Sweden

Abstract

Background: Exhaled breath temperature (EBT) has been suggested as a marker of airway inflammation in asthma. **Objectives:** The aim of the present study was to investigate EBT in asthmatic subjects compared to healthy controls after an exercise challenge test, and in subjects with exercise-induced bronchoconstriction compared to subjects without, and to compare with body temperatures. **Methods:** A total of 21 healthy controls and 20 asthmatics were included. Forced expiratory volume in 1 s (FEV_1), EBT and oral, axillary and auricular temperatures were measured before and after an exercise challenge test. **Results:** FEV_1 % predicted (%p) was significantly lower in asthmatic subjects compared to healthy controls at all time points after exercise. The largest drop in FEV_1%p correlated with EBT after 5 min. EBT increased markedly 5 min after exercise and remained high for at least 60 min. In asthmatics whose FEV_1 dropped by >10%, EBT was higher after 60 min compared to the remaining asthmatics. EBT correlated with oral temperature at all time points after exercise, with axillary temperature only at 15, 30 and 60 min, and not at all with auricular temperature. **Conclusions:** EBT is increased after exercise, and elevated EBT correlated with a drop in FEV_1%p. The immediate increase in EBT did not differ between asthmatics and controls but remained elevated in the asthmatics whose FEV_1 dropped by >10%, indicating a different vascular response.

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Introduction

Asthma is characterized by an inflammation of the airways, with increased hyperreactivity and vascularization of the bronchial smooth muscle. Several factors, such as allergens, exercise and cold or hot air, may give rise to bronchoconstriction. During bronchoconstriction, the airways narrow and asthmatic patients experience respiratory distress.

During exercise, the respiratory rate increases, leading to an exertion of the airways that may result in bronchial constriction and a decline in lung function, measured as forced expiratory volume in 1 s (FEV_1). Asthmatic patients are reported to respond in this fashion to varying degrees, a condition referred to as exercise-induced asthma (EIA). The presence of EIA in an asthmatic patient indicates that inflammatory processes are active, with in-

H.S. and D.N. contributed equally to the study.

Table 1. Subject characteristics

	Controls (n = 21)	Asthmatics (n = 20)
Male/female, n	13/8	11/9
Age, years	23 (21–25)	22 (21–25.3)
Baseline FEV_1%p	107.5 (101.8–114.0)	99.7 (94.8–105.7)
Baseline EBT, °C	33.82 (33.65–34.30)	34.10 (33.82–34.30)
FEV_1 drop >10%, n	6	7
FEV_1 maximum drop, %	5 (4–13)	8 (5–13)
Taste of blood after exercise, n	6	7
Atopy, n	7	16
ICS, n	0	10
Exhaled NO, ppb	18.0 (11.5–23.5)	14.5 (10.3–29.8)

Data are expressed as medians (interquartile range), where appropriate. ICS = Inhaled corticosteroids (400–800 µg of budesonide/day).

creased activity and concentration of eosinophils in the sputum [1].

In addition, exercise-induced bronchoconstriction (EIB) has been suggested to describe the narrowing of the airways occurring in otherwise healthy persons with no symptoms of asthma. The prevalence of EIB in the non-asthmatic general population is believed to be over 10% [2] and is markedly increased among endurance sports athletes [3, 4]. It is currently unclear what similarities and disparities exist between EIA and EIB [5]. EIA and EIB are usually defined on the basis of a fall of >10% from baseline FEV_1 after a standardized exercise challenge test [6]. The pathogenesis of EIA and EIB remains elusive, although evidence points to dehydration of the airways with eosinophil and mast cell degranulation as central mechanisms behind the phenomenon [7–9], increasing both bronchial muscle constriction and blood vessel dilatation. The latter could be the explanation for an increase in EBT and would possibly be even more pronounced in subjects with concomitant airway inflammation. Prolonged hyperventilation of cold or dry air can cause the dehydration, suggesting an explanation for the high prevalence of the condition among cross-country skiers, cyclists and other endurance sports athletes. Furthermore, exercising in cold air may temporarily decrease the levels of exhaled nitric oxide (NO), reducing its bronchodilating effect [10].

Previous studies have reported that asthmatics present an elevated exhaled breath temperature (EBT) compared to healthy controls [11], and it has been suggested to be a

new biomarker in asthma control [12, 13]. The elevation of exhaled NO as an inflammatory marker, seen in asthmatics [14], has previously been shown to correlate with increased EBT [15]. EBT is believed to be affected by the increased vascularization of the airway mucosa shown in asthmatics, leading to increased heat exchange during expiration [16]. The vasodilatation thought to occur in EIA and EIB might lead to further increased heat exchange during expiration. However, research on EBT is currently very limited, and a potential relationship between EIA/EIB and EBT needs further investigation.

By exercising, an inflammatory response of the airways, with vasodilatation and bronchial smooth muscle constriction, is induced in asthmatic subjects. We hypothesized that the EBT of asthmatic subjects would increase more compared to the EBT of healthy controls after exercise due to increased vascularization. The aim of the present study was to investigate how EBT is affected by exercise in asthmatic subjects compared to healthy controls, as well as in those who show signs of EIB compared to those who do not. A secondary aim was to compare EBT with other body temperature measures, to set an increase in EBT in relation to an overall increase in body temperature [17] during exercise.

Methods

Subjects

Twenty subjects with a doctor's diagnosis of mild asthma according to the guidelines of the Global Initiative for Asthma [18] were investigated (table 1). Twenty-one healthy subjects without respiratory symptoms, diagnosed asthma or dyspnea during exercise were used as controls. Subjects with respiratory tract infection (within the previous 2 weeks) or other medical conditions (apart from asthma) affecting their health were excluded from the study. The subjects were not allowed to drink coffee for at least 2 h prior to the study, to conduct any form of exercise on the same day or to use any exhausting means of transportation to get to the laboratory. All asthmatic subjects refrained from using β_2-agonists, as well as inhaled corticosteroids for at least 48 h before the study. Subjects filled out a form concerning their previous and present health prior to the study. All subjects gave written informed consent, and the study was approved by the Local Ethics Committee of Lund University (LU-357/2008).

Study Design

After at least 15 min of rest, exhaled NO, spirometry, oral temperature, EBT and axillary and auricular temperatures were measured (in the given order). Four ECG leads were used to measure the heart rate throughout the exercise challenge test. The subjects were not allowed to warm up beforehand. The exercise challenge test was divided into 3 phases conducted on a treadmill, as follows: (1) during the first 2 min of running, the speed and level of upward slope were set to increase the heart rate of the subject to
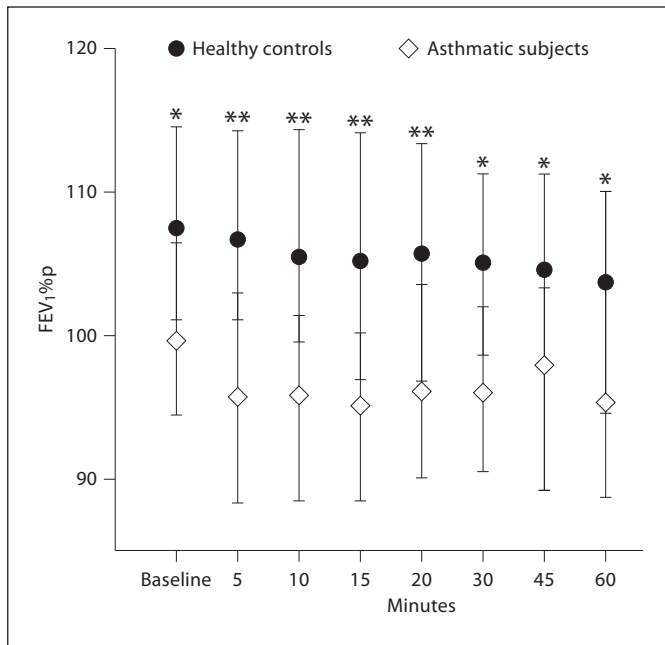
Fig. 1. FEV$_1$%p in controls and asthmatic subjects before (baseline) and 5, 10, 15, 20, 30, 45 and 60 min after exercise. * p < 0.05, ** p < 0.01: significant difference between controls and asthmatics.

Fig. 2. Correlation between change in FEV$_1$%p (ΔFEV$_1$%p; defined as lowest FEV$_1$%p after exercise – baseline FEV$_1$%p) and EBT at 5 min after exercise. ● = Healthy controls; ◇ = asthmatic subjects.

approximately 90% of the calculated maximum capacity (defined as 220 beats per min – age in years); (2) the speed and level of upward slope were adjusted to keep the subject's heart rate steady at that level for 4 min, and (3) during the last 2 min, the speed and level of upward slope were increased to push the subject's heart rate above the level of the previous phases and induce maximum exertion. Thereafter, the subjects rested for 60 min, and spirometry, oral temperature, EBT and axillary and auricular temperatures were measured repeatedly as described below. The environmental conditions were a stable temperature of 20–23°C and a relative humidity of 25% throughout the study.

Exhaled NO Measurement
Exhaled NO was measured before exercise by a handheld device (NIOX Mino, Aerocrine, Sweden) according to the American Thoracic Society and European Respiratory Society recommendations [19] with an exhalation flow rate of 50 ml/s.

Lung Function Test
Flow volume spirometry (Jaeger MasterScope, Würzburg, Germany) measuring FEV$_1$ was performed 3 times before (baseline) and once 5, 10, 15, 20, 30, 45 and 60 min after the exercise challenge test. FEV1 % predicted (%p) was calculated according to the reference spirometric values of Crapo et al. [20].

EBT Measurement
EBT was measured using an X-halo (Delmedica Investments, Singapore) once before and 5, 15, 30, 45 and 60 min after the exercise challenge test. Subjects were instructed to breathe tidally,
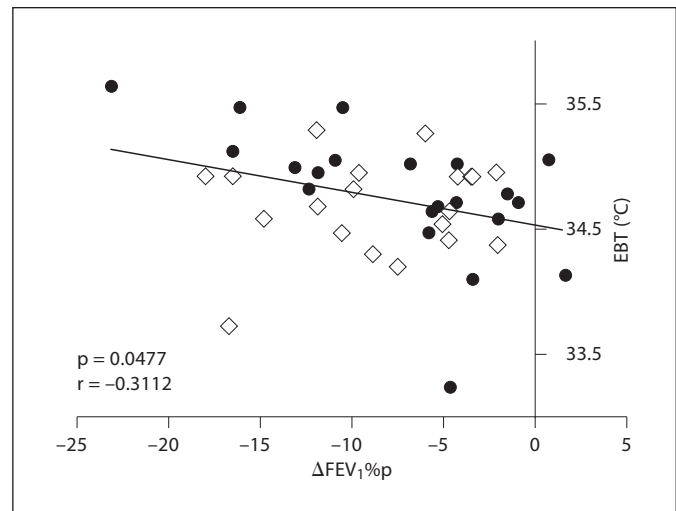
inhaling through the nose and exhaling through the mouth into the device. Measurements took 1–5 min to complete. Between measurements, the X-halo was stored in an incubator at 32°C, in order to maintain a stable starting temperature.

Body Temperature Measurement
Oral and axillary temperatures were measured once, and auricular temperature was measured 3 times (the median of each set of measurements was used), before and 5, 15, 30, 45 and 60 min after the exercise challenge test.

Statistical Analyses
GraphPad Prism version 5.0 was used for statistical analysis. The results are expressed as medians (interquartile range). Correlations were calculated by Spearman's correlation test. Comparisons between groups were made by the Mann-Whitney test. Wilcoxon's test was used for analysis of paired measurements. A p value of <0.05 (two tailed) was considered significant.

Power calculation was performed based on two independent groups of 20 evaluable subjects. With an expected standard deviation of 0.5, it was possible to detect a difference of 0.5°C with a power of 87%.

Results

Subjects
All subjects were able to complete the exercise challenge test. FEV$_1$ dropped more than 10% compared to baseline in 7 asthmatics and 6 healthy controls (table 1). The Asthma Control Test score among the asthmatics
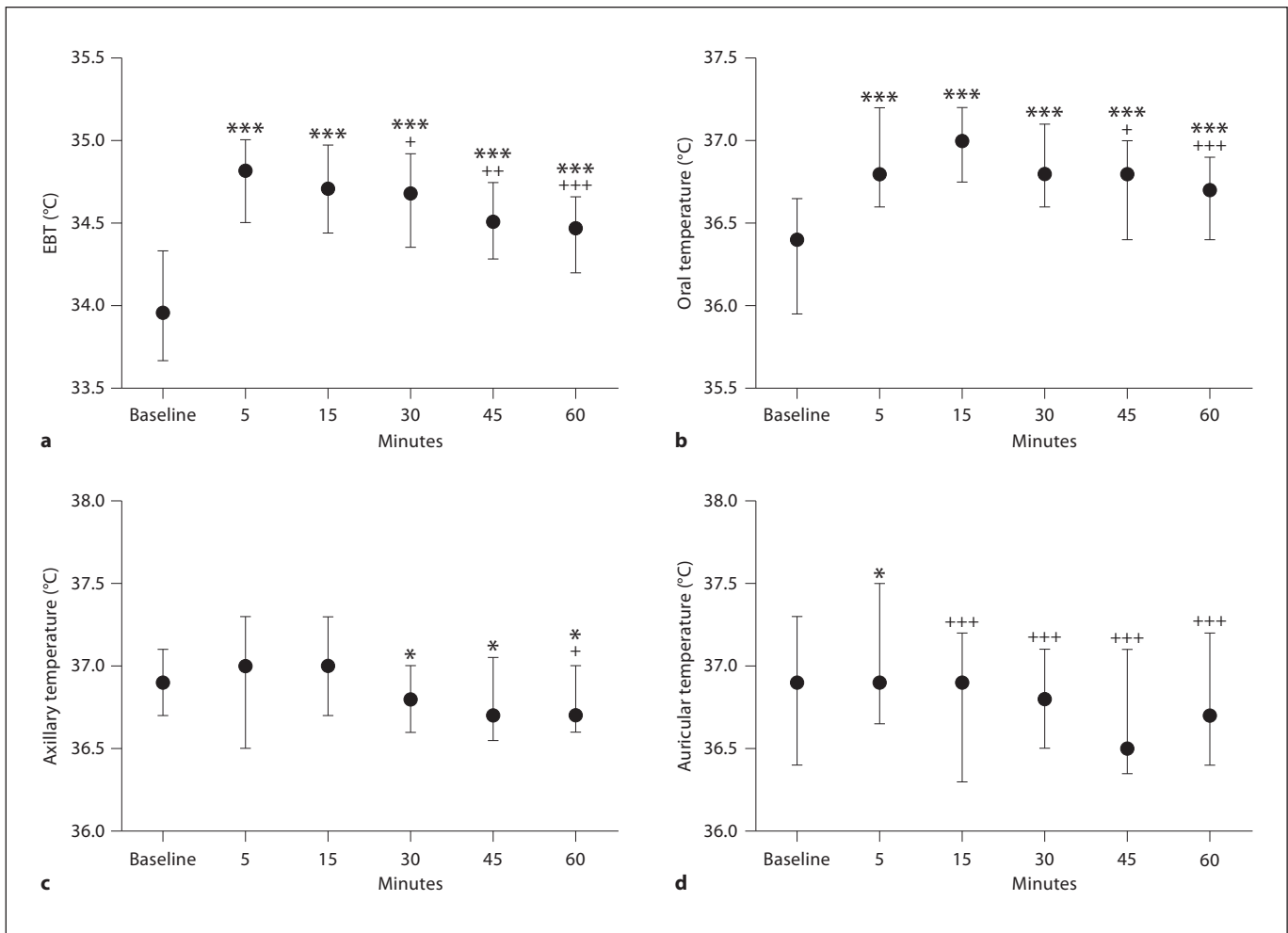
Fig. 3. EBT (**a**) and body temperatures [oral temperature (**b**), axillary temperature (**c**) and auricular temperature (**d**)] before (baseline) and 5, 15, 30, 45 and 60 min after exercise in all subjects. * p < 0.05, ** p < 0.01, *** p < 0.001: significant difference compared to baseline; + p < 0.05, ++ p < 0.01, +++ p < 0.001: significant difference compared to the measurement at 5 min.

was 23 (21–24), and it did not differ between those whose FEV_1 dropped more than 10% after the exercise test and those in whom it did not.

Lung Function

Levels of $FEV_1\%p$ were significantly lower in asthmatic subjects compared to healthy controls at baseline (p = 0.020) and 5 (p = 0.0020), 10 (p = 0.0018), 15 (p = 0.0028), 20 (p = 0.0088), 30 (p = 0.011), 45 (p = 0.031) and 60 min (p = 0.034) after exercise (fig. 1).

The fall in $FEV_1\%p$ after exercise (defined as lowest $FEV_1\%p$ after exercise – baseline $FEV_1\%p$) correlated negatively with EBT after 5 min [all subjects: p = 0.048, r = –0.31 (fig. 2); asthmatics: p = 0.82, r = 0.053; controls:

p = 0.0049, r = –0.59]. The fall in $FEV_1\%p$ did not correlate with EBT measured at other time points or with the change in EBT (difference in EBT compared to baseline) at any time point (either for asthmatics, controls or all subjects together).

No significant difference in $FEV_1\%p$ was seen between male and female subjects or between steroid-treated and non-steroid-treated asthmatic subjects (data not shown).

Exhaled Breath Temperature

EBT was significantly elevated in all subjects 5, 15, 30, 45 and 60 min after the exercise challenge test compared to baseline (p < 0.0001; fig. 3a). The EBT was highest 5 min after exercise and decreased thereafter.
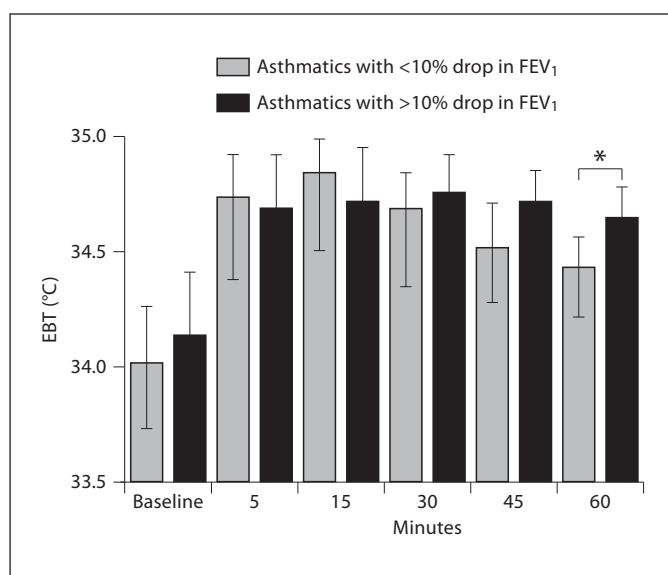
Fig. 4. EBT before (baseline) and 5, 15, 30, 45 and 60 min after exercise in asthmatic subjects whose FEV$_1$ dropped by >10% compared to asthmatic subjects in whom it did not. * p < 0.05.

Table 2. EBT in healthy controls and asthmatics after exercise

	EBT, °C		p value
	controls (n = 21)	asthmatics (n = 20)	
Baseline	33.82 (33.65–34.36)	34.10 (33.73–34.35)	0.51
5 min	34.82 (34.61–35.05)	34.75 (34.43–34.92)	0.21
15 min	34.64 (34.41–35.02)	34.82 (34.47–34.93)	0.89
30 min	34.64 (34.29–34.95)	34.72 (34.37–34.87)	0.91
45 min	34.51 (34.41–34.75)	34.58 (34.27–34.79)	0.84
60 min	34.44 (34.10–34.66)	34.47 (34.28–34.67)	0.68

Data are expressed as medians (interquartile range).

Table 3. EBT in subjects with or without a >10% drop in FEV$_1$ after exercise

	EBT, °C		p value
	no drop in FEV$_1$ >10% (n = 28)	drop in FEV$_1$ >10% (n = 13)	
Baseline	33.96 (33.70–34.27)	34.13 (33.57–34.41)	0.99
5 min	34.75 (34.49–34.95)	34.92 (34.53–35.21)	0.29
15 min	34.64 (34.43–34.87)	34.82 (34.42–35.09)	0.32
30 min	34.53 (34.31–34.84)	34.88 (34.66–34.95)	0.051
45 min	34.44 (34.28–34.71)	34.71 (34.34–34.87)	0.15
60 min	34.44 (34.18–34.60)	34.47 (34.42–34.73)	0.27

Data are expressed as medians (interquartile range).

EBT measured after 60 min was higher in asthmatic subjects whose FEV$_1$ dropped by over 10% compared to asthmatic subjects in whom it did not [34.64°C (34.47–34.78) vs. 34.41°C (34.19–34.55); p = 0.032; fig. 4]. This difference was not seen when comparing measurements of EBT taken at other time points. Moreover, EBT was not statistically different when comparing all asthmatic subjects with healthy controls at any time before or after the exercise challenge test (table 2). In addition, no differences were seen when comparing all subjects whose FEV$_1$ dropped more than 10% compared to baseline at any time after the exercise challenge test to those in whom it did not (table 3).

EBT was higher in asthmatic subjects treated with steroids compared to non-steroid-treated asthmatic subjects at baseline [34.13°C (34.04–34.42) vs. 33.78°C (33.52–34.27); p = 0.034] and after 5 min [34.92°C (34.65–34.95) vs. 34.53°C (34.28–34.85); p = 0.036]. After 15–60 min, this difference was no longer seen. No significant difference in EBT was seen when comparing male with female subjects.

There was a significant correlation between EBT and oral temperature measured at the same time points, i.e. 5 (p = 0.0020), 15 (p = 0.0024), 30 (p = 0.033), 45 (p = 0.013) and 60 min (p = 0.0083) after the exercise challenge test. However, baseline EBT did not correlate with baseline oral temperature (p = 0.081). EBT also correlated with

axillary temperature at 15 (p = 0.0016), 30 (p = 0.0092) and 60 min (p = 0.0024) but not with auricular temperature at any time point.

Body Temperatures

Oral temperature, when compared to baseline, did increase significantly 5, 15, 30, 45 and 60 min after exercise (p ≤ 0.0001 for all; fig. 3b). There was no difference in oral temperature when comparing asthmatic subjects with controls or all subjects whose FEV$_1$ dropped more than 10% compared to baseline with those in whom it did not. Neither was there any difference specifically between asthmatic subjects whose FEV$_1$ dropped by over 10% and asthmatic subjects in whom it did not.

Axillary temperature did not change 5–15 min after exercise but decreased significantly 30 (p = 0.047), 45 (p = 0.028) and 60 min (p = 0.035) after exercise compared to baseline (fig. 3c). The axillary temperature was higher in those whose FEV$_1$ dropped by over 10% compared to

those in whom it did not at 45 min [36.9°C (36.8–37.2) vs. 36.7°C (36.5–37.0); p = 0.027] but not at any other time point. There was no difference in axillary temperature when comparing asthmatics with controls or asthmatic subjects whose FEV_1 dropped by over 10% with asthmatic subjects in whom it did not.

Auricular temperature was significantly elevated 5 min after exercise compared to baseline (p = 0.0019) but not at any other time point (fig. 3d). Auricular temperature did not differ between asthmatic subjects and controls, between those whose FEV_1 dropped more than 10% and those in whom it did not, nor specifically between asthmatic subjects whose FEV_1 dropped by over 10% and asthmatic subjects in whom it did not.

Exhaled NO
NO levels were not statistically different in asthmatic subjects compared to healthy controls (table 1), in asthmatic subjects whose FEV_1 dropped by over 10% compared to asthmatic subjects in whom it did not, nor in asthmatic subjects using inhaled corticosteroids compared to those not on inhaled corticosteroids. The levels of NO in exhaled air did not correlate with levels of EBT or the greatest increase in EBT after exercise.

Discussion

This study showed that EBT was markedly increased 5–60 min after exercise and that no corresponding increase in body temperature was seen (measured as axillary and auricular temperature). We also showed that the fall in FEV_1%p after exercise correlated negatively with EBT after 5 min, demonstrating that subjects with a larger fall in FEV_1%p have a higher EBT after exercise. Our primary hypothesis was that asthmatics or even subjects with EIB would be expected to have increased EBT in response to exercise, due to the idea that airways with established inflammation would experience a higher increase in EBT after exercise. Interestingly, this could not be confirmed by this study, as there was no difference between subjects with previously diagnosed asthma and healthy controls, nor between subjects with and without EIB. This suggests that EBT changes related to exercise may reflect a normal physiological response in the airways rather than being due to pathology. While the increase in EBT changes after exercise did not differ between healthy subjects and the entire group of asthmatics, EBT remained higher 60 min after exercise specifically in asthmatics with EIB. This delayed recovery

could possibly reflect some degree of inflammation in the airways.

It has previously been hypothesized that dehydration of the airways with increased osmolarity in the mucosal surface liquid leads to mast cell degranulation [21, 22], releasing bronchoconstricting mediators, such as bradykinin and histamine, and activating endothelial nitric oxide synthase [23], which exerts a vasodilating effect. This could explain the increase in EBT after exercise, suggesting that elevated EBT is a potential marker of a temporary effect on the airways, such as bronchoconstriction or swelling due to vasodilatation, rather than of the long-term inflammation of the asthmatic disease.

A particular finding in our study is that some subjects, irrespective of the degree of exercise or exhaustion, reported the sensation of a 'taste of blood' directly after the test. The origin for this sensation is not known, but it is a widely recognized response to vigorous exercise. Among our subjects who were positive for a taste of blood, most reported that the sensation started directly after the exercise and became more manifest after 10–15 min. A greater drop in FEV_1%p was seen in the subgroup positive for the blood taste compared to those negative for it [–11.9%p (–16.3 to –7.2) vs. –4.9%p (–10.5 to –3.4); p = 0.013]. Baseline levels of FEV_1%p were also significantly higher in asthmatic subjects who reported a taste of blood after exercise compared to asthmatic subjects who did not [104.9%p (104.2–114.2) vs. 95.2%p (92.4–101.6); p = 0.032]. In addition, a larger difference in EBT from baseline to directly after the exercise test (at 5 min) was seen in those subjects who reported a taste of blood compared to those who did not [0.99°C (0.77–1.49) vs. 0.65°C (0.39–0.94); p = 0.0088]. The sensation of a taste of blood may therefore be explained by a vascular response with increased blood circulation and increased heat emission through vasodilatation, followed by an increased temperature in the airways. It is known that exercise per se may alter taste sensation; thus subjects reporting a taste of blood may have a much more pronounced response in the entire respiratory tract, including the nose [24].

Our secondary aim was to investigate the relationship between EBT and body temperatures, which are known to be increased after exercise [17]. In fact, there was a substantially greater increase in EBT than body temperature, measured as axillary and auricular temperature. In addition, while EBT and oral temperature were elevated compared to baseline for at least 60 min after exercise, axillary and auricular temperatures were not. EBT correlated considerably with oral temperature but not with axillary or auricular temperature. One explanation could be that

EBT and oral temperature most probably also reflect changes in the systemic temperature and are more accurate than axillary or auricular temperature. However, we believe that the elevation of EBT and oral temperature after exercise is primarily related to the airways and/or the oral cavity, as both are part of the respiratory tract and are affected differently than systemic temperature during exercise.

Exhaled NO is currently used as a marker of inflammatory processes in the airways, and previous studies have shown a higher level of NO in exhaled air of asthmatic patients [14]. In this study, no significant differences were found between asthmatic subjects and healthy controls, nor between those on steroid treatment and those not treated with steroids. This could be due to the fact that a population of subjects with mild, well-controlled asthma was used in this study, as confirmed by Asthma Control Test scoring. In addition, there is no consistency of increased NO in various asthmatic phenotypes; in particular, in the group of asthmatics with EIA there is no clear increase in NO. However, our asthmatics had significantly lower levels of $FEV_1\%p$ than the controls at baseline and at all time points after the exercise challenge test, showing impaired lung function among the asthmatics.

In this study, we conclude that EBT was increased after exercise. Oral temperature was similarly increased after exercise but not axillary or auricular temperatures. We also found a relationship between a drop in lung function and elevated EBT, as subjects with a larger fall in $FEV_1\%p$ had a higher EBT 5 min after exercise. The level of reduction in lung function after exercise, as well as EBT measured after exercise, were significantly higher in subjects who felt a taste of blood compared to those who did not. Interestingly, there was no difference between subjects with previously diagnosed asthma and healthy controls, nor between subjects with and without EIB. This suggests that EBT in relation to exercise may primarily reflect a normal physiological response in the airways. While the immediate response was the same in asthmatics and controls, EBT remained elevated only in asthmatics with EIB, suggesting some relation to persistent inflammation.

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respiratory MEDICINE

Increase of club cell (Clara) protein (CC16) in plasma and urine after exercise challenge in asthmatics and healthy controls, and correlations to exhaled breath temperature and exhaled nitric oxide

CrossMark

Ellen Tufvesson*, Henning Svensson, Jaro Ankerst, Leif Bjermer

*Respiratory Medicine and Allergology, Dept. of Clinical Sciences, Lund University,
221 84 Lund, Sweden*

Summary

Exercise is known to affect the airway epithelium through dehydration, followed by a release of mediators, such as club cell (Clara) protein (CC16). The aim of this study was to follow the CC16 levels at repeated time points in plasma and urine after exercise in asthmatic subjects and controls, and to relate the findings to exhaled breath temperature (EBT) and exhaled nitric oxide (NO).

Twenty-two asthmatics and 18 healthy subjects performed an exercise challenge test on a treadmill. Lung function, CC16 in plasma and urine, EBT and fractional exhaled NO were investigated before and repeatedly for 60 min after the exercise.

The increase in CC16 concentration in plasma was seen already one minute after exercise ($p < 0.001$) and increased further after 20 ($p = 0.009$) until 60 min ($p = 0.001$). An increase in urinary levels of CC16 peaked after 30 min ($p < 0.001$), and declined after 60 min but were still higher than baseline ($p = 0.002$). There were no differences in plasma or urine CC16 levels between asthmatics and controls, but males had higher plasma levels compared to females ($p < 0.001$) at all time points. EBT peaked at 15 min ($p < 0.001$) and thereafter declined, and $FE_{NO}50$ ($p < 0.0001$), alveolar NO concentration ($p = 0.049$) and bronchial flux of NO ($p = 0.0055$) decreased after exercise.

In conclusion, this study shows that CC16 in plasma increased during 60 min after exercise, not synchronized with CC16 levels in urine. CC16 levels in plasma correlated to EBT and

* Corresponding author. Tel.: +46 46 222 78 28.
  E-mail address: Ellen.Tufvesson@med.lu.se (E. Tufvesson).

exhaled NO, reflecting an overall epithelial involvement. There was no difference between asthmatics and healthy controls, showing a physiological rather than pathophysiological response.

Introduction

Exercise is known to affect the airway epithelium, and trigger an exercise-induced bronchoconstriction (EIB) due to the cooling and osmotic effects caused by airway dehydration [1]. The prevalence of asthma and EIB is markedly increased in elite athletes [2], and are most probably due to an epithelial injury arising from breathing poorly conditioned air at high flows for long periods of time [3]. Evaporative water loss from the airway surface, in conditioning the inspired air, is believed to be the stimulus for exercise-induced bronchoconstriction. An acute effect of this dehydration is the release of mediators which can stimulate smooth muscle contraction or protect the airways [4,5].

An important factor that is involved in this process is club cell (Clara) protein (CC16), a protein secreted primarily from the club cells (Clara) which are found predominantly in the terminal bronchioles [6]. CC16 is suggested to be a protective mediator in the airway inflammatory process [7,8]. In the airways of asthmatics, fewer CC16-positive epithelial cells have been found compared to controls [9]. The number of CC16 containing cells in the respiratory mucosa correlates with levels found in plasma [10], and lower levels of CC16 in serum from asthmatic children [11] and adults [9,12] have been shown accordingly. There are also studies showing polymorphism in the CC16 gene associated with asthma [13−15]. We have previously shown that epithelial stress occurs during exercise by demonstrating that CC16 is increased in urine after exercise [16,17] and eucapnic voluntary hyperventilation [18] in athletes. The levels of CC16 are also known to increase in serum after exercise [19−21]. However, even though CC16 is known to increase both in serum and in urine, a more detailed time kinetic view is lacking. Thus by frequent sampling closely after exercise it might be possible to clarify if the increase in CC16 levels in plasma is due to direct epithelial leakage or increased secretion/production. Whether the increase in CC16 is due to the osmotic stress and/or the mechanical stress generated within the airways when large quantities of unconditioned air are inhaled remains unknown. However, we know that it is dependent on inspired air conditions and is more abundant in dry compared to humid air [16].

The aim of this study was thus to investigate the epithelial involvement during exercise by repeatedly measure the levels of CC16 in plasma and urine in asthmatic subjects and healthy controls. Levels of CC16 in urine have previously shown very large individual variations, and by using complementary plasma levels we aimed to minimize these variations and possibly detect more distinct differences between asthmatics and healthy controls. With a tight schedule of plasma sampling we wanted to investigate a detailed time kinetic of the CC16 response after exercise.

A secondary aim was to investigate how exercise affects the relation between CC16 involvement, exhaled breath temperature (EBT) and exhaled nitric oxide (NO).

Materials and methods

Subjects

Twenty-two subjects with mild asthma according to Gina guidelines [22] and regular asthma symptoms were investigated (Table 1). Eighteen healthy subjects without respiratory symptoms or diagnosed asthma were used as controls. All subjects were non-smokers without respiratory tract infection within three weeks prior to the investigation. Caffeine was withheld two hours prior to the investigation, strenuous physical exercise was not allowed on the same day, and no form of exhausting mean of transportation to get to the laboratory was allowed. All asthmatic subjects refrained from using β_2-agonists and inhaled corticosteroids for at least 48 h before the study. No subjects were on anti-leukotriene treatment. The study was approved by the Regional Ethical Review board of Lund, and all subjects gave informed consent.

Study design

At arrival in the clinic, participants emptied their bladder and were asked to drink a glass of water. Thereafter, exhaled NO was measured, spirometry performed and blood sample, temperatures and urine sample were taken. Four ECG leads were used to measure the heart rate throughout the exercise challenge test. The subjects were not allowed to warm up beforehand. The exercise challenge test was divided into three phases (a−c) conducted on a treadmill: a) during the first two minutes of running, speed and level of upward slope were set to increase the heart rate of the subject to approximately 90% of calculated maximum capacity (defined as 220 beats per minute − age in years), b) the speed and level of upward slope were adjusted to keep the subject's heart rate steady at that level for four minutes, c) during the last two minutes, the speed and level of upward slope were increased to push the subject's heart rate above the level of the previous phases, and induce a maximum exertion. Hereafter, the subjects were resting for 60 min while blood and urine samples were taken, spirometry performed and EBT, oral and auricular temperatures were measured repeatedly as described below.

Flow-volume spirometry (Jaeger MasterScope, Wüzburg, Germany) measuring FEV_1 was performed three times before (baseline) and once 5, 10, 15, 20, 30, 45 and 60 min after the exercise challenge test. FEV_1% predicted (%p) was calculated according to Crapo [23].

Table 1 Patient characteristics and exhaled NO levels at baseline and post exercise.

Patient characteristics	Healthy controls $n = 18$	Asthmatics $n = 22$
Sex, male/female	9/9	12/10
Age, years	25 (22—35)	26 (24—33)
FEV_1, l	4.0 (3.3—4.7)	3.8 (2.9—4.5)
FEV_1, %p	101 (94—112)	95 (87—109)
Positive exercise test, n	1	9
Fall in FEV_1 during test, %	4.6 (0—6.8)	6.1 (3.0—18.8)
Atopy, n	4	12
ICS, n	0	15
Exhaled NO levels		
$FE_{NO}50$ baseline, ppb	15.8 (9.5—35.1)	21.8 (15.1—37.3)
$FE_{NO}50$ post exercise, ppb	13.1 (7.7—35.9)*	18.8 (11.2—27.7)***
CANO, baseline, ppb	2.7 (2.1—3.6)	3.2 (2.3—5.0)
CANO post exercise, ppb	2.6 (1.8—3.2)	2.6 (1.9—3.3)
$J'awNO$ baseline, nl/s	1.0 (0.3—1.8)	0.9 (0.6—1.5)
$J'awNO$ post exercise, nl/s	0.7 (0.3—1.7)*	0.9 (0.5—1.3)

Data are shown as median (IQR). FEV_1 = forced expiratory volume in 1 s, ICS = inhaled corticosteroids, $FE_{NO}50$ = fractional exhaled NO at flow 50 ml/s, CANO = alveolar NO concentration, $J'awNO$ = bronchial flux of NO, * = flagging statistical difference compared to corresponding baseline (*$p < 0.05$, ***$p < 0.001$).

CC16 analysis

Baseline plasma and urine samples were collected just prior to the exercise test. Following completion of the exercise tests, plasma samples were collected after 1, 5, 10, 15, 20, 30, 45 and 60 min, and urine samples were collected after 30 and 60 min. Because CC16 is also produced in small amounts in the male urogenital tract, the first 100 ml of each male urine collection was discarded in the present study, to minimize contamination [24]. All samples were stored at −80 °C and analysed within two months [21]. CC16 was measured using the Human Clara Cell Protein ELISA kit from BioVendor (Modrice, Czech Republic). All urine samples were corrected for creatinine. Urine levels of creatinine, albumin, immunoglobulin G and protein HC were measured at the Clinical Chemistry at Lund University Hospital, using a COBAS 6000 system.

Exhaled breath temperature

Exhaled breath temperature was measured in a sub fraction of the subjects ($n = 23$; 16 asthmatics and 7 non-asthmatics; 15 males and 8 females, same FEV_1 %p, exercise response, CC16 levels and NO levels as the total study

population), using an Xhalo (Delmedica Investments, Singapore), before and 15, 30, 45 and 60 min after the exercise challenge test. Subjects were instructed to breathe tidally, inhaling through the nose and exhaling through the mouth into the device. Measurements took 1—5 min to complete.

Oral and auricular temperatures were measured once before and 15, 30, 45 and 60 min after the exercise challenge test.

Exhaled nitric oxide concentration

NO measurements were performed using a NIOX Flex (Aerocrine, AB, Stockholm, Sweden). Patients were comfortably seated; inhaled NO depleted ambient air, and exhaled at different flow rates (50 (giving $FE_{NO}50$), 100, 200 and 300 ml/s) 3—4 times. Alveolar NO concentration (CANO) and bronchial NO flux ($J'awNO$) was approximated by plotting NO-output against exhaled flow (at 100—300 ml/s). The slope and intercept of this linear regression approximated CANO and $J'awNO$, respectively [25,26].

Statistical analyses

All statistical calculations were performed using SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL). CC16 results followed Gaussian distribution (Shapiro—Wilks test) and are therefore presented as mean (SEM), and statistical analyses were done using parametric tests (unpaired and paired t-test). All other results and correlations are expressed as median (interquartile range (IQR)), and statistical analyses were done using non-parametric tests (Mann—Whitney test for unpaired data, Wilcoxon's test for paired data and correlations were calculated by Spearman's correlation test). Unpaired tests were used for comparing cases (asthmatics versus healthy, males versus females, etc) and paired tests were used to compare repeated measures of CC16, EBT and exhaled NO. A p-value of <0.05 was considered significant.

Results

Exercise test

Nine asthmatics and one control subject had a positive exercise test (defined as a fall in $FEV_1 \geq 10\%$). There were no significant differences between asthmatics and healthy controls (or between asthmatics on ICS treatment compared to those who were not on ICS treatment) regarding age, sex, baseline FEV_1, FEV_1 %p or fall in FEV_1 after the exercise test (Table 1).

CC16 in plasma and urine

There was an increase in CC16 concentration in plasma already one minute after the exercise test compared to baseline ($p < 0.001$) in all samples (Fig. 1A). These higher levels thereafter remained and increased further after 20 ($p = 0.009$) until 60 min ($p = 0.001$). No difference between asthmatics and controls was seen, nor between all subjects who had a positive exercise test (including the healthy control subject with a positive exercise test) and those who did not.
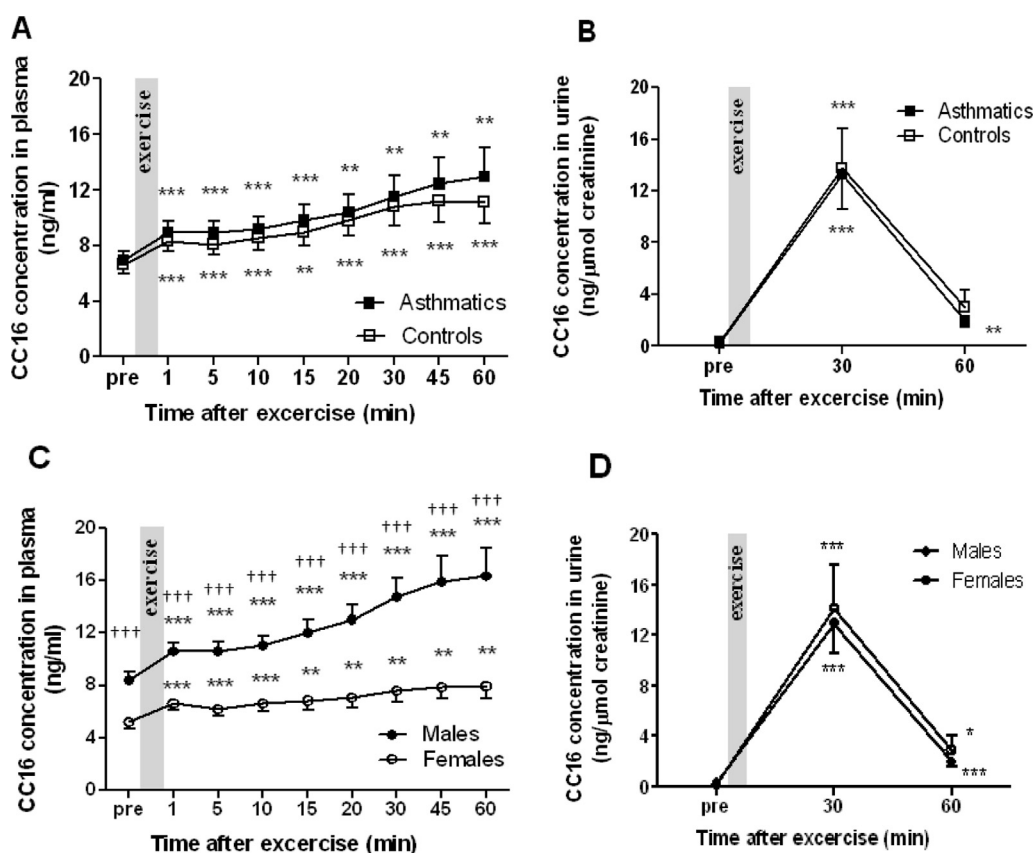
Figure 1 CC16 concentrations in plasma (A, C) and urine (B, D) at baseline (pre) and 1—60 min after exercise challenge in (A, B) in asthmatics (■) and healthy controls (□) and in (C, D) in males (●) and females (○). CC16 levels in urine were corrected for creatinine levels. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ show significance compared to baseline, and ††† = $p < 0.001$ show significant difference between males and females.

There was a homogenous increase also in urinary levels of CC16 at 30 min after the exercise test ($p < 0.001$), which decreased after 60 min ($p < 0.001$ compared to 30 min) but was still higher than baseline ($p = 0.002$). Similar to plasma, there were no differences in urine levels of CC16 between asthmatics and controls (Fig. 1B).

There were no correlations between fall in FEV_1 and CC16 levels in either plasma or urine.

Interestingly, males had significantly higher plasma levels of CC16 compared to females ($p < 0.001$) at all time points (Fig. 1C). However, there were no differences between males and females in relative increase (values at each time point divided by baseline values) in CC16 levels. No similar differences could be seen in urine levels of CC16 between males and females (Fig. 1D).

Exhaled breath temperature

There was an increase in EBT from baseline to 15 min ($p < 0.001$). The temperature thereafter declined, but remained higher than baseline for 45 min (Fig. 2A). No difference in EBT was seen between asthmatics and healthy controls, or between males and females, and there was no correlation between fall in FEV_1 and EBT at any time point.

A correlation was seen between CC16 levels in plasma 10—45 min after the test and ΔEBT (=defined as the difference between baseline EBT and the highest EBT after the exercise challenge), ($p = 0.027-0.049$; $r = 0.48-0.42$) (Fig. 2B). In addition, a reverse correlation was seen between all relative CC16 values in plasma from 1 to 60 min and EBT at 60 min after exercise ($p = 0.011-0.034$, $r = -0.56$ to -0.46).

Exhaled NO

Overall there was a lowering of exhaled NO levels after exercise. When including all subjects there was a decrease in $FE_{NO}50$ ($p < 0.0001$), CANO ($p = 0.049$) and $J'awNO$ ($p = 0.0055$) after exercise. After subdivision into healthy controls and asthmatics (Table 1), both groups showed a significant reduction in $FE_{NO}50$ (healthy controls: $p = 0.021$ and asthmatics: $p = 0.001$), and the controls in $J'awNO$ ($p = 0.019$) and asthmatics closely in CANO ($p = 0.058$). No significant differences in $FE_{NO}50$, CANO or $J'awNO$ could be seen between healthy controls and asthmatics.

Correlations between all relative CC16 values in plasma from 5 to 60 min and the differences in $FE_{NO}50$ from baseline to post-exercise ($ΔFE_{NO}50$) were seen (p-values ranging from 0.004 to 0.041 and r-values ranging from 0.48 to 0.39). Furthermore, CC16 levels in plasma from 10 to 60 min after the test and ΔCANO correlated significantly (p-values ranging from 0.004 to 0.023 and r-values ranging from 0.48 to 0.38).
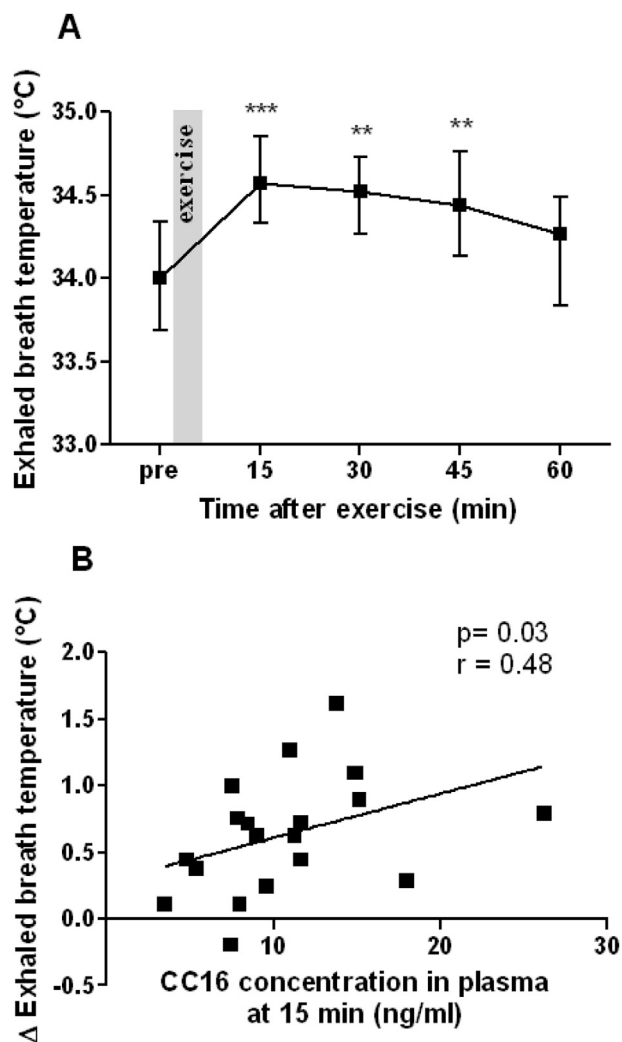
Figure 2 (A) EBT in all subjects baseline (pre) and 15—60 min after exercise challenge. (B) Correlation between ΔEBT (=defined as the difference in EBT from baseline to the highest EBT after exercise challenge) and CC16 concentrations in plasma at 15 min after exercise. ** = $p < 0.01$ and *** = $p < 0.001$ show significance compared to baseline.

Discussion

This is the first study comparing plasma to urine response in CC16 after exercise. This study showed that CC16 levels in plasma were increased directly after exercise, and continued to increase during at least 1 h. No difference in CC16 levels could be seen between asthmatics and healthy controls, but males showed higher levels than females. CC16 levels in urine peaked 30 min after exercise, declined close to baseline levels after 60 min, and did not differ between asthmatics and healthy controls or between males and females. In addition, CC16 levels in plasma after exercise correlated to both an increase in EBT and decreases in total exhaled and alveolar NO levels.

No difference in CC16 levels could be seen between asthmatics and healthy controls, which may be due to the response being physiological rather than pathophysiological. CC16 is suggested to be a protective mediator in the airway inflammatory process, but could serve as a protective mechanism during exercise induced by airway epithelial water loss independently of concurrent inflammation, i.e. asthma. Mild asthmatics and healthy control subjects are investigated in this study, and it is possible that more severe asthmatics, assumed to have a greater degree of airway epithelial damage at baseline, would behave differently. Additionally, type of regular sport activity may affect epithelial conditions, and especially swimmers' epithelia and CC16 levels are known to be altered [27,28]. In our study, the majority of the subjects were endurance sport athletes, mostly runners and floorball players, and only one swimmer (healthy control) was included. We therefore believe that our subject population was rather homogenous in the context of epithelial damage.

Possible explanations for a CC16 increase in plasma are leakage through increased permeability in the airway epithelium, increased production/secretion of CC16 in the lung or reduced renal clearance. Most probably, there is a direct exercise induced effect on the epithelium caused by dehydration. It has previously been shown that increased microvascular permeability after exercise is related to the severity of EIB [29]. However, in our study there was no difference between asthmatics and healthy controls, which may suggest that the increase in CC16 in plasma would not only be due to increased leakage over the epithelium.

An interesting finding was the higher levels of CC16 in plasma from males compared to females, while the relative increase of CC16 was the same after exercise. The reason for this is not known, but may be due to an alternative source in males. CC16 is known to be produced in the prostate, but an increase in CC16 levels would then most probably be seen in male's urine which was not the case. A higher relative amount of club cells (Clara) in male lungs, not directly related to lung function within sexes, could explain the larger absolute increase (Fig. 1C).

The increase of CC16 in plasma was in fact larger than directly measured, because a portion of CC16 was excreted in the urine. About 40 ng CC16/ml urine was lost during the first hour, and estimated calculations show that this corresponded to about 2 ng/ml CC16 in plasma (based on about 300 ml urine). The levels of CC16 in Fig. 1A should thereby be elevated about 2 ng/ml during the first part of the curve, giving another pattern of earlier increase going into a stable phase prior to what could directly be measured in plasma.

The portion of CC16 excreted in the urine was the same in males and females. Urinary excretion did therefore not explain the gender differences in plasma levels. Moreover, sputum and BAL levels of CC16 have been measured in another study, revealing no differences between males and females (unpublished data). The amount of CC16 secreted into the airway lumen was therefore most probably not depending on sex. In addition, no difference in EBT was seen between males and females, as shown both in this study and in a previous study [30]. This reflects a sex neutral epithelial effect, at least regarding perfusion.

The urine levels of CC16 displayed large inter-individual differences after exercise, which has been observed also in previous publications [16,18]. Because this could be due to differences in glomerular properties, the urine specimens

from a subfraction of the subjects ($n = 13$) were also analysed for clinical glomerular markers, such as albumin, immunoglobulin G and protein HC (=alpha-1-microglobulin). None of these markers differed between males and females, or between asthmatics and healthy controls. All markers were increased 30 min after exercise. Most prominently albumin increases, similarly to CC16, confirming that exercise per se increases glomerular permeability, being the main reason for high CC16 levels in urine [31]. The minor increase in protein HC demonstrates that the effect is not due to changes in tubular reabsorption. Another interesting point is that the molecular weight of CC16 is close to the glomerular threshold for protein filtration. In this study, the relative increase in CC16 was significantly higher than albumin, immunoglobulin G and protein HC and therefore more sensitive to changes in glomerular permeability. This closeness to glomerular threshold may be the explanation for the large inter-individual changes in urinary CC16 levels. In addition, the increase in plasma CC16 was thereby not an effect of reduced renal clearance.

EBT has previously been shown to increase after exercise [30,32], which is demonstrated also in this study. In addition, the correlation between CC16 levels in plasma and the increase in EBT after exercise demonstrate a link between epithelial effect and an increase in perfusion. In this study, EBT was measured in only a sub fraction of the subjects (matched for FEV_1 %p, exercise response, CC16 levels and NO levels), but because there was no gender difference in EBT or difference between asthmatics and healthy subjects in EBT (also reported previously [30]), the smaller group of subjects most probably reflect the total study population.

Overall, there were decreases in all exhaled NO parameters after exercise. This has been observed earlier for $FE_{NO}50$ [33,34], but not investigated for CANO and $J'awNO$. A possible explanation is the increased ventilation of the airways. Exhaled NO is to a large extent produced by the airway epithelium, and a correlation between $\Delta FE_{NO}50$ and all relative CC16 values in plasma from 5 to 60 min, shown in this study, suggest an overall related epithelial effect. In addition, $\Delta CANO$ correlated to the CC16 levels in plasma, which might also be due to the epithelial effect giving rise to a narrowing of the airways thereby impeding the outflow of the alveolar air.

In conclusion, this study shows that CC16 had an increasing pattern in plasma 1—60 min after exercise, together with an increase in urine after 30 min. The increase in plasma may be due to both an altered pulmonary epithelial permeability after intense exercise, as well as increased production/secretion of CC16 from the club cells (Clara). These plasma levels were higher in males compared to females, while the relative increase of CC16 was the same. The large inter-individual variation in urine levels of CC16 was most probably due to individual differences in increased glomerular permeability. In addition, EBT was increased and exhaled NO was decreased following exercise, and correlations to CC16 levels in plasma were most probably reflecting an overall epithelial involvement. There was no difference in CC16 levels between asthmatics and healthy controls, showing a physiological rather than pathophysiological response.

Financial disclosure and conflicts of interest

Acknowledgement

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Respiration

Exhaled Breath Temperature in Asthmatics and Controls after Eucapnic Voluntary Hyperventilation and a Methacholine Challenge Test

Henning Svensson   Leif Bjermer   Ellen Tufvesson

Respiratory Medicine and Allergology, Department of Clinical Sciences, Lund University, Lund, Sweden

Abstract

Background: It has been suggested that exhaled breath temperature (EBT) is increased in asthmatic subjects. ***Objectives:*** Our aim was to investigate EBT in asthmatics compared to healthy controls before and after eucapnic voluntary hyperventilation (EVH) and a methacholine challenge test (MCT). ***Methods:*** A total of 26 asthmatics and 29 healthy controls were included. Forced expiratory volume in 1 s (FEV_1), EBT and oral, axillary and auricular temperatures were measured before and after EVH and MCT. ***Results:*** FEV_1 % predicted (%p) was significantly lower in asthmatic subjects compared to healthy controls at all time points. EBT was significantly increased in all subjects 15–30 min after EVH and 5–45 min after MCT. Oral temperature displayed a similar pattern of increase, in contrast to axillary and auricular temperature, and correlated with EBT before and after both of the challenge tests. EBT after 5 min correlated with the largest drop in FEV_1%p after EVH in asthmatic subjects. No significant differences or changes in EBT were found when comparing asthmatics to healthy controls before or after any of the tests. ***Conclusions:*** EBT is increased after both EVH and MCT, possibly reflecting a vascular response. This is related to both the fall in FEV_1 and to oral temperature, suggesting an effect on the whole respiratory tract including the oral cavity. No differences in EBT are seen between asthmatics and healthy controls, indicating that the increase in EBT is mainly physiological rather than pathophysiological.

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Introduction

Asthma is characterized by inflammation of the airways, with inflammatory cells present as well as airway remodeling, increased vascularization and bronchial hyperresponsiveness (BHR). Provocation testing for detecting BHR in asthmatics has gained importance for diagnosis and for monitoring disease activity, guiding the dosing of inhaled steroids [1].

Exhaled breath temperature (EBT) is a potential biomarker for airway inflammation. It has been shown to relate to disease exacerbations [2–4] as well as to vascular [5] and fibrous remodeling [6] of the airways, and to correlate with levels of exhaled nitric oxide (NO) in childhood and adult asthma [7, 8]. Sub-epithelial blood vessel density is higher in asthmatic subjects than in healthy controls [9], and the hypothesis is that increased blood flow during airway inflammation would result in heating of the ventilated air to a higher temperature [5]. Some studies have shown that EBT is elevated in asthmatics compared to healthy controls [2, 8, 10], but research on the relationship between EBT and BHR is limited. In a previous study, we found that an increase in EBT corre-

Henning Svensson
Respiratory Medicine and Allergology
Department of Clinical Sciences, Lund University
S–221 84 Lund (Sweden)
E-Mail Henning.Svensson@med.lu.se

lated with a decrease in forced expiratory volume in 1 s (FEV_1) in both asthmatics and controls after an exercise challenge test [11]. A similar correlation has been found in asthmatic children [12]. In addition, we found that there was a prolonged increase in EBT after exercise in asthmatics for whom the FEV_1 fell during the test compared to asthmatic subjects for whom it did not fall. This indicates a link between EBT and airway inflammation, but the potential of a clinical implication of EBT is yet to be determined. Increased baseline levels of 8-isoprostane, a marker of oxidative stress, have been found in exhaled breath condensate of asthmatic children with BHR after an exercise challenge test, supporting the notion that persistent airway inflammation is important for exercise-induced bronchoconstriction to occur [13].

Eucapnic voluntary hyperventilation (EVH) is an indirect challenge test, similar to the exercise challenge test, where the inflammatory cells of the airways are triggered to release mediators [14, 15], causing smooth muscle cell constriction and vasodilatation. The triggering factor for this release is believed to be an increased osmolarity of the periciliary fluid covering the respiratory mucosal membrane [16], due to increased loss of water to the air when there is a high rate of ventilation. The specificity of an indirect challenge test is considered high, as, in theory, inflammation is required for a positive result to occur.

Methacholine is believed to act directly on the muscarinic receptors [17] of smooth muscle cells, endothelial cells and mucus-producing cells, inducing bronchial obstruction, vasodilatation and mucus production. The sensitivity of a methacholine challenge test (MCT) is high, while a positive test has a low diagnostic specificity. The presence of inflammation and chronic remodeling of the airways are both believed to increase reactivity to methacholine [18].

The aim of this study was to investigate changes in EBT after EVH and MCT, and to compare it to changes in body temperature. EVH is believed to trigger bronchoconstriction and vasodilatation through the release of inflammatory mediators, similar to an exercise challenge test. Methacholine was used as an additional challenge, reflecting another pathophysiological mechanism. We hypothesized that those subjects who responded to EVH with bronchoconstriction would increase their EBT to a greater extent than those who did not, due to the probable vasodilatation following the release of mediators from airway inflammatory cells. An increase in EBT would also theoretically be seen after MCT, where the pharmacological effect of methacholine acts not only on smooth muscle cells but also induces vasodilatation. A secondary aim was to investigate whether an increase in airway resistance would affect the change in EBT, due to narrowing of the airways.

Table 1. Subject characteristics

	Controls (n = 29)	Asthmatics (n = 26)
Males/females, n	16/13	16/10
Age, years	25 (23–26)	24 (22–27)
Baseline FEV_1%p	104 (97–107)	95 (90–98)
FEV_1 drop ≥10% after EVH, n	6	14
Fall in FEV_1%p after EVH, percentage units	6 (4–9)	10 (6–15)
FEV_1 drop ≥20% after MCT, n	7	16
Fall in FEV_1%p after MCT, percentage units	10 (7–20)	21 (13–28)
Atopy, n	7	20
ICS (400–800 µg budesonide/day), n	0	20
Exhaled NO, ppb	14.7 (9.9–23.5)	17.1 (14.9–26.4)

Data are expressed as a median (interquartile range), where appropriate. For baseline FEV_1%p and exhaled NO values, a mean of the two registrations before each test is used for each subject.

Materials and Methods

Subjects

Twenty-six subjects with a doctor's diagnosis of asthma according to the guidelines of the Global Initiative for Asthma [19] were investigated (table 1). Twenty-nine healthy subjects with no diagnosis of asthma or any respiratory symptoms were used as controls. All subjects were interviewed concerning their previous and present health, and asthmatic subjects filled out an Asthma Control Test questionnaire. Subjects with respiratory tract infections (within the last 3 weeks) or any other medical conditions (apart from asthma) affecting their health were excluded, as were subjects with a history of smoking. The subjects were not allowed to drink coffee for at least 6 h prior to either challenge test or to conduct any form of exercise on the same day. All asthmatic subjects refrained from using short-acting β2-agonists for at least 8 h and long-acting β2-agonists and inhaled corticosteroids (ICS) for at least 24 h prior to any part of the study. All subjects gave written informed consent and the study was approved by the regional Ethics Review Board, Lund.

Study Design

We conducted tests from the 14 July 2011 to the 1 August 2012. Two different respiratory tract provocation tests, EVH and MCT, were performed in random order by all subjects. At least 48 h passed between tests. Prior to each test, and after at least 5 min of rest, exhaled NO, spirometry, EBT and axillary, auricular and oral temperatures were measured (in the given order). Thereafter, the subjects performed a provocation test. Afterwards, measurements of spirometry, EBT, axillary, auricular and oral temperatures were

performed repeatedly during a period of rest, as described below. After completing the measurements, 60 min after the provocation test, 400 μg of salbutamol was inhaled by the subject. After another 15 min of rest, a final set of measurements (spirometry, EBT and body temperatures) was conducted.

Eucapnic Voluntary Hyperventilation

The subjects hyperventilated for 6 min through a mouthpiece connected to a container administering dry hypercapnic air (5% CO_2) via a dry air ventilation device (Aiolos Medical AB, Karlstad, Sweden). The air was at an ambient temperature. A nose clip was used, and the flow was set to $26 \times FEV_1$ (baseline value) l/min. The subjects were supervised at all times and guided by a reservoir balloon attached to the ventilation device, ensuring that the rate and depth of breathing were adequate. The test was considered positive if FEV_1 dropped by ≥10% (compared to the baseline value) at any measurement within 30 min of the end of the hyperventilation.

Methacholine Challenge Test

For the MCT, a tidal-volume-triggered device (Aerosol Provocation System, APS; Erich Jaeger GmbH) was used. One inhalation of 9 mg/ml NaCl was performed first as a negative control. Methacholine was then inhaled repeatedly, with a spirometry performed 2 min after each inhalation and the next inhalation following directly thereafter. Five inhalations were administered with increasing doses (50, 150, 300, 600 and 900 μg), resulting in a maximal cumulative dose of 2,000 μg of methacholine. A positive test was defined as a drop of FEV_1 by ≥20% to baseline value, in which case the challenge test was considered completed and measurements were made as described below.

Exhaled NO Measurement

Exhaled NO was measured prior to each of the tests using a NIOX Flex (Aerocrine AB, Stockholm, Sweden) according to recommendations of the American Thoracic Society and European Respiratory Society [20] with an exhalation flow rate of 50 ml/s.

Lung Function Test

Flow volume spirometry (Jaeger MasterScope, Würzburg, Germany) measuring FEV_1 was performed before and 5, 10, 15, 20, 30, 45 and 60 min after the end of each of the provocation tests, and 15 min after inhaling salbutamol. FEV_1 % predicted (%p) was calculated according to the reference spirometric values of Crapo et al. [21].

EBT Measurement

EBT was measured using an X-Halo (Delmedica Investments, Singapore) once before and 5, 15, 30, 45 and 60 min after each of the tests, as well as 15 min after inhaling salbutamol. Subjects were instructed to breathe tidally, inhaling through the nose and exhaling through the mouth into the device. Measurements took 2–6 min to complete.

Body Temperature Measurements

Body temperatures were measured before and 5, 15, 30, 45 and 60 min after each of the provocation tests as well as 15 min after inhaling salbutamol. Oral and axillary temperatures were measured with digital clinical thermometers C402 and C202, respectively (Terumo, Leuven, Belgium). Auricular temperature was measured using a ThermoScan type 6013 (Braun, Kronberg, Germany).

Impulse Oscillometry System

A Jaeger MasterScreen impulse oscillometry system (IOS; Erich Jaeger GmbH) was used to test a subgroup consisting of 22 of the subjects (9 asthmatics and 13 healthy controls). IOS was performed before MCT and after each inhalation during the challenge prior to spirometry, as previously described [22]. Subjects used a nose clip and were instructed to press the palms of their hands against their cheeks in order to decrease the influence of movements of the upper airways on IOS parameters. Oscillometric pressure impulses with a pulse sequence of 5 per second and a frequency spectrum between 5 and 35 Hz were superimposed on tidal breathing for at least 30 s. Airway resistance at 5 Hz (R5) and 20 Hz (R20), reactance at 5 Hz (X5), resonant frequency (Fres) and area of reactance integrated from 5 Hz to Fres (AX) were determined, and R5–R20 was calculated.

Statistical Analyses

GraphPad Prism version 4.0 was used for statistical analysis. The results are expressed as median (interquartile range) where appropriate. Correlations were calculated using Spearman's correlation test. Comparisons between groups were made by using the Mann-Whitney test. Analysis of paired measurements was made by using Wilcoxon's test. A p value of <0.05 (two-tailed) was considered significant. Power calculation was performed based on 2 independent groups of 26 evaluable subjects. With an expected standard deviation of 0.5, it was possible to detect a significant difference with a power of 94%.

Results

Subjects

Twenty subjects (14 asthmatics and 6 controls) had a positive EVH test and 23 (16 asthmatics and 7 controls) had a positive MCT (table 1). Seventeen of the subjects (12 asthmatics and 5 controls) were positive for both challenge tests. There were no significant differences in EBT or ΔEBT (defined as the difference between baseline EBT and the highest EBT after the challenge test) when comparing those who were positive for both challenge tests to those who were positive for one test only or negative for both tests. The Asthma Control Test score among asthmatic subjects was 21.5 (20–23), and it did not differ between subjects who had a positive EVH or MCT and those who did not.

Lung Function

As expected, levels of FEV_1%p were significantly lower in asthmatic subjects than controls at baseline (p = 0.002–0.006) and 5–60 (p = 0.001–0.006) min after both tests, and also after inhaling salbutamol (p = 0.003–0.007).

All subjects (both asthmatics and controls) experienced a drop in the level of FEV_1 after both EVH and MCT (p < 0.001), and FEV_1 increased postsalbutamol
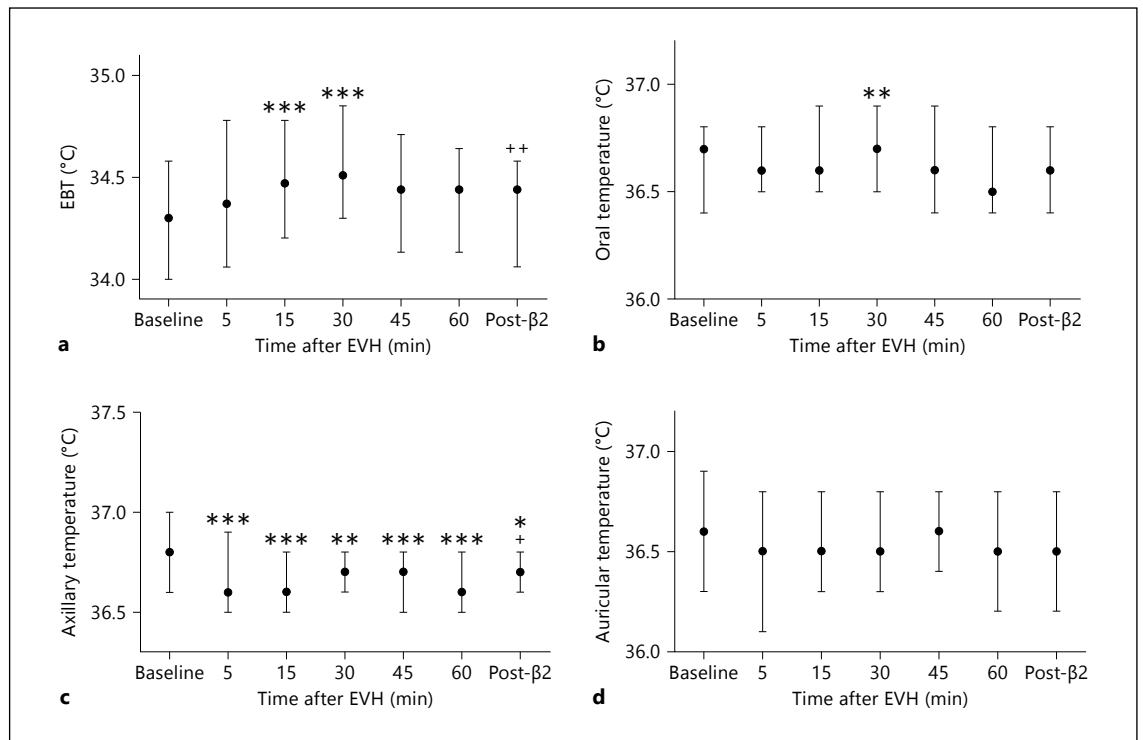
Fig. 1. EBT (**a**), oral temperature (**b**), axillary temperature (**c**) and auricular temperature (**d**) before (baseline) and 5, 15, 30, 45 and 60 min after EVH and postsalbutamol (Post-β2) in all subjects. * p < 0.05, ** p < 0.01, *** p < 0.001: significant difference compared to baseline. + p < 0.05, ++ p < 0.01: significant difference between measurement after 60 min and postsalbutamol.

compared to at both baseline (p < 0.001 after EVH; p = 0.040 after MCT) and 60 min after the challenge tests (p < 0.001). A majority of the subjects had their greatest drop in FEV_1 at 5–10 min after both EVH (n = 34) and MCT (n = 42). FEV_1%p in all subjects was lowest 5 min after EVH [90.5% (83.4–100.2)] and 5 min after MCT [83.4% (74.7–100.4)].

During EVH, FEV_1%p was significantly lower at all time points (except for 45 min) in asthmatic subjects with ICS treatment than in those not treated with ICS. Following MCT, FEV_1%p was lower in asthmatic subjects treated with ICS after 15 min and postsalbutamol, but not at any other time point. There was no difference in FEV_1%p between male and female subjects.

Exhaled-Breath Temperature
EBT was significantly increased compared to baseline following EVH in all subjects after 15 min, and it peaked at 30 min (p < 0.001; fig. 1a). After MCT, EBT was significantly increased in all subjects compared to baseline at 5–45 min (p < 0.001–0.006; fig. 2a). EBT peaked 15 min after MCT.

There were no significant differences between asthmatics and healthy controls in EBT or ΔEBT at any time point during the challenge tests (fig. 3) or between subjects with and without a positive challenge test. There were no differences in baseline EBT or ΔEBT between asthmatic subjects with and without ICS treatment, between atopic and nonatopic subjects or between male and female subjects.

EBT Correlation with Lung Function
ΔFEV$_1$%p (defined as the maximum decrease in FEV_1%p: baseline FEV_1%p – lowest FEV_1%p after the challenge test) after EVH correlated with EBT measured after 5 min in asthmatic subjects (p = 0.048, r = 0.392), but not in controls (p = 0.641, r = –0.091), all subjects (p = 0.421, r = 0.111; fig. 4a) or when examining into subjects with a positive or negative EVH challenge test (data not shown).

No similar correlation between ΔFEV$_1$%p and EBT 5 min was seen after MCT in the asthmatics, the controls or all subjects (fig. 4b).

There was no correlation between ΔFEV$_1$%p and ΔEBT after any of the challenge tests.
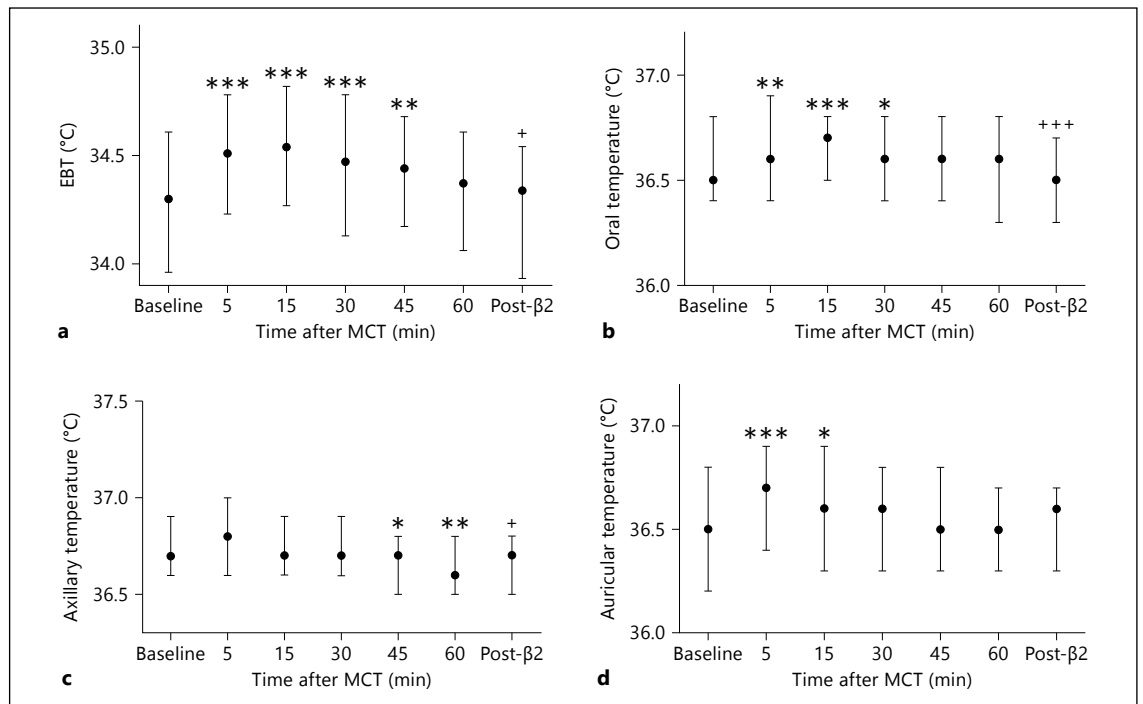
Fig. 2. EBT (**a**), oral temperature (**b**), axillary temperature (**c**) and auricular temperature (**d**) before baseline and 5, 15, 30, 45 and 60 min after MCT and postsalbutamol (Post-β2) in all subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significant difference compared to baseline. + $p < 0.05$, +++ $p < 0.001$: significant difference between measurement after 60 min and postsalbutamol.
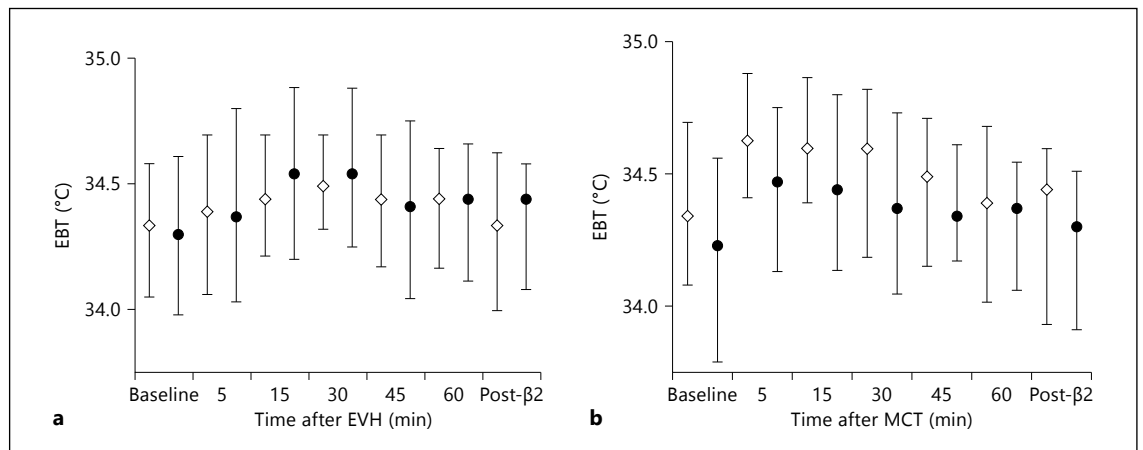

Fig. 3. EBT in asthmatics and controls before baseline and after 5, 15, 30, 45 and 60 min and postsalbutamol (Post-β2) after EVH (**a**) and MCT (**b**). ◊ = Asthmatic subjects; ● = healthy controls.

Body Temperatures and Correlations with EBT

Oral temperature displayed an increase after both EVH and MCT, similar to what was seen in EBT. Oral temperature was significantly elevated in all subjects 30 min after EVH ($p = 0.003$; fig. 1b). After MCT, oral temperature was elevated compared to baseline after 5–30 min ($p < 0.001$–0.046; fig. 2b).

Neither axillary nor auricular temperatures showed a similar pattern of increase after EVH (fig. 1). On the contrary, axillary temperature was decreased and auricular
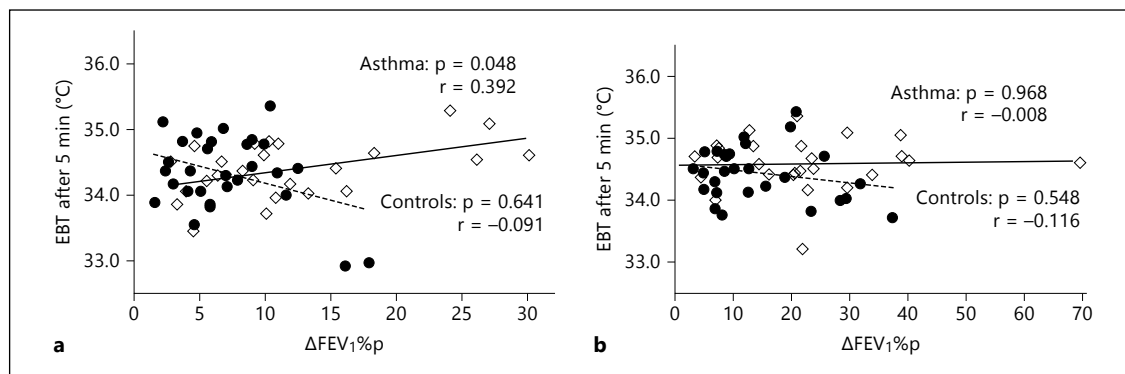
EBT after Airway Provocation

5

Fig. 4. Correlation between maximum decrease in FEV_1%p (ΔFEV_1%p; defined as baseline FEV_1%p – lowest FEV_1%p after the challenge test) and EBT at 5 min after EVH (**a**) and MCT (**b**). ◊ = Asthmatic subjects; ● = healthy controls; —— = correlation in asthmatic subjects; ---- = correlation in controls.
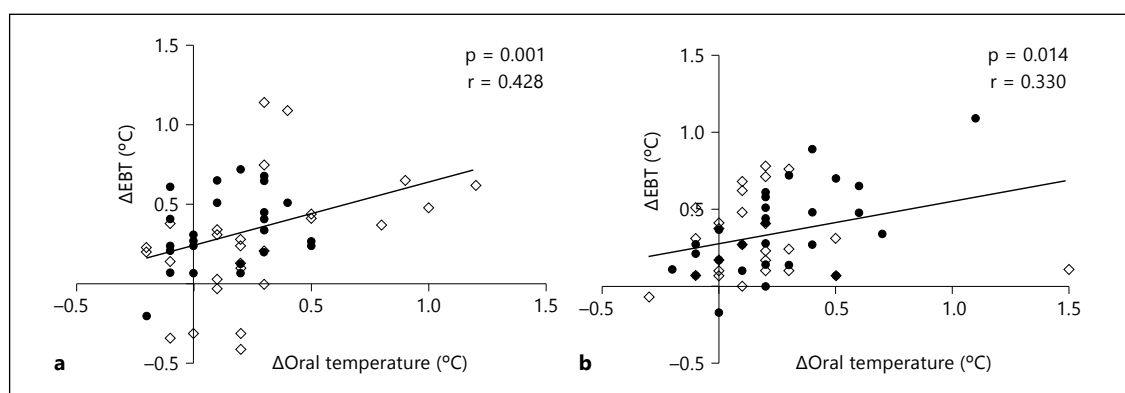

Fig. 5. Correlation between change in EBT (ΔEBT; defined as highest EBT after the challenge test – baseline EBT) and change in oral temperature (Δoral temperature; defined as highest oral temperature after the challenge test – baseline oral temperature) after EVH (**a**) and MCT (**b**). ◊ = Asthmatic subjects; ● = healthy controls; —— = correlation in all subjects.

temperature was not affected. After MCT, auricular temperature was increased at 5 min ($p < 0.001$) and 15 min ($p = 0.013$) compared to baseline value (fig. 2d), while axillary temperature instead dropped after 45 min ($p = 0.020$) and 60 min ($p = 0.009$; fig. 2c).

EBT correlated with oral temperature at baseline ($p = 0.044$, $r = 0.270$), before EVH and 5 min ($p = 0.032$, $r = 0.288$), 45 min ($p = 0.005$, $r = 0.372$) and 60 min ($p = 0.029$, $r = 0.292$) afterwards as well as postsalbutamol ($p < 0.001$, $r = 0.501$). EBT and oral temperature also correlated before and after MCT at baseline ($p = 0.003$, $r = 0.390$) and after 15–60 min ($p = 0.001$–0.015, $r = 0.325$–0.424). Peak oral temperature coincided with peak EBT after both EVH (fig. 1) and MCT (fig. 2).

ΔEBT and Δoral temperature (defined as the difference between baseline oral temperature and maximum oral temperature after the challenge test) correlated significantly after EVH ($p = 0.001$, $r = 0.428$) and MCT ($p = 0.014$, $r = 0.330$; fig. 5). No corresponding correlations were seen when comparing ΔEBT to axillary or auricular temperature (data not shown).

Exhaled Nitric Oxide

Baseline levels of exhaled NO did not differ between asthmatic subjects and controls, between atopic subjects and nonatopic subjects, between asthmatic subjects with or without a positive challenge test or between asthmatic subjects with or without ICS treatment. Levels of exhaled NO did not correlate with EBT before or after any of the challenge tests except postsalbutamol after MCT ($p = 0.041$, $r = 0.277$). It did not correlate with ΔEBT.

Impulse Oscillometry System

No significant correlations were found between EBT (including ΔEBT) and IOS parameters at any time point, or between EBT and the change in any IOS parameter compared to at baseline.

As expected, increases in R5, R20, R5–R20 and AX, and a decrease in X5, were seen in all subjects when comparing baseline values to those measured after MCT. Fres was increased in controls only. R5–R20%p was significantly higher in asthmatic subjects than in healthy controls after MCT (data not shown), but no other IOS parameters differed at baseline or after MCT.

Discussion

This study showed that EBT increased significantly 15–30 min after EVH and 5–45 min after MCT, and that no difference in EBT between asthmatic subjects and healthy controls could be detected. Levels of FEV_1%p were significantly lower in asthmatic subjects than in controls at all time points; this confirmed that the selection of subjects was adequate. Furthermore, no difference in EBT was seen when comparing subjects with and without BHR after any of the challenge tests.

The increase in EBT seen after EVH and MCT may be a result of increased blood flow following vasodilatation. However, vascular tone alone does not explain the changes in EBT, seeing as EBT decreased from the time point of 60 min to postsalbutamol after both challenge tests, even though salbutamol is known to increase bronchial blood flow [23]. Other factors such as bronchodilation may also affect EBT, possibly masking the effects of increased blood flow on EBT. Similar studies should be undertaken with the aim of assessing the effects of EVH and MCT on markers of inflammation in exhaled breath condensate, including metabolites [24, 25], leukotriene B4 [26] and isoprostanes [27, 28] in patients with asthma. Likewise, it would be worth studying the effects of these challenges on e-nose breathprints [29, 30].

A significant increase in EBT was seen already at 5 min after MCT, in contrast to at 15 min after the end of hyperventilation. Inhalation of methacholine affects vascular tone and increases bronchial blood flow [18], possibly leading to an early response in EBT. Increased blood flow after EVH is dependent on dehydration of the periciliary fluid and on the release of inflammatory mediators, which might delay the reaction somewhat and give rise to a relatively mild response in both EBT and decrease of lung function. EBT was elevated for a longer period of time following MCT compared to EVH. The duration of an elevation of EBT after airway provocation tests might be related to the intensity of the challenge and the airway response. For example, EBT was elevated for an even greater period of time (at least 60 min) following a standardized exercise challenge test [11]. A greater increase in body temperature in general after exercise would not form a satisfactory explanation for this difference, since EBT was elevated independently of the axillary and auricular temperature. The maximum response to exercise (peak EBT) was greater than that after EVH, in spite of the fact that the respective durations of hyperventilation in the two challenge tests are comparable. The increase in cardiac output, with a possible effect on blood flow of the airway mucosa during and after exercise, may provide an explanation for these differences. During EVH, heart rate increases only marginally and blood flow would therefore not be increased to the same extent. Another possibility is that a standardized exercise challenge is a better method of ensuring that ventilation, with subsequent dehydration of the airways, is adequate. While subjects performing EVH may subtly decrease their rate of ventilation to a level perceived as more comfortable, a standardized exercise challenge test performed on a treadmill at approximately 90% of maximum heart rate ensures that the respiratory rate increases considerably.

In this study, we showed that the maximum change in FEV_1%p correlated with EBT in asthmatic subjects 5 min after EVH, but not in the controls. A similar pattern was seen 5 min after the exercise challenge test [11] in all subjects and in the controls, but not when looking at asthmatics separately. The similarities of correlations between EBT and decrease in lung function support the theory that EVH is equivalent to an exercise challenge test, at least in this perspective. However, while EBT reached its peak after 5 min following the exercise challenge test, maximum median EBT after EVH was seen after 30 min.

The maximum increase in oral temperature correlated with a maximum increase in EBT after both EVH and MCT. Oral temperature displayed the same pattern as EBT after both the provocation tests that we used as well as after exercise, proving that the two temperature measurements are closely related. Although the X-Halo has been validated in previous studies [2, 4], one must take into consideration the possibility that since measurements reflect the plateau of the breath temperatures of several expirations registered during a time period of a few minutes, oral temperature in itself may have some effect on the result. This would be in contrast to the single-breath method described in other studies [5–8]. Auricu-

lar temperature increased 5–15 min after MCT, which may possibly be explained by a spread of heat to the ear through the Eustachian tube. However, peak auricular temperature did not coincide with the peaks of EBT and oral temperature after MCT. Axillary temperature was decreased after EVH and to a lesser extent after MCT. The explanation for this is not known, but it is possible that the time of rest following arrival at the laboratory was insufficient, resulting in an elevated axillary temperature at baseline. However, in this case, baseline axillary temperature before MCT would most probably also have been affected.

In summary, our findings suggest that EBT is directly related to oral but not to axillary or auricular temperature, regardless of the type of challenge test or level of physical activity. We believe that this connection is a result of increased blood flow due to vasodilatation taking place only in the airways and/or the oral cavity, both being part of the respiratory tract.

Some of the controls in our study displayed BHR after one or both of the airway challenge tests. The prevalence of positive methacholine challenge tests in the general population has been reported to range from <10% to >40% in various studies, and normal variations in BHR can be expected even among subjects selected for the absence of known potential causes of a positive challenge test, such as asthma [31]. Similar variations are most probable even for indirect tests of BHR, even though they normally display a higher specificity [32].

Exhaled NO has been shown to correlate with EBT in some studies [7, 8], but this was not one of our findings. We also found no differences in levels of exhaled NO between asthmatics and controls, between asthmatics with and without ICS treatment or between atopic and nonatopic subjects. As seen by the results of the Asthma Control Test, this study used subjects with relatively mild and well-controlled asthma, possibly representing a different phenotype from that used in studies showing elevated levels of NO in asthmatics. The fact that there was a significant difference in $FEV_1\%p$ at all times should nevertheless confirm that our selection of asthmatic subjects was representative. EBT has also recently been suggested to correlate with body height [33], but no such correlations were found in our study.

One hypothesis was that airway narrowing, measured as airway resistance by IOS, would affect changes in EBT. However, no such correlations could be seen.

To conclude our findings, we have shown an increase in EBT after both EVH and MCT, reflecting a normal physiological response which is similar in asthmatic subjects and healthy controls. EBT is probably affected by vasodilating agents and increases when lung function declines, although significant correlations between the two are limited. EBT and oral temperature both increase and display significant correlations before and after EVH and MCT, probably reflecting the same processes affecting temperature in the respiratory tract, independently of body temperature in general.

Financial Disclosure and Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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