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From sneeze to wheeze: Non-invasive studies on asthma and rhinitis

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2009



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To Cilla & Hillevi

**If you know the enemy and know yourself, your
victory will not stand in doubt;
if you know Heaven and know Earth, you may
make your victory complete.**

Sun Tzu

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LIST OF PAPERS¹

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-V):

I. Aronsson D, Tufvesson E, Bjerner L

Allergic rhinitis with or without concomitant asthma: difference in perception of dyspnoea and levels of fractional exhaled nitric oxide. *Clin Exp Allergy*. 2005 Nov;35(11):1457-61.

II. Tufvesson E, Aronsson D, Ankerst J, George SC, Bjerner L

Peripheral nitric oxide is increased in rhinitic patients with asthma compared to bronchial hyperresponsiveness. *Respir Med*. 2007 Nov;101(11):2321-6.

III. Tufvesson E, Aronsson D, Bjerner L

Cysteinyl-leukotriene levels in sputum differentiate asthma from rhinitis patients with or without bronchial hyperresponsiveness. *Clin Exp Allergy*. 2007 Jul;37(7):1067-73

IV. Aronsson D, Tufvesson E, Ankerst J, Bjerner L Allergic rhinitis with hyper-responsiveness differ from asthma in degree of peripheral obstruction during metacholine challenge test. *Clin Physiol Funct Imaging*. 2008 Mar;28(2):81-5.

V. Aronsson D, Tufvesson E, Bjerner L

Characterization of airway reactivity to methacholine, mannitol and eucapnic hyperventilation in mild asthmatic. *Submitted to Clin Resp Journal*.

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ABBREVIATIONS

| | |
|--------------------------|--|
| AHR ² | Airway hyperresponsiveness |
| APC | Antigen presenting cells |
| BHR ² | Bronchial hyperresponsiveness |
| CA _{NO} | Alveolar concentration of nitric oxide |
| CD | Cluster of differentiation |
| Cys-LTs | Cysteinyl leukotrienes |
| DTT | Dithiothreitol |
| ECP | Eosinophil cationic protein |
| EIB | Exercise induced bronchoconstriction |
| EVH | Eucapnic voluntary hyperventilation |
| FENO | Fractional exhaled nitric oxide |
| FEV ₁ | Forced expiratory volume in one second |
| Fres | Resonant frequency |
| FVC | Forced vital capacity |
| ICS | Inhaled corticosteroid |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IOS | Impulse oscillometry |
| <i>J</i> _{awNO} | Proximal nitric oxide flux |
| LT | Leukotriene |
| MCh | Methacholine |
| NO | Nitric oxide |
| PBS | Phosphatebuffered saline |
| PEF | Peak expiratory flow |
| R | Resistance |
| RAST | Radioallergosorbent test |
| SPT | Skin prick test |
| X | Reactance |

² In paper I-IV the term bronchial hyperresponsiveness is used and in paper V the term airway hyperresponsiveness is used. For the purpose of this thesis the terms are interchangeable. For the sake of simplicity the term airway hyperresponsiveness is used when not referring to a specific paper.

INTRODUCTION

Allergic rhinitis

Allergic rhinitis is a global problem that causes major disability and illness in all ethnic groups and ages. The economic impact on the society is often hard to estimate due to low direct costs, but the indirect cost is substantial, since allergic rhinitis affects work performance, sleep, school and social life [1]. For example, the total expenditures 2005 to treat (health care and prescription treatment) allergic rhinitis were estimated to \$11.2 billion for USA alone [2]. Prevalence of allergic rhinitis can be as high as 25-40 % in some countries and seems to be rising, especially in parts of the world with previously low prevalence numbers [3-8]. In Sweden, studies on military recruits show an increase in prevalence of nasal symptoms of allergic rhinitis from 4 % during the fifties to over 15 % in the mid-seventies [9]. The most common aeroallergens in Sweden are pollen (birch, timothy, mugwort), animal dander (cat, dog, horse), house dust mites and moulds.

Rhinitis is defined as an inflammation of the lining of the nose and is characterized by nasal symptoms including anterior or posterior rhinorrhoea, sneezing, nasal blockage and/or itching of the nose. These symptoms occur during two or more consecutive days for more than 1 hour on most days [10]. The most common cause of rhinitis is most likely infection (i.e. common cold). *Allergic* rhinitis is clinically defined as a symptomatic disorder of the nose, caused by an IgE-mediated inflammation of the nasal membranes, and is often associated with ocular symptoms [1].

Genetic as well as environmental factors influence development of allergic rhinitis. The patterns of inheritance are complex and the recent increase in the prevalence of allergic rhinitis cannot be explained by genetic factors alone [11]. Exposures to inhaled allergens cause allergic rhinitis, while food allergens rarely are the cause of isolated nasal symptoms. Other suggested risk factors include exposure to air pollutants, birth weight, prematurity, ethnicity and various lifestyle and environmental factors in the western industrial areas [12-16]. In 1989, Strachan proposed that infection and unhygienic conditions may protect against development of allergy [17]. This so-called “hygiene-hypothesis” has since then been developed and explored but no unified concept has yet emerged [18]. Like for the risk factors mentioned above, further research is needed.

Traditionally, allergic rhinitis has been subdivided into *seasonal*, *perennial* and *occupational*, based on the time of exposure and following symptoms, where seasonal allergic rhinitis is most commonly caused by outdoor allergens such as molds and pollen. Perennial allergic rhinitis on the other hand is associated with indoor allergens (eg house dust mites) [19]. However, this classification is to a large degree based on the causing allergens, and is not entirely satisfactory as a majority of the patients are sensitized to many different allergens, and symptoms may vary. Therefore, this classification has been gradually abandoned in favour of the terms *intermittent* and *persistent allergic rhinitis*, which is solely based on the duration of symptoms [1].

The diagnosis of allergic rhinitis is based mainly on patient symptom history. Diagnosis can be aided by objective tests based on the

demonstration of allergen-specific IgE in the skin (e.g. skin prick test) or in the blood (e.g. RAST) [20].

Asthma

Asthma is a serious global health problem. It has been defined, based on its functional consequences:

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early in the morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment [21].

Asthma is a problem worldwide, with an estimated 300 million affected individuals. Prevalence ranges from 1-18 %, depending on location. Annual worldwide deaths from asthma have been estimated at 250000 and mortality does not appear to correlate well with prevalence. [21, 22]. Recently, a decrease in prevalence has been recorded in North America and Western Europe. However, increasing asthma symptom prevalence in Africa, Latin America and parts of Asia indicate that the global burden of asthma is continuing to rise, but the global prevalence differences are lessening [23]. The rate of asthma seems to increase as communities adopt western lifestyles and become urbanised.

The international patterns of asthma prevalence are not explained by the current knowledge of the causes of asthma. Research into the causes of

asthma and the efficacy of primary and secondary intervention strategies represent key priority areas in the field of asthma research [22].

As with allergic rhinitis, both genetic and environmental factors play a role in the development of the disease. Asthma has a heritable component, but the mechanisms seem complex [24, 25]. A specific gene connected to asthma is yet to be found [26]. Rather, several genes associated to asthma have been identified [27]. Interestingly, genes that influence the response to asthma treatment, such as glucocorticosteroids, have been identified [28].

A number of environmental factors have been suggested as influencing the risk of developing asthma, eg indoor and outdoor allergens, infections, tobacco smoke, diet, air pollution and various occupational sensitizers [29-34]. Protective factors include being raised in a rural setting, having older siblings and being exposed to certain infections [35-37]. This is in line with the “hygiene hypothesis” mentioned above.

Diagnosis of asthma is to a large degree based on medical history where symptoms such as episodic breathlessness, wheezing, chest tightness and cough are key indicators of the disease. Seasonal variability of symptoms, family history of asthma, childhood eczema and exercise related symptoms are other factors that may indicate asthma [38]. Lung function testing such as spirometry and peak expiratory flow provides possibilities to further strengthen the diagnosis. Typical for asthma is the reversibility of lung function abnormalities [39, 40]. Another hallmark of asthma is the propensity of the airways to react with narrowing to non-allergic stimuli such as cold air, smoke or heavy perfumes. This is referred to as airway hyperresponsiveness and can be demonstrated in the clinic with various provocative agents, such as methacholine (MCh) or

histamine [41, 42]. Further investigations include exploration of possible allergies and, recently, testing to establish presence of allergic inflammation in the airways.

The Airways

When inhaling, the air passes through the mouth or the nose down the pharynx and the larynx. Together with the paranasal sinuses, these anatomical features constitute the *upper airways* (*fig 1*). The air then enters the tracheobronchial tree (*lower airways*), starting with the trachea. The first 16 branchings, or *generations*, of the airways are called the *conducting zone*, since no gas exchange takes place here. The *transitional zone* runs through generation 17-19 and consists of the respiratory bronchioles, where the functional unit of the gas exchange in the lung, the *alveoli*, first appears. The *respiratory zone* (generation 20-23) contains alveolar ducts and alveolar sacs, and this is where most of the gas exchange takes place. The bronchioles beyond generation 7-8, where the diameter is less than 2 mm, are sometimes referred to as the *small airways*. The small airways provide only 10 % of total airway resistance, even though it accounts for approximately 80 % of the total lung surface area [43].

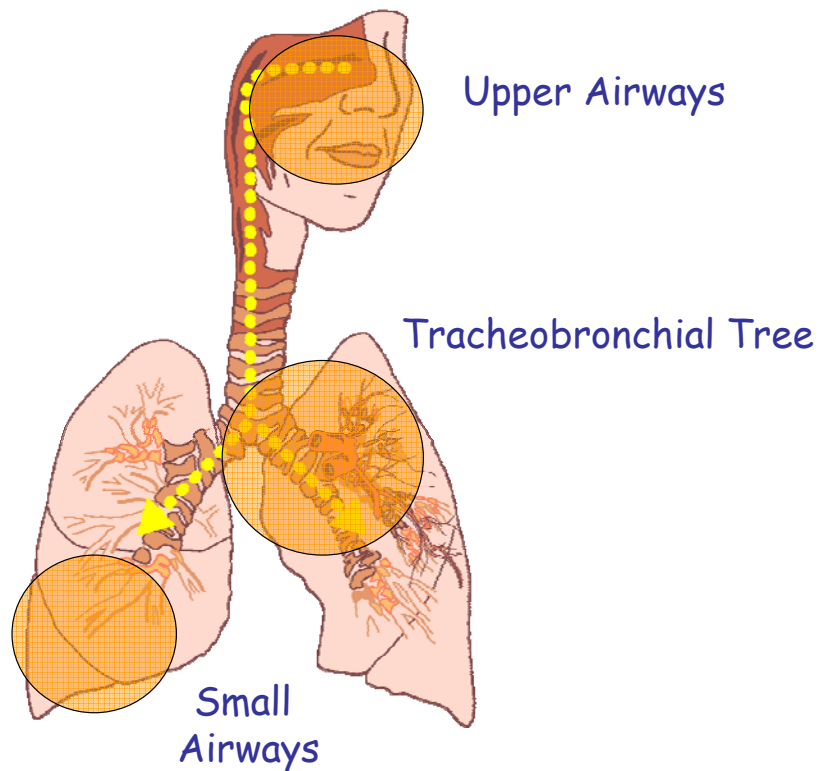


Fig 1. Anatomical features of the airways.

The allergic reaction

Sensitisation

In the allergic subject, the allergic immune response begins with sensitisation (*fig 2*). When exposed to allergens, antigen presenting cells such as *dendritic cells* or *Langerhans cells* in the epithelium lining the airways of the lungs and nose, express these allergens on their cell surface. This in turn will activate other cells involved in the immune response, particularly T-lymphocytes. Through a series of complex cell interactions, involving mediators such as interleukins, B-lymphocytes are

transformed into antibody secretory cells - *plasma cells*. In the allergic response, the plasma cell produces IgE-antibodies, primed for the specific allergen. Once formed and released into the circulation, IgE binds to high affinity receptors on *mast cells*, leaving its allergen specific receptor site available for future interaction with allergen. The immune system is now sensitised for the specific allergen. Other cells known to express high-affinity receptors for IgE include basophils, Langerhans cells and activated monocytes.

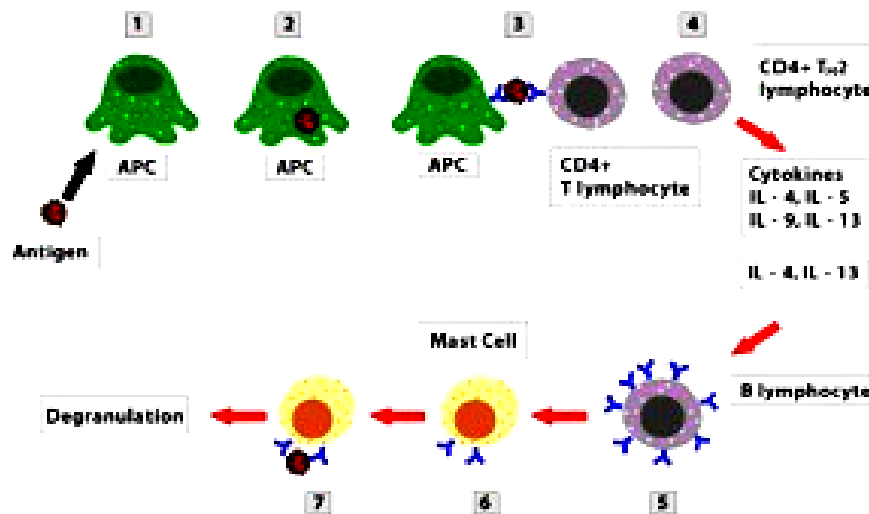


Fig 2. The Sensitisation. Antigen presenting cells (APC) express antigen on their surface, thereby triggering B-lymphocytes, which produces IgE-antibodies. The antibodies then bind to mast cells, priming them for the specific antigen. Figure from AnaesthesiaUK.

Early and late response

When the now sensitised subject is re-exposed to the allergen, binding of the allergen to IgE triggers the immune system to initiate a more aggressive and rapid memory response. The early-phase allergic response is that which occurs within 30 minutes of allergen exposure. Cross-linking of a sufficient number of mast cell/basophil-bound IgE antibodies by allergen initiates a process of intra-cellular signalling which leads to degranulation of cells and release of primary inflammatory mediators, such as *histamine* and *cysteinyl leukotrienes*. The symptoms induced are dependent on the affected organ, and include bronchoconstriction in the lower airways, wheal-and-flare reaction in the skin and rhinorrhea in the nose.

A late-phase response commonly occurs 3-8 hours after allergen exposure. The phase is dominated by recruitment, tissue infiltration and activation of eosinophils, macrophages and lymphocytes [44]. Mechanisms involved in the initiation of the late-phase cellular response are not entirely clear, but most likely involve multiple cells and mediators. T helper 2 cells have been suggested to have a central role in directing the allergic inflammation [45, 46].

Airway inflammation and the united airways concept

United airways concept

The increase in the prevalence of asthma has been associated with an increase in atopic sensitisation, and is paralleled by similar increases in other allergic disorders such as eczema and rhinitis [47]. Most patients with asthma have rhinitis [48]. Of 7219 patients with asthma in the UK, 76 % reported symptoms of rhinitis. Of this 76 %, half said that their

rhinitis made their asthma worse [49]. Many patients with allergic rhinitis have an increased bronchial reactivity to methacholine or histamine [50, 51]. It is also known that patients with rhinitis have an increased risk of developing clinical asthma over time [52]. The presence of airway hyperresponsiveness together with atopic manifestations in childhood increases this risk [53]. This close connection has led to the concept of “one airway one disease” or *united airways* [54, 55]. One model that has been proposed is that the two conditions are manifestations of one syndrome and that the more severe the rhinitis, the more severe the asthma [56]. However, it is not clear whether allergic rhinitis represents an earlier clinical manifestation of allergic disease in atopic subjects who will later go on to developing asthma or whether the nasal disease itself is causative for asthma [1].

Thus, allergic rhinitis and asthma are commonly associated, and the nasal and bronchial mucosa is similar in many ways. There are also differences. The nose and bronchi have different embryologic origin, and smooth muscle is present only in the bronchi [57]. Still, segmental bronchial provocation can induce nasal inflammation in patients with allergic rhinitis and, conversely, nasal allergen challenge can induce bronchial inflammation [58, 59]. Different theories have been suggested on how this distant interaction can be explained. For example, locally produced inflammatory mediators could affect distant leukocytes through systemic circulation, or circulating leukocytes could become activated when passing through the affected tissue [60].

In both allergic rhinitis and asthma, inflammation of the airways is strongly associated with airway hyperresponsiveness and symptoms. The acute inflammatory response includes well known reactions such as

bronchoconstriction, plasma exudation and mucus hypersecretion in the lungs, and itching, sneezing, rhinorrhea and blockage in the nose [61, 62]. The inflammation involves infiltration of inflammatory cells such as activated mast cells, eosinophils and T cells in the airway wall and at the airway surface [46, 63]. In asthma, over 100 different mediators are recognized to be involved and mediate the inflammatory response in the airways [64]. Even structural cells of the airways such as epithelial cells, smooth muscle cells and fibroblasts have been shown to synthesize and release inflammatory mediators [65-67]. The eosinophil cationic protein (ECP) is a secretory ribonuclease, which is found in the eosinophilic leukocyte [68]. Levels of ECP can be measured in various body fluids (eg sputum, serum, saliva) and have been shown to correlate well with airway inflammation but not airway hyperresponsiveness. Thus, it can be useful in assessing asthma severity, compliance with anti-inflammatory asthma therapy and as a guide to tailing down inhaled corticosteroid therapy [69].

Overproduction of IgE plays a critical role in the inflammatory process in both allergic rhinitis and asthma, and is the result from complex interaction between B-cells, T-cells, mast cells and basophils through various inflammatory mediators [70, 71]. Key mediators are the cysteinyl leukotrienes (CysLTs), a family of inflammatory lipid mediators synthesized from arachidonic acid by several cells, including mast cells, eosinophils and macrophages. Receptors for CysLTs can be found in both bronchial and nasal mucosa, and production of CysLTs is increased in patients with allergic rhinitis and asthma. They appear to play a role in both the early and late phase of the allergic reaction, and are involved in recruitment and maturation of inflammatory cells [72, 73].

While the acute inflammation phase has previously been in focus, it is being increasingly recognized that chronic inflammation is an important aspect of asthma [74]. This chronic inflammation may result in structural changes in the airway, referred to as airway remodeling. These structural changes include fibrosis resulting from deposition of extra cellular matrix components such as collagen, smooth muscle cell hyperplasia and hypertrophy, hyperplasia of mucus-secreting cells, and new vessel formation (angiogenesis) [75]. This remodeling may explain the irreversible lung function abnormalities experienced in some asthmatics, even in remission [76]. Glycosaminoglycans are essential extracellular matrix molecules which regulate tissue flexibility. Hyaluronan is a glucosaminoglycan, and as such an important part of early connective tissue repair. Hyaluronan deposition around and internal to the smooth muscle would be expected to oppose the effect of smooth muscle contraction [77]. Elevated levels of hyaluronan are commonly seen in bronchoalveolar lavage in patients with fibrosing inflammatory conditions, and thus can be regarded as a potential marker of tissue remodelling [78, 79].

In allergic rhinitis, remodeling is still poorly understood and the pathological extent of nasal remodeling as well as its clinical consequences is unclear [80, 81].

The role of the small airways

As mentioned above, the small airways provide only 10 % of the total airway resistance [43]. This has led to the small airways being termed “the silent zone” since airflow obstruction within them causes little change in conventional tests of lung function [82]. However, it is known that asthmatic inflammation is present in the small airways [83]. Although inflammation in the large central airways has been the subject of numerous asthma studies, inflammation in the small distal airways remains largely unexamined because of the relative inaccessibility of these structures. However, growing evidence suggest that small airway inflammation is not clinically silent in asthma. By the use of a fiberoptic bronchoscope wedged into a subsegmental bronchus, Wagner et al found a sevenfold increase in peripheral airway pressure in mild asthmatics compared to healthy subjects, even though the lung function appeared normal [84]. Increased numbers of lymphocytes and eosinophils have been shown to be uniformly distributed throughout the large and small airways of mild and severe asthmatic persons as compared with control cases [85]. Small airway remodeling may be the explanation for the development of irreversible airflow obstruction [86]. Nocturnal asthma is associated with an increase in night-time distal lung inflammation, as evidenced by the accumulation of alveolar tissue eosinophils, macrophages and CD4+ lymphocytes. Interestingly, only alveolar (and not central airway) eosinophilia correlated with overnight reduction in lung function [87, 88]. The presence of an enhanced inflammatory process in the small airways is consistent with an increase in the peripheral airway resistance [89]. The involvement of the small airways

seems to be particularly prominent in fatal asthma [90]. Distal lung disease appears to increase the risk of recurrent asthma exacerbation [91]. The introduction of high-resolution computed tomography allows assessment of the contribution of small airways to deficits in lung function. Results of such imaging suggest that the small airways may play a significant role in airway hyperresponsiveness in asthmatics [92, 93].

In conclusion, all these findings suggest that the small airways are of utmost importance in the development and progress of asthma, and subsequently also plays an important role in the treatment of the disease. The clinical implications of small airways disease on the united airway concept are still not clear.

Monitoring airway inflammation

Invasive vs. non-invasive techniques

The nature and extent of pulmonary diseases can be assessed by direct invasive bronchoscopy with bronchial washings, biopsy, and/or bronchoalveolar lavage. While bronchoscopy can provide valuable information, it requires well trained personnel, and can be demanding on the patient. The last few decades, new promising non-invasive techniques to monitor lung function and airway inflammation have been developed:

Nitric oxide

Nitric oxide (NO) was initially described as an endothelium-derived relaxing factor [94]. It can be measured in exhaled air, and is produced in the nose and paranasal sinuses, as well as in the bronchial tree [95, 96].

Levels of exhaled NO increase during active asthma and allergic rhinitis [97, 98]. Thus, high levels of NO may reflect ongoing inflammation in the airways of the patients, and can therefore be regarded as a non-invasive potential clinical tool to monitor asthma [99]. Indeed, exhaled NO has been shown to correlate with other inflammation indicators, such as induced sputum eosinophilia and bronchial reactivity, in steroid-naïve asthmatics [100]. Exhaled NO arises from the airway and alveolar compartments, and recently, new analytical methods have been developed to characterize these sources [101]. Through models of the NO exchange dynamics, the exhalation flow rate dependence of the exhaled NO concentrations have been explained. This allows for discrimination of the NO contribution in the different compartments of the lung. Put simply, by measuring NO at different exhalation flows it is possible to approximate the NO concentration in the peripheral region as well as the conducting airways. Thus, exhaled NO may provide further pathophysiological understanding of the pattern of inflammation in various airway diseases.

Nasal NO concentrations are very high relative to the lower respiratory tract in humans, and has been proposed as a surrogate marker for inflammation in allergic rhinitis, but results have not been as consistent as in asthma [97, 102, 103].

Induced Sputum

The aim of sputum induction is to collect a sample of secretions from the lower airways in subjects who do not produce sputum spontaneously, which allows access to cell subsets and inflammatory biomarkers which

may help in the diagnosis and monitoring of the airway disease. Nebulised isotonic or hypertonic solutions are used to induce production of expectorate. The expectorate can then be processed and analysed for biomarkers of disease. The method has been well validated and reference values for healthy adults have been published [104, 105]. It is well known that the level of ECP and eosinophilic cell count in sputum are higher in asthmatics than in healthy subjects, and eosinophil cell count has been used as a successful tool to guide asthma treatment adjustment [45, 69, 106, 107]. Induced sputum is also a potential tool for phenotyping asthma: high percentages of lymphocytes have been found in ski asthma, whereas eosinophils and neutrophils were increased in asymptomatic swimmers and runners respectively [108-110]. Future research may find novel biomarkers [111].

Evaluation of lung function

Spirometry

While patient history and clinical examination are important in the diagnosis of asthma, they do not provide any reliable information on the extent of the airway obstruction. Spirometry is the traditional method for measuring lung function and has been used for decades, and recommendations on standardisation have been published [39, 112]. It is a physiological test that measures how an individual inhales or exhales volumes of air as a function of time. This is most commonly expressed as the forced vital capacity (FVC), which is the volume delivered during an expiration made as forcefully and completely as possible starting after full inspiration, and the forced expiratory volume in one second (FEV₁), which is the volume delivered during the first second of the FVC

manoeuvre. These, and other parameters, can be used to evaluate the subject's lung function compared to reference values, and also to some degree characterize the type of impairment (eg obstructive or restrictive lung disease). It can also be used to assess the reversibility of the obstruction, by comparing results before and after treatment with bronchodilators or inhaled steroids [39]. This greatly enhances diagnostic confidence, as patients with asthma frequently have poor perception of symptom severity, especially if their asthma is longstanding [113]. Thus, spirometry can provide complementary information about different aspects of asthma control.

Impulse Oscillometry

The forced oscillation technique is a non-invasive method with which to measure respiratory mechanics, and was first used in the fifties [114, 115]. Impulse oscillometry (IOS) is a variant of this technique, and it measures airway impedance by sending a pulse-shaped sound wave produced by a loudspeaker into the lungs of a spontaneously breathing subject and looking for changes in flow in response to the dilatory effect of the applied energy. The oscillations provide a measure of total airway impedance, which reflects both resistive elements of the airways (resistance, R) and viscoelastic and inertive forces in the lungs and the chestwall (reactance, X). By applying sound waves of different frequencies during different phases of the respiratory cycle, the instrument can measure resistance, defined as the opposition of the respiratory system to the flow of air, at different levels in the respiratory tree. Reactance is the sum of inertance, which is the inertive force of the

air column in the conducting airways, and capacitance, which reflects the elastic properties of the peripheral lung. The inertive force of the air column is a physical property of air, and is normally not of any interest in human studies. The inertive part of the total reactance increases with higher frequencies of the sound pulse (the air column must be moved more frequently). Resonant frequency (F_{res}) is the frequency where the inertance and capacitance are equal in magnitude and opposite in sign (phase), and is measured in Hz. Thus, inertive forces dominate at frequencies above F_{res} whereas elastic forces are increasingly related to frequencies below F_{res} . Low frequent reactance is usually reported as X5 (reactance at 5Hz). X5 reflects changes to the lung periphery and is non-specific. Increased negative values can be seen both in restrictive and obstructive disease.

While spirometry is a valuable method for measuring lung function, it is effort dependent and careful instructions to the patient on how to perform the expiratory manoeuvre is required. The forced oscillation technique, on the other hand, requires minimal cooperation from the patients, since the forced oscillations are superimposed on the normal breathing, thus avoiding the need for any special breathing manoeuvre or any noticeable interference with respiration. The minimal influence on respiratory properties is particularly important when assessing airway hyperresponsiveness. The forced oscillation technique also provides the possibility to assess airflow obstruction in the peripheral airways, something that conventional lung function tests can not do. However, one should be aware of the fact that the IOS model is based on theoretical assumptions. Very few physiological correlations between forced oscillation technique parameters and direct evidence of airway function

have been published. Therefore, one has to be careful of drawing too extensive conclusions from the results.

Airway hyperresponsiveness

It is not unusual for patients with asthma to have a normal spirometry, yet experience airway narrowing in response to a stimulus that would be innocuous in a healthy person [41, 116]. This hyperreactivity of the airways is termed bronchial or airway hyperresponsiveness.

The mechanisms behind airway hyperresponsiveness are not completely understood. Excessive contraction of airway smooth muscle due to increased contractility of smooth muscle cells could be one important factor [117]. The thickness of the airway wall from necropsy specimens is greater in subjects with fatal asthma than in those with milder disease and in non-asthmatics, and oedema and structural changes in the airway wall could amplify airway narrowing due to contraction of smooth muscle for geometric reasons [90, 118, 119]. Airway remodeling changes could decrease the radial constraint provided by connective tissue elements, allowing excessive airway smooth muscle shortening, even though some data actually suggest that airway remodeling may provide protection against airway narrowing [119, 120]. Finally, epithelial damage from ongoing inflammation may allow greater amounts of bronchoconstrictor mediators to reach smooth muscle cells, sensory nerves or other cells involved in airway narrowing [121].

While it is not entirely clear what drives the chronic airway hyperresponsiveness, fluctuations in the extent of eosinophilic inflammation may underlie changes in the degree of hyperresponsiveness seen during the course of the disease. Eliminating eosinophilic

inflammation by glucocorticoid treatment improves airway hyperresponsiveness, although it does not eliminate it completely [122]. Likewise, avoiding allergens that may trigger inflammation only improves, but does not eliminate, airway hyperresponsiveness [123]. Almost all asthmatics exhibit increased responsiveness, especially during symptomatic episodes. Airway hyperresponsiveness has also been described in patients with allergic rhinitis, as well as in other pulmonary diseases such as chronic obstructive pulmonary disease [50, 124, 125]. Hence, the presence of airway hyperresponsiveness does not necessarily mean that the patient has asthma. However, lack of airway hyperresponsiveness to a large degree excludes asthma. Thus, measurement of the degree of hyperresponsiveness may help establish a diagnosis of asthma [126, 127].

Challenge testing

Airway hyperresponsiveness can be demonstrated by several different provocation tests. They are usually divided into two groups: direct and indirect provocation. In direct challenge the provoking substance is assumed to act directly on the receptors of effector cells such as smooth muscle cells, endothelial cells and/or mucus producing cells, and hereby inducing bronchial obstruction. The effect is believed to be only partly dependent on present inflammation, and it may be present even in patients with chronic changes, i.e. remodelling [128]. Indirect challenge on the other hand, is presumed to be acting on inflammatory cells, causing them to release mediators, which in turn triggers smooth muscle

cell constriction [42, 129]. Thus, in theory, a positive result to indirect challenge requires inflammation present in the airways.

Methacholine chloride (acetyl- β -methylcholine) is a parasympathomimetic synthetic analog of acetylcholine. It stimulates muscarinic receptors, causing bronchial smooth muscle constriction [130]. Methacholine challenge is a commonly used direct test and has been shown to identify airway hyperresponsiveness with high sensitivity (*fig 3.*) [41, 131, 132]. A negative test can to a high degree exclude asthma as the cause of a patient's symptom, while a positive test has less diagnostic specificity.

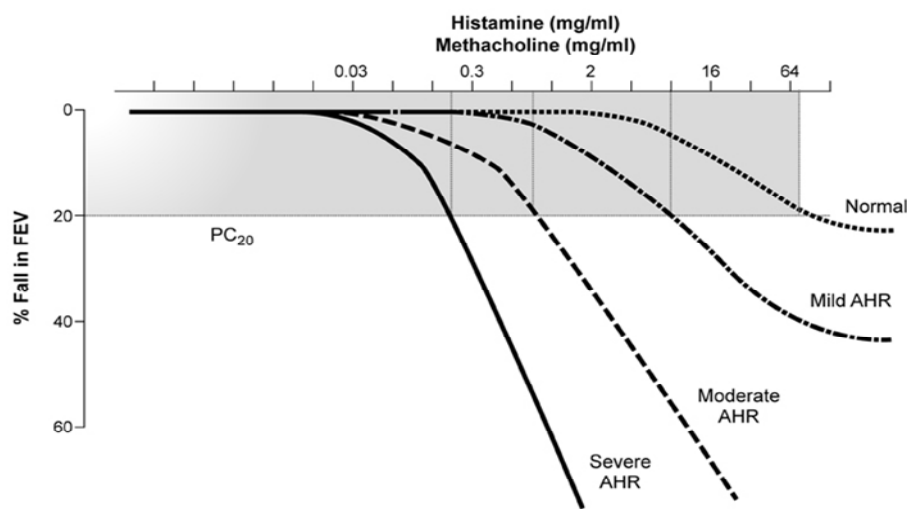


Fig 3. Example of reaction patterns to direct challenge testing (e.g. methacholine or histamine). The concentration of the inhaled provocative substance that triggers a 20 % fall in FEV₁ (PC₂₀) determines the degree of airway hyperresponsiveness.

Indirect tests on the other hand are generally less sensitive. The triggering mechanism in exercise induced bronchoconstriction (EIB) is believed to be the loss of water via evaporation from the airway surface.

This water loss is believed to cause airway narrowing through thermal and osmotic effects of the dehydration [133, 134]. Cold, dry air is more provocative than warm, humid air [135].

Eucapnic voluntary hyperventilation (EVH) simulates the hyperventilation achieved during exercise and it is assumed that individuals sensitive to the provocation are reacting to the increased ventilation per se, possible due to drying of the airway surface liquid and increased osmolarity. EVH have shown high sensitivity in identifying patients with EIB [136].

Mannitol challenge is a fairly new method of applying an osmotic stimulus that mimics the effects of the dehydration caused by hyperventilation during exercise. Mannitol is a polyol (sugar alcohol), and is a potent osmotic stimulus [137]. Mannitol hyperresponsiveness have proven to predict the response to corticosteroid therapy in asthmatics [138]. It can be used to identify patients with asthma with EIB [139]. However, it has been shown to be less sensitive than MCh in identifying airway hyperresponsiveness [140].

AIMS

The overall aim of this thesis was to study the distribution of inflammation and obstruction in asthmatics, by using non-invasive methods, and to compare the results to results from patients with allergic rhinitis. Five studies are included in this thesis with the following specific aims:

- I.** To investigate whether patients with allergic rhinitis and asthma differed from rhinitis with or without bronchial hyperresponsiveness in degree of perception of dyspnoea and airway inflammation, measured as fractional exhaled nitric oxide.
- II.** To assess peripheral and proximal NO concentration in rhinitic subjects, and to correlate the peripheral NO concentration to the peripheral obstruction in response to methacholine.
- III.** To measure induced sputum Cys-LT, as well as markers of remodelling and eosinophilic inflammation in sputum from patients with rhinitis with or without BHR, comparing the results with patients with rhinitis and clinical asthma.
- IV.** To compare the degree of involvement of the peripheral airways during methacholine challenge test in asthmatics and patients with allergic rhinitis with or without BHR by using the impulse oscillometry technique.

- V. To investigate whether different direct and indirect stimuli induces different patterns of obstruction, recorded as central and peripheral resistance. Also to see whether baseline resistance could predict a positive response to direct or indirect provocation.

METHODS

Study populations

For **paper I**, patients with seasonal allergic rhinitis were recruited and investigated with methacholine challenge testing with impulse oscillometry, fractional nitric oxide and induced sputum, both during and outside pollen season.

For **paper II-IV**, the size of the study population in paper I was increased by further recruitment. All subjects underwent the testing detailed above. Only those patients that were able to produce sputum were used in paper III.

For **paper V**, mild asthmatics were recruited and investigated with MCh, EVH and mannitol challenge testing, as well as impulse oscillometry and fractional nitric oxide.

All patients attended the outpatient clinic of the department of lung medicine in Lund. All subjects gave written informed consent, and the ethical committee in Lund approved the studies.

Study populations are described in table 1.

| | allergic rhinitis, total (female) | asthma | controls |
|------------------|--|---------------|-----------------|
| Paper I | 29 (17) | 11 | 14 |
| Paper II | 51 (30) | 26 | 12 |
| Paper III | 41 (26) | 16 | 13 |
| Paper IV | 53 (30) | 26 | 13 |
| Paper V | | 34 | 14 |

Table 1. Study populations

Subject characterization

All subjects were non-smokers without upper respiratory tract infection within three weeks prior to the investigation.

Healthy controls (paper I-V)

Healthy controls had no history of allergic symptoms. A skin prick test, SPT, (Alk Abello, Copenhagen, Denmark) was used to screen for sensitization to a standard panel of 10 common airborne allergens (birch, timothy, mugwort, cat, dog, horse, *d. pteronyssinus*, *d. farinae*, *aspergillus* and *cladosporium*). Controls with positive skin prick test were excluded. None of the controls included were hyperresponsive to methacholine (negative challenge on a cumulative dose of 2000 microg). Their age ranged from 19 to 56 (paper I-IV) and 24 to 61 (paper V).

Patients with seasonal allergic rhinitis (paper I-IV)

Subjects with symptoms of allergic rhinitis were recruited and tested with SPT. Only those with pure seasonal allergy were investigated, ie those who had a positive SPT to birch, timothy and/or mugwort. Those with confirmed sensitization to perennial allergens (animal dander, dust mites or moulds) were excluded. Sensitization to animal dander was allowed if the patient were not exposed to animals.

Patients with allergic rhinitis was subdivided into patients with allergic rhinitis and no bronchial hyperresponsiveness, patients with allergic rhinitis and bronchial hyperresponsiveness but no symptoms of asthma and patients with allergic rhinitis and doctor's diagnosed asthma.

Patients with asthma (paper I-V)

In paper I-IV, some of the patients with allergic rhinitis had concomitant asthma. They had symptoms of airway obstruction and were clinically diagnosed mild asthmatics according to global initiative for asthma (GINA) standards. Three of the asthmatics, used in paper II, inhaled corticosteroids daily (200-400 microg budesonide).

In paper V, the disease group consisted of patients with clinically diagnosed mild asthma. Inhaled corticosteroid (ICS) treatment was allowed with a maximum daily dose equivalent of 800 microg budesonide.

Spirometry

Flow-volume spirometry was used to assess pulmonary function in all papers. A MasterScope spirometer, software version 4.5 (Erich Jaeger GmbH, Wurzburg, Germany) was used for the flow-volume spirometry, which was done according to the guidelines of the European Respiratory Society [112]. The reference values were obtained from Crapo et al. [141]. The better of two measurements of forced expiratory volume in 1 s (FEV₁) with less than 4% variation was recorded as baseline.

Borg Symptom Score

Borg symptom score results are presented in paper I.

Before every flow-volume measurement the subjects were asked to grade their perception of dyspnoea on a 10-grade scale, with 0 being no dyspnoea at all and 10 being maximal dyspnoea (*fig 4*) [142]. All subjects had a baseline dyspnoea of 0. The subjects were blinded to their

lung function response. Borg scores were plotted against percentage of fall in FEV₁ from baseline and linear regression analysis was used to calculate a FEV₁/Borg slope (Slope-BorgMCh) for every individual, which was used as an index of dyspnoea.

| | |
|------------|-----------------------------------|
| 0 | nothing at all |
| 0,5 | extremely weak |
| 1 | very weak |
| 2 | weak |
| 3 | moderate |
| 4 | somewhat strong |
| 5 | strong (heavy) |
| 6 | |
| 7 | very strong |
| 8 | |
| 9 | |
| 10 | extremely strong (maximal) |

Fig 4. The Borg Symptom Scale.

Methacholine challenge testing

Results from methacholine challenge testing are presented in all papers.

Presence of airway hyperresponsiveness was measured with a methacholine challenge test. First, baseline FEV₁ was assessed with flow-volume spirometry, described above. If the baseline value was below 70 % of the predicted value, the challenge was aborted. The test was carried out with tidal volume triggered equipment (Aerosol Provocation System, APS, Erich Jaeger GmbH, Wurzburg, Germany). The APS delivered a cumulative dose of 2000 microg MCh in five increments (50, 150, 300, 600 and 900 microg) following an initial dose of 0.9 % NaCl. The

challenge was discontinued if the FEV₁ declined more than 20 % during the protocol. A positive test was defined as the cumulative dose that caused a decline in FEV₁ by 20 % or more (PD₂₀FEV₁) from baseline. The PD₂₀FEV₁ was determined by interpolation by the last two points on the log dose-response plot. The amounts of MCh given during every increment of the challenge test were plotted against the corresponding percentage fall in FEV₁ from baseline. Linear regression analysis was used to calculate a MCh/FEV₁ slope (Slope-FEV₁^{MCh}) which was used as an index of airway hyperresponsiveness. When FEV₁ fell below 80 % of the baseline value or when the total amount of 2000 microg MCh was delivered, 400 microg of salbutamol were given to all subject immediately after finishing the provocation. After 10-15 minutes a new flow-volume spirometry was carried out, to ensure that the subjects were recuperating properly.

Exhaled Nitric Oxide

Data obtained from Exhaled Nitric Oxide measurements are presented in paper I, II and V.

NO measurements were performed in accordance with International American Thoracic Society recommendations, using a NIOX, nitric oxide gas analyser (Aerocrine, AB, Stockholm, Sweden) [143]. Patients were comfortably seated, inhaled NO depleted ambient air, and exhaled at different flow rates (paper I-IV: 10, 50, 100 and 400 ml/s; paper V: 50, 100, 200 and 400 ml/s) 2–4 times depending on divergence.

Peripheral NO concentration (or alveolar concentration, CA_{NO}) and proximal maximal NO flux (*J*_{awNO}) was approximated by plotting NO-

output (product of concentration and flow) against exhalation flow (at flow 100-400 ml/s) [101]. The slope and intercept of this line approximate CA_{NO} and J'_{awNO} , respectively [144, 145]. Calculations using the flow 50 ml/s were also performed, but were not used as an increase in slope and a decrease in the intercept were observed confirming previous reports that linearity between NO-output and flow is valid only for approximately above 99 ml/s [146]. All NO measurements were done prior to bronchial challenge test.

Impulse Oscillometry

Results from impulse oscillometry are presented in paper II, IV and V.

During each challenge test, impulse oscillometry was used to provide further information on the magnitude and site of obstruction in the airways.

A Jaeger MasterScreen Impulse Oscillometry System (Erich Jaeger GmbH, Wuerzburg, Germany) was used. Oscillometry was performed before the challenge and after each step of the challenge, prior to the spirometry, to avoid the influence of deep inspiration and subsequent maximal forced expiratory maneuvers on IOS parameters. The subjects used nose clips and were told to press the palms of their hands against the cheeks to decrease the upper airways shunt. For about 30 seconds, oscillometric pressure impulses were superimposed on the tidal breathing of the subject, having a pulse sequence of 5 per second and a frequency spectrum between 5-35 Hz. Airway resistance at 5 Hz and 20Hz (R_5 , R_{20}), reactance at 5 Hz (X_5), resonant frequency (F_{res}) and area of reactance integrated from 5 Hz to F_{res} (AX) were determined. During

MCh and Mannitol challenge test, IOS were performed 45 s. after each challenge step, while FEV₁ was performed 75 s. after each challenge step. During the EVH challenge, IOS was performed 1, 3, 5, 7.5, 10, 15 and 20 min post challenge, with FEV₁ performed immediately after IOS at each step.

Induced sputum

Data obtained from induced sputum are presented in paper III.

Sputum induction

Sputum was induced by inhalation of nebulized isotonic saline solution (0.9% NaCl) for 0.5, 1, 2 and 4 min, followed by a hypertonic solution (4.5% NaCl) for 0.5, 1, 2 and 4 min. Lung function (PEF) was measured 1 min after each induction time-point, and induction was interrupted if lung function was decreased $\geq 20\%$. Subjects were asked to rinse their mouth and blow their nose, and try to cough between each dose of nebulized saline. Sputum induction was continued until adequate sample volume was obtained (mean time: 7.8min, SD: 4.4), and there was no difference in sputum induction time among the patient groups.

Sputum processing

Sputum plugs were sorted out, and treated with four volumes of 0.65 mM dithiothreitol (DTT) in phosphatebuffered saline (PBS) for 1 h in 4 °C. Additional four volumes of PBS were added, followed by filtration through a 60 mm filter and a final centrifugation (1000 g for 5 min),

which separated the supernatant from the cells. The supernatant was frozen until later analysis.

Sputum analysis

Sputum was analyzed for cysteinyl-leukotrienes and LTB₄ using EIA (detection limit 13 and 6 pg/mL, respectively) from Cayman Chemical (Ann Arbor, MI, USA). Before analysis of subsequent assays, sputum was dialysed to PBS to eliminate the amount of DTT. ECP was measured using the UniCap ECP kit (detection limit 0.5 ng/mL, Pharmacia Diagnostics, Uppsala Sweden), IL-8 and IL-13 using Quatikine (detection limit 3.5 and 32 pg/mL, respectively, R&D Systems, Abingdon, UK), hyaluronan and laminin using ELISAs (detection limit 10 ng/mL for both assays) from Echelon Biociences incorporated (Salt Lake City, UT, USA) and Chemicon International (Temecula, CA, USA), respectively. The total protein concentration was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories.

Inc., Hercules, CA, USA). All values were adjusted to the total amount of protein in sputum (and presented as amount per microgram total protein) to abolish differences due to sputum heterogeneity. Samples were run in duplicate with a maximum in between variation of 5%. All tests were commercially standardized and further standardization for the use of sputum analysis was performed.

Mannitol challenge testing

Results from mannitol challenge testing are presented in paper V.

Pre-challenge spirometry was performed as described for MCh challenge above. A mannitol powder kit (Aridol™; Pharmaxis, Frenchs Forest, Australia) was used in conjunction with a dry powder inhaler device to administer a cumulative dose of 635 mg in 8 increments according to the manufacturer's instructions. The challenge was discontinued if the FEV₁ declined more than 15 % from baseline or if a between-dose fall of >10 % occurred, which was considered a positive test [147]. After the challenge, the subject received an inhalation of 400 microg salbutamol and a new spirometry was performed 10-15 minutes later to ensure that the subject was recuperating properly. The PD₁₅FEV₁ was determined by interpolation by the last two points on the log dose-response plot. The Mannitol/FEV₁ slope was calculated in the same way as the slope for MCh (see above).

Eucapnic Voluntary Hyperventilation

Results from Eucapnic Voluntary Hyperventilation are presented in paper V.

Pre-challenge spirometry was performed as described for MCh challenge above. The patients were instructed to hyperventilate for 4 minutes, at 85 % of maximum voluntary ventilation (30 x Baseline FEV₁), guided by a reservoir balloon. In order to maintain eucapnia, the dry air ventilation device (Ailos Medical AB, Karlstad, Sweden) administered hypercapnic air (5 % CO₂). The air inspired was dry and at room temperature. The spirometry was repeated together with IOS post challenge at 1, 3, 5, 7.5, 10, 15 and 20 minutes. Thereafter the subject received an inhalation of 400 microg salbutamol and a new spirometry was performed 10-15

minutes later to ensure that the subject was recuperating properly. A drop of $FEV_1 > 10\%$ compared to baseline was regarded as a positive test (EVH 10) [127].

Statistical Analysis

Generally, as the data could not be assumed to have a normal distribution, non-parametric tests were used. The Mann-Whitney U-test was used for comparison between two groups (paper I-V). Statistical comparison between more than two groups was done with Kruskal-Wallis test for independent samples (paper I-V). Spearman correlation coefficient was used to determine correlation between groups (paper I-III, V). In the case of paired samples, Wilcoxon's test was used (paper I-III). A p-value of less than 0.05 was considered significant.

RESULTS AND COMMENTS

Paper I - Allergic rhinitis with or without concomitant asthma: difference in perception of dyspnoea and levels of fractional exhaled nitric oxide

It is well established that allergic rhinitis and asthma are closely linked entities and more than 75 % of the patients with asthma reports concomitant rhinitis [148]. Asthma is also closely associated to BHR, and a large part of patients with rhinitis alone show a reactive pattern in bronchial provocation tests, even though they have no symptoms of clinical asthma [50]. The fact that patients can react with airway obstruction to bronchial challenge, which is a hallmark of asthma, without experiencing symptoms is curious and could possible be explained by difference in degree and/or geographical distribution of inflammation. In this study, we aimed to investigate the degree of perception of dyspnoea and airway inflammation in patients with allergic rhinitis with or without concomitant asthma, both during and outside pollen season.

We found that 12 out of 18 patients with allergic rhinitis without asthma had bronchial hyperresponsiveness to methacholine, which is in line with previous observations. We also found increased inflammatory activity, measured as FENO, during pollen season in asthma patients, but not in those with rhinitis alone. There was a correlation between the degree of inflammation and the degree of BHR in the asthma patients, but not in patients with allergic rhinitis and BHR. This may indicate that the pathogenesis of BHR is dependant on several factors, and that ongoing inflammation is more linked to BHR in asthmatics.

Interestingly, patients with asthma had a greater perception of the obstruction induced during the methacholine challenge test, compared to patients with rhinitis and BHR (*fig 5*). No correlation of symptoms and FENO levels could be found, indicating that the presence or absence of symptoms could not be explained by degree of inflammation alone. Thus, the reason for this difference in perception is still unknown. Psychological factors could play a role, and possibly could awareness of obstruction increase over time. Another explanation could be that symptoms may be dependent on geographical distribution of inflammation, specifically the degree of peripheral airway involvement.

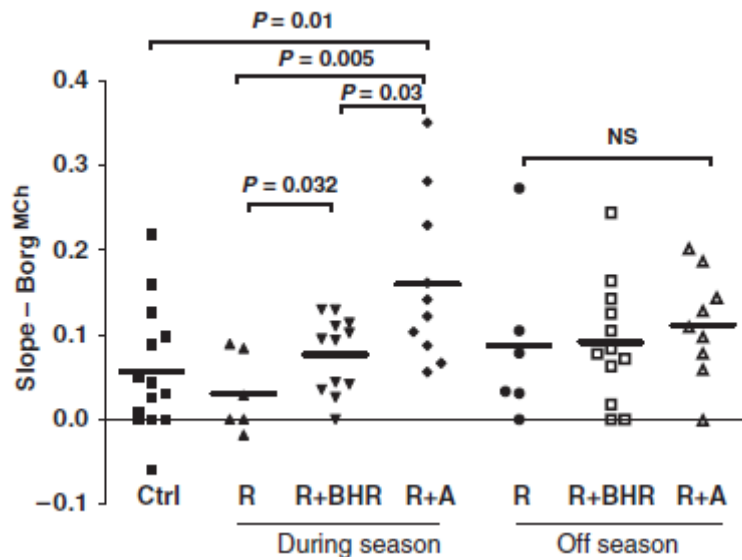


Fig 5. Slopes of Borg/FEV1 for controls, patients with rhinitis alone with and without bronchial hyper-responsiveness (BHR) and patients with rhinitis and asthma during season and off season.

Paper II - Peripheral nitric oxide is increased in rhinitic patients with asthma compared to bronchial hyperresponsiveness

Based on the conclusions in paper I, in this paper we hypothesised that involvement of the peripheral airways differs between patients with rhinitis and concomitant asthma and patients with (or without) BHR. In recent years it has been possible to measure NO at different exhalation flow, and approximate the NO concentration in the peripheral region as well as the conducting airways [144]. Thus, in theory, it is possible to study the geographical distribution of inflammation in the airways. Involvement of peripheral airways can also be estimated by impulse oscillometry, where different responsive patterns to different frequencies reflect peripheral properties of the respiratory tract [149].

We found increased peripheral NO concentrations in patients with rhinitis and concomitant asthma compared to patients with rhinitis only, while patients with rhinitis and BHR represented an intermediate step between those with rhinitis only and those with asthma (*fig 6*). Increased proximal NO concentrations was also seen in asthmatics, but not in patients with rhinitis and BHR. Furthermore, we found a correlation between peripheral NO concentration and degree of peripheral obstruction during methacholine challenge test, while no correlation were seen between proximal NO concentrations and peripheral obstruction parameters. Thus, those with highly reactive peripheral airways also seemed to have a higher degree of ongoing small airway inflammation. Overall, results from this study seem to strengthen our hypothesis that asthmatics have more widespread inflammation, which includes the small airways. Interestingly, the three subjects in the asthmatic group that had

anti-inflammatory treatment (ICS), still showed signs of a high level of peripheral inflammation as well as peripheral obstruction.

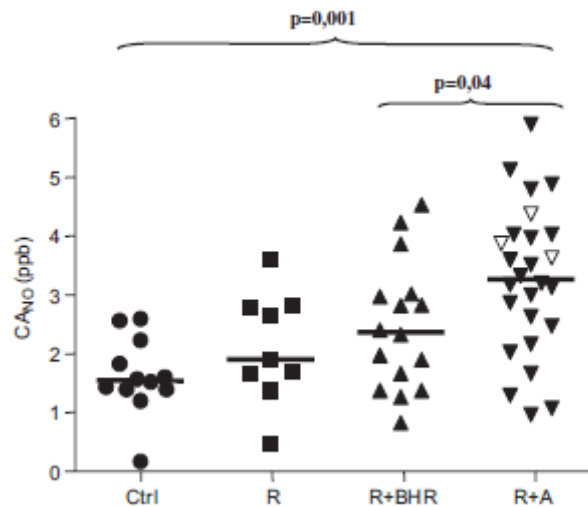


Fig 6. Peripheral NO concentration. Concentration of peripheral NO ($C_{A_{NO}}$) assessed by measuring exhaled NO at several exhalation flow rates in patients with rhinitis (R), rhinitis with bronchial hyperresponsiveness (R+BHR), rhinitis and concomitant asthma (R+A) and healthy controls (Ctrl).

Paper III - Cysteinyl-leukotriene levels in sputum differentiate asthma from rhinitis patients with or without bronchial hyperresponsiveness

The transition from allergic rhinitis to clinical asthma is probably a gradual one, with bronchial hyperresponsiveness possibly representing an intermediate step. Our findings in paper II seem to strengthen this theory. Previous studies have shown that levels of eosinophils and eosinophilic cationic protein (ECP) in induced sputum are increased in patients with rhinitis and BHR, but not as high as in patients with rhinitis and asthma

[150, 151]. It is well known that both the level of ECP and eosinophilic cell count in sputum are higher in asthmatics than in healthy subjects, with a correlation to disease severity [45] [69, 152].

Cysteinyl-leukotrienes (Cys-LTs) are actively involved in the inflammation in asthma and rhinitis, Cys-LTs are known to be elevated in sputum from asthmatics and have been shown to be correlated to eosinophil cell count [153, 154].

Hyaluronan is a glucosaminoglycan, and as such an important part of early connective tissue repair, and can be regarded as a potential marker of tissue remodelling [78, 79].

In this paper we wanted to measure induced sputum Cys-LTs, as well as markers of remodelling and eosinophilic inflammation in sputum from patients with rhinitis with or without BHR, comparing the results with patients with rhinitis and clinical asthma.

We found increased levels of Cys-LT and hyaluronan in sputum in asthmatics compared to patients with rhinitis with or without BHR (*fig 7*). Asthmatics had a slightly higher concentration of ECP compared to patients with rhinitis and BHR, but the difference was not significant. This indicates that there is more inflammatory turnover of the connective tissue in rhinitis patients with asthma compared with BHR only, and that Cys-LT driven inflammation is present in the asthmatic group. While patients with rhinitis and BHR had significantly lower levels of Cys-LT compared to asthmatics, they still had slightly higher levels of ECP and Cys-LT compared to patients with rhinitis only. This might indicate an initiated inflammatory process in the airways that may later lead to the development of asthma, strengthening our hypothesis that transition from

rhinitis only to clinical asthma is probably a gradual one, with BHR representing an intermediate step.

The levels of Cys-LT significantly decreased in asthmatics after pollen season, while there were no significant changes in the levels of hyaluronan and ECP concentration during and after pollen season. Possibly, this is due to Cys-LT levels reflecting an ongoing inflammation, while tissue matrix turnover is a process that is occurring over time and therefore may not change as rapidly.

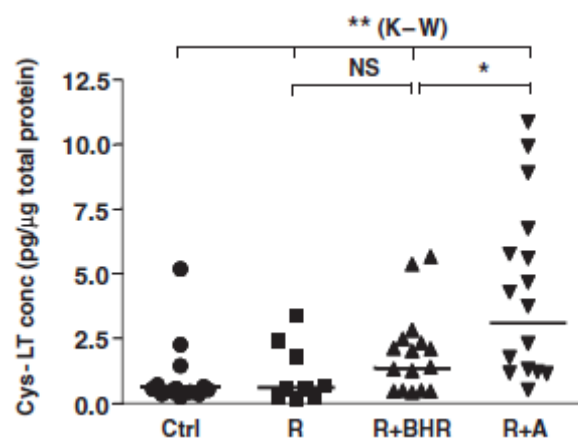


Fig 7. Concentration of cysteinyl-leukotrienes (Cys-LT) in sputum from patients with rhinitis (R), rhinitis with bronchial hyperresponsiveness (R+BHR), rhinitis and concomitant asthma (R+A) and healthy controls (Ctrl).

Paper IV - Allergic rhinitis with hyperresponsiveness differ from asthma in degree of peripheral obstruction during methacholine challenge test

So far, our studies have shown increasing evidence of peripheral airway involvement in asthmatics. However, peripheral airways only accounts for 10 % of the total airway resistance, which means that conventional

tests of the lung function (e.g. FEV₁) fail to accurately reflect changes in peripheral resistance [43, 155, 156]. Hence, the term “silent zone” is sometimes used for the small airways [157]. Impulse oscillometry is a forced oscillation technique that allows for discrimination between central and peripheral obstruction [158].

In this paper we wanted to compare the degree of involvement of the peripheral airways in asthmatics and patients with allergic rhinitis with or without BHR, specifically the degree of peripheral airway obstruction during methacholine challenge test, by using impulse oscillometry.

We found that while patients with rhinitis and asthma and patients with rhinitis and BHR showed similar reactivity to methacholine, asthmatics had significantly more increase in parameters indicating peripheral obstruction (i.e. dR5-R20, AX, X5) during the methacholine challenge test (*fig 8*). The proximal resistance (i.e. R20) followed a similar pattern in all groups. Thus, both patients with asthma and patients with rhinitis and BHR reacted to methacholine with decrease in FEV₁, and while the degree of obstruction in the bronchi seemed to be similar, asthmatics had signs of a higher degree of peripheral involvement. Possibly, this could explain our previous findings that asthmatics have a greater perception of bronchial obstruction.

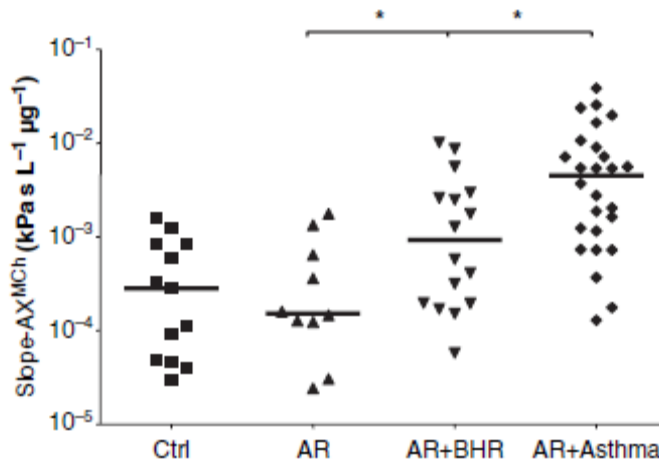


Fig 8. Slope-AX^{MCh} for controls, patients with AR with or without BHR and patients with AR and concomitant asthma.

Paper V - Characterization of airway reactivity to methacholine, mannitol and eucapnic hyperventilation in mild asthmatics

In the previous papers we have found evidence of peripheral airway obstruction in asthmatics, triggered by methacholine challenge tests. Provocation testing for identifying airway hyperresponsiveness in asthmatics has become increasingly important in the diagnosis of asthma and for monitoring the effect of treatment. Methacholine challenge is a well established provocation test; it is a direct test, ie it acts directly on the receptors of effector cells such as smooth muscle cells, endothelial cells and/or mucus producing cells, and hereby inducing bronchial obstruction. Hence, it is believed to be only partly dependent on present inflammation. Indirect challenge on the other hand, is presumed to be acting on inflammatory cells, causing them to release mediators, which in turn triggers smooth muscle cell constriction. Indirect challenges could

thus possibly provide more information about underlying inflammation in the airways.

While methacholine challenge testing is a very sensitive tool for detecting airway hyperresponsiveness, not all asthmatics react to indirect testing. Specifically, exercise induced obstruction is a common feature in asthma, but not all asthmatics suffer from it. Exercise challenge is believed to cause obstruction through hyperventilation, which could cause drying of the airway surface liquid and increased osmolarity. Two other examples of indirect challenges are eucapnic voluntary hyperventilation and mannitol provocation test, which both apply osmotic stimuli to the airways. Since EIB seems to occur more in peripheral airways than in central airways [159], it is plausible to assume that different pattern of reaction to various challenge tests may identify different asthma phenotypes.

In this study we compare the reactive pattern during direct and indirect challenge testing in patients with mild asthma, by using impulse oscillometry. We also investigated whether baseline resistance could predict the outcome of either challenge test.

We found that 5 patients were negative to all tests. Of the remaining 29 patients, 27 were positive to direct testing (methacholine) and 23 were positive to indirect testing (either EVH or mannitol). Thus, even in mild asthmatics, a majority of the patients are positive to indirect testing. Interestingly, even though EVH and Mannitol challenges are thought to trigger the same mechanisms, not all patients positive to EVH were positive to mannitol. This indicates that the tests are not fully interchangeable. However, the limits for what constitutes a positive result differ between the tests (10 % fall in FEV₁ for EVH and 15 % fall for

mannitol), and when changed to 10 % fall in FEV₁ for both tests, the result became more similar.

No difference in broncho-constrictive pattern could be identified during the different provocation tests; the obstruction induced seemed to follow the same geographical pattern regardless of the triggering stimuli. However, those with a positive mannitol provocation had a lower FEV % pred and signs of more peripheral airway involvement at baseline. This supports the idea that peripheral airway involvement is an important predictor of asthma airway reactivity.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Asthma is a serious global health problem that has increased rapidly in prevalence in the western world during the last decades, and is now increasing rapidly in the developing world as well. The main challenge for asthma researchers today is to find a way to prevent this increase in prevalence. While factors influencing the development and expression of asthma are known, and the pathological features of asthma are increasingly well described, the exact link between exposure to risk factors and the development of chronic airway inflammation are not yet fully understood. Until we know more, the possibilities to stop the development of asthma with pharmacological intervention will be limited, and focus will be on identifying and reducing exposure to risk factors. Hopefully, future research results will shed more light on the pathophysiological connection between allergic rhinitis and asthma. The findings in this thesis suggest that advanced allergic airway disease includes involvement of more peripheral parts of the lung. If indeed the progress from allergic rhinitis to asthma is dependent on geographical spreading of the airway inflammation to the peripheral airways, it might be theoretically possible to stop this progress with pharmacological therapy, thus hindering the development of asthma. This, of course, is dependant on gaining knowledge of the specific mechanisms driving the inflammation, which already are under extensive scrutiny from asthma researchers around the world. However, it should be of particular interest to elucidate the process behind the involvement of the peripheral airways.

Is this the step that completes the progress from allergic rhinitis with airway hyperresponsiveness to full blown asthma?

Interestingly, airway hyperresponsiveness is fairly common in patients with allergic rhinitis without concomitant clinical asthma. In paper I, we show that these rhinitis patients do not experience symptoms from bronchial obstruction to the same degree as asthmatics do. Thus, the obstruction of the large airways alone cannot explain the dyspnoea experienced by asthmatics. Indeed, the relationship between inflammation in the airways of a patient and either the symptoms of asthma or airway hyperresponsiveness is not simple. Dyspnoea is multifactorial and the exact mechanism that causes dyspnoea in asthmatics is not fully understood. The sense of respiratory effort, chemoreceptor stimulation, mechanical stimuli arising in lung and chest wall receptors, and neuroventilatory dissociation may all contribute to the sensation of dyspnoea [160]. In asthma, it is speculated that hyperinflation of the lung is a great contributor to dyspnoea [161]. In our studies, it is unclear if asthmatics had more hyperinflated lungs compared to patients with rhinitis and airway hyperresponsiveness. We did find a higher degree of peripheral involvement during the methacholine challenge in the asthmatics. It is tempting to try to explain the difference of dyspnoea in the two groups by referring to difference in peripheral involvement of the peripheral airway, especially since it is in the peripheral airways that the actual primary function of the lungs, the gas-exchange, takes place. However, we found no correlation between degree of peripheral airway involvement and degree of dyspnoea. The reason for the higher degree of symptoms in the asthmatic group remains unclear.

Current guidelines recommend “that patients with persistent allergic rhinitis should be evaluated for asthma by history, chest examination and, if possible and when necessary, assessment of airflow of obstruction before and after bronchodilator” [1], to catch the development of asthma in patient with allergic rhinitis. With emerging insight in the importance of the small airways, small airway involvement should be considered in patients with asthma. Monitoring the small airways is not an easy feat, though. We have seen in this thesis that IOS can provide information on resistance and other properties of the small airways. Fractional exhaled NO can be used to evaluate presence of ongoing peripheral inflammation. These tools are as of yet not easily implemented in the clinical practice. Development of new techniques, or improvement of current technology, could in the future facilitate a more comprehensive assessment of the airways.

Asthma is a heterogenous disease. Different variants include exercise-induced bronchoconstriction and cough-variant asthma. Correct characterization of the disease could have implications for the treatment and exploring the degree of peripheral involvement could be an important part of phenotyping the airway inflammation. In paper V, we found that different provocation tests were not fully interchangeable, and that positive results may reflect different phenotypes. For example, patients with a positive mannitol challenge generally had more evidence of peripheral airway involvement at baseline. Further research on this area is needed. In our study, we tested a broad sample of mild asthmatics. It would be of special interest to investigate specific variants of the asthma disease to elucidate if the pattern of inflammation and airway resistance differs between different asthma groups.

Ideally, we should strive for the ability to cure asthma, if we cannot fully prevent it. While no curative treatment exist today, it is possible to reverse the bronchospasm with bronchodilators. We have access to a variety of anti-inflammatory drugs that block parts of the inflammatory response, in particular the inhaled corticosteroids which have been the mainstay in asthma treatment for over 30 years. Corticosteroids accomplish its effect by inducing the recruitment of the nuclear enzyme histone deacetylase 2 (HDAC2) to multiple activated inflammatory genes, which leads to deacetylation of the hyperacetylated genes, thereby suppressing inflammation [162]. However, not all patients with asthma respond to treatment with corticosteroids, even in high doses. Neither do corticosteroids seem to prevent reduction of lung function over time, which indicates that the remodelling process in the airways is not affected by corticosteroid treatment [163]. It is of particular interest that most current ICS are delivered in a suspension with a particle size of $>2\mu\text{m}$. Thus, it is possible to have an untreated, persistent inflammation in the small airways despite high-dose ICS treatment [164]. Also, treatment with intra-nasal corticosteroids for concurrent rhinitis in asthmatics has been found to have a limited benefit in reducing asthma morbidity in some studies [165, 166]. Current guidelines recommend treatment of not only the lower but also the upper airways [1]. In light of this, the increased involvement of peripheral airways in asthmatics found in this thesis would further stress the need for treating the entire airway system. Since long-term treatment with oral steroids has severe side-effects, and presence of steroid resistant inflammation makes it less effective in some instances, new alternatives are desirable. There already exists other oral treatments in the form of antileukotrienes (e.g.

montelukast), but although antileukotrienes have had some clinical effect in asthma, they are generally less effective and more expensive than inhaled corticosteroids [167]. Anti-IgE treatment (omalizumab) affects the underlying allergic response and is used in patients with elevated serum levels of IgE, specifically as an add-on treatment for severe asthma which is uncontrolled on inhaled corticosteroids [168]. There have been various attempts to block specific mediators and cytokines, but so far the results have not been convincing [169]. The growing knowledge of the mechanisms behind asthma provides more possible targets, and new potential drugs are under development. Future asthma and allergy treatment will probably include not only one but two or more disease-modifying agents administered to the same patient. The possibility for developing a complete cure for asthma is remote, and would probably require an almost complete understanding of the function and regulation of the immuno-system. Until we are there, focus should be on treating the entire asthma disease, including the small airways!

In conclusion, we have found signs of peripheral airway involvement in asthmatics. The next step for me would be to try to further map the distribution of airway inflammation in the airways. New technology could possibly provide new information. For example, High-resolution computer tomography scanning have already made it possible to measure regional air-trapping that accompany changes in small airway calibre [170]. Positron emission tomography and functional magnetic resonance imaging are interesting techniques that may give both anatomic and metabolic information. Inert gas washout techniques may be used to detect not only the degree of pulmonary ventilation inhomogeneity, but also to gain important insight into the location of the underlying disease

process [171]. By using these techniques and other tools previously used in the thesis on different variants of asthma (eg allergic vs non-allergic asthma) I hope to be able to further characterize the different phenotypes, thus getting one step closer to understanding the disease!

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Allergisk rinit (hösnuva) är ett globalt hälsoproblem som orsakar nedsatt funktionsförmåga och sjukdom i alla åldersgrupper. Prevalensen (andel sjuka) av allergisk rinit kan ligga så högt som 25-40 % i vissa länder och verkar vara i stigande.

Astma är spritt över hela världen, med ett uppskattat antal insjuknade på ca 300 miljoner. Prevalensen varierar från 1 % i de mest skonade områdena, till 18 % i de hårdast drabbade.

Det är klarlagt att det finns nära samband mellan allergisk rinit och astma. Bland annat finns det en uttalad samsjuklighet; mer än 75 % av astmatikerna har allergisk rinit. Astma är tätt kopplat till bronkiell hyperreaktivitet, dvs en benägenhet hos de stora luftvägarna att dra ihop sig vid retning. Även en stor andel patienter med diagnosen rinit uppvisar bronkiell hyperreaktivitet vid provokationstest med retande stimuli, trots att de inte har några astmasymptom. Bronkiell hyperreaktivitet är normalt ett kännetecken för astma, och det faktum att rinitpatienter kan ha luftvägsobstruktion utan symptom är anmärkningsvärt. Möjligen kan detta förklaras av en skillnad i utbredning och/eller grad av luftvägsinflammation.

I denna avhandling var målet att studera utbredning, typ och grad av inflammation samt utbredning av luftvägsobstruktion i astmatiker jämfört med patienter med allergisk rinit med eller utan bronkiell hyperreaktivitet.

Vi fann att patienter med astma hade större förmåga att känna av den luftvägsobstruktion som inducerades under provokation med det luftvägsretande ämnet metakolin. Patienter med rinit och en bronkiell hyperreaktivitet av samma grad som astmatikerna fick mindre symptom från sina luftvägar. Genom att använda icke-invasiva metoder såsom sputuminduktion, mätning av kväveoxid i utandningsluften och impulsoscillometri i tillägg till provokationstest kunde vi identifiera tecken på ökad inflammatorisk aktivitet i de perifera luftvägarna i den astmatiska gruppen. Astmatikerna uppvisade också större resistansökning perifert i lungan under provokationstest, trots ungefär samma grad av central resistansökning som gruppen av rinitpatienter med bronkiell hyperreaktivitet. Överlag visade astmatikerna tecken på en mer aktiv och spridd inflammation i luftvägarna, jämfört med rinitpatienterna. Intressant nog så hade patienter med rinit och bronkiell hyperreaktivitet något högre nivåer av inflammationsmarkörer i sputum, jämfört med rinitpatienter utan bronkiell hyperreaktivitet. Sammantaget har jag i denna avhandling visat att astmatiker har ett engagemang av de perifera luftvägarna, något som inte finns i samma grad hos rinitpatienter. Detta kan möjligen delvis förklara skillnaden i förmåga att känna av luftvägsobstruktion. Övergången från rinit till astma är förmodligen gradvis, och utvecklandet av bronkiell hyperreaktivitet kan vara ett steg på vägen. Det perifera luftvägsengagemanget hos astmatiker medför implikationer för framtida behandlingstrategier, som bör innefatta hela luftvägsträdet.

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APPENDIX