Towards restoring the physiological protection against airway narrowing in asthma : take a deep breath!

Slats, A.M.

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Take a deep breath!

Annelies Slats

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Take a deep breath!

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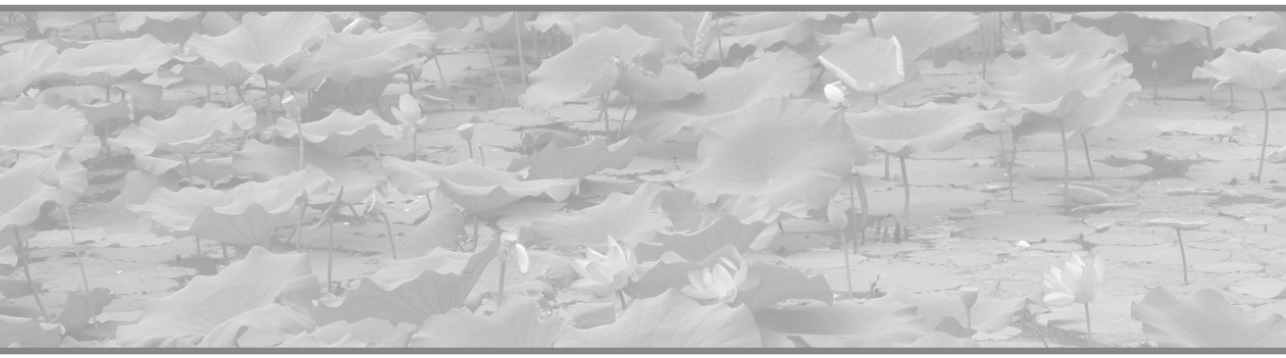
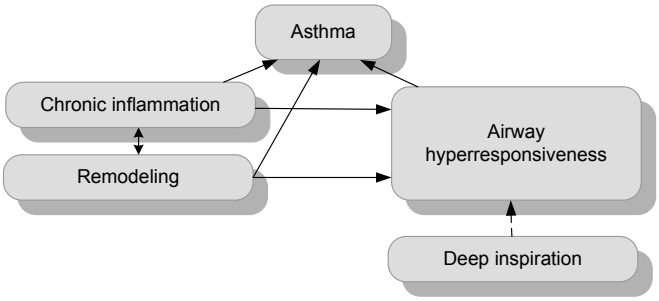


Chapter 1

General introduction

1. Introduction

Asthma is defined as a chronic inflammatory disorder of the airways, associated with an increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, and cough[1]. Airway hyperresponsiveness is a term used to indicate that the airways narrow too easily and too much in response to different provoking stimuli, such as allergens or cigarette smoke. Although in this definition inflammation and hyperresponsiveness are causally related, the mechanism underlying this association has not been elucidated. Deep inspirations affect airway narrowing, and may therefore play a role in airway hyperresponsiveness in asthma **(Figure 1)**.



1.1. Chronic airway inflammation and remodeling in asthma

The role of airway inflammation in asthma has been established by findings in resection material in patients with fatal asthma[2-4], but also from analyses of bronchial biopsies of patients with milder forms of asthma[5]. These inflammatory changes were found throughout the central and peripheral airways[6-8], and often vary with the severity of the disease[9]. The changes observed can be divided into inflammatory cell infiltration in the airway wall and structural changes of the airway wall in response to, or in parallel with, chronic inflammation (remodeling).

1.1.1. Inflammatory cells

Cell types that have been identified as characteristic for asthmatic inflammation are degranulated mast cells, eosinophils and lymphocytes. More recently, infiltration of the smooth muscle bundles by mast cells have been found to be characteristic for asthma as well[10,11]. *Mast cells* release histamine in response to IgE binding to the high-affinity receptor on the cell. Histamine induces smooth muscle contraction leading to airway narrowing. *Eosinophils* are a source of inflammatory proteins[6,9], including the major basic protein, that can damage the epithelium, degranulate mast cells and intensify bronchial hyperresponsiveness. The eosinophil is also a rich source of leukotrienes that contract smooth muscle and increase vascular permeability[12]. *T lymphocytes* may play a role in asthmatic inflammation by the release of cytokines. There are two types of CD4+ T helper(h) cells. Type 1 Th cells produce interleukin (IL)-2 and interferon

(IFN)-γ, which are essential for cellular defense mechanisms. Type Th 2 cells produce cytokines (IL-4, 5,6,9 and 13) that mediate allergic inflammation[13,14]. Observations in animal models and patients with asthma[15] suggest that allergic inflammation in asthma results from a Type 2 mediated mechanism.

Inflammatory cells and mediators may thus influence the response of airway smooth muscle to direct or indirect stimuli, by either directly activating the smooth muscle cells or inducing a cytokine and chemokine driven pathway that mediates both inflammation and smooth muscle function.

1.1.2. Structural changes

Changes in the structural components of the airway wall are often referred to as the remodeling process. Characteristic observations of structural changes are epithelial disruption, thickening of the basal membrane (collagen deposition beneath the basal membrane), hyperplasia and hypertrophy of airway smooth muscle[16-18], changes in extracellular matrix in- and outside the airway smooth muscle bundles[3,19], increased number of vessels and vascular permeability, and hyperplasia of goblet cells.

In principal, remodeling is thus a repair mechanism. However, in asthma it may be inappropriate since it alters the structural properties of the airway wall in a way that it affects the physiology of airway mechanics:

- Epithelial disruption may lead to an easy access of stimuli to the smooth muscle cells, and is related to airway hyperreactivity[20]
- Thickening of the basal membrane may provide load against smooth muscle contraction by stiffening the airway wall, or favors airway closure by preventing mucosal foldings[21-24].
- Hyperplasia or hypertrophy of airway smooth muscle may increase force generation and shortening capacity[25,26].
- Increased vascular permeability may lead to airway wall edema that thickens the airway wall and with that reduce the lumen and increases the outer perimeter. It may also lead to stiffening of the airway wall.
- Deposition of extracellular matrix may increase the load on airway smooth muscle contraction when situated on the inside of the smooth muscle layer[27,28], but may also reduce the parenchymal load when present in the outer layer of the airway wall.
- Collagen deposition within the airway smooth muscle bundles may modulate force transference among the contractile cells[24,29].

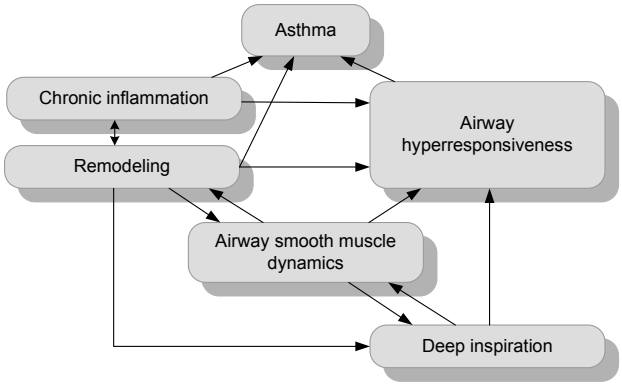
Therefore, not only acute and chronic inflammatory processes may lead to airway hyperresponsiveness, also the structural changes alter the mechanical properties of the airway wall and thus play a large role in the net effect of airway smooth muscle contraction.

1.2. Airway hyperresponsiveness

Individuals without asthma respond less to non-specific contractile agonists. This is reflected by a maximal response plateau on a dose-response curve with only modest airway narrowing at the highest dose[30-32]. In patients with asthma the plateau is elevated or completely abolished, indicating excessive airway narrowing. Also the dose-response curve is often shifted to the left, indicating a higher sensitivity to the contractile agent.

The parenchyma provides an elastic load against which the smooth muscle must shorten. The elastic recoil pressure is lung volume dependent, meaning that the elastic load presented to the airway smooth muscle increases as lung volume increases[33]. And vice versa the maximal response to a given agonist decreases as lung volume increases[34].This suggests that elastic load on airway smooth muscle is the principal determinant of maximal airway smooth muscle shortening in normal subjects. Although this load is appreciable it may not be sufficient to explain the plateau observed in healthy subjects. If the airways are sufficiently stimulated with contractile agonists, complete closure of even large cartilaginous conducting airways can readily occur within the lung at FRC[35]. Thus the elastic load of the parenchyma is not enough to prevent full airway closure at a static low lung volume. What other determinant of lung volume could then explain the protective mechanism of maximal response? It is speculated that the target of airway modulation by lung volume change is the airway smooth muscle cell itself[36,37], leading to less contractility[38]. Changes in the mechanism that modulates smooth muscle function by lung volume changes, can therefore indirectly change airway responsiveness to contractor stimuli. Therefore, it appears that the dynamic environment in the lung, as a result of continuously stretching forces during tidal breathing and occasional deep breaths, plays a major role in maintaining this environment in a condition where excessive airway narrowing is unfeasible or at least can be reversed by the system itself.

Taken together, in order to understand the pathophysiology of asthma one needs to clarify the role of stretch, i.e. deep inspirations, in the dynamic environment of the lung, and its interaction with airway smooth muscle, airway hyperresponsiveness, remodeling processes and chronic airway inflammation **(Figure 2)**.



2. Deep inspirations

2.1. History

Every subject takes a deep breath every 3 to 6 times an hour. This is usually interpreted as a sign of tiredness or boredom..., but they appear to have a physiological meaning as well. Already in 1859, the respiratory physician Henry Salter[39], observed that deep inspirations had different effects on the breathing pattern of his asthmatic patients:

"The spasm may be broken through, and the respiration for the time rendered perfectly free and easy, by taking a long, deep, full inspiration. In severe asthmatic breathing this cannot be done".

It was not until 1948 that Melville and Caplan[40] demonstrated in dogs that a maximal inflation of the lungs overcomes experimentally induced bronchoconstriction. In 1961 Nadel and Thierney[41] showed that in non-asthmatic humans deep inspirations also temporarily reduced airway narrowing. Since then, many studies have been performed to either examine the different effects of deep inspiration on airway narrowing in health and disease, but also to explore the pathophysiological mechanism of deep inspiration-induced bronchodilation.

2.2. Measuring airway responses to deep inspiration

Up till now, no standardized methodology has been described to measure airway responses to deep inspiration. Thus, many modified bronchoconstrictor challenges and different methods of lung function evaluation have been developed to do so[42]. The modified challenges are used to induce airways obstruction in healthy subjects, but also to measure airway responses to deep inspiration without previously performed deep inspirations[43,44]. Modified spirometry is used to compare flow rates on the expiratory flow-volume curve following a partial inhalation or a maximal inhalation (M/P ratios) and to observe the change in this ratio during a standard challenge[32,45]. Measurements of respiratory resistance by forced oscillation technique enables researchers to continuously measure resistance during tidal breathing and deep inspiration maneuvers[46,48], and more importantly for a longer period following a deep inspiration giving insights in the airway dynamics after deep inspirations. High resolution computed tomography (HRCT) scans visualizes the narrowing and distention of airways up to 3 mm following methacholine and deep inspirations, respectively[49]. These different techniques and study designs make it challenging to compare results between studies[50], but if these considerations are taken when reading study results on airway responses to deep inspiration a fair comparison can be made.

- Consider differences in airways obstruction (baseline vs. induced obstruction vs. spontaneous obstruction).
- Consider differences in method of measuring the response. FEV_1 includes a deep inspiration in the measurement and therefore may affect the response directly, but partial flow volume curves are less reproducible.

- Consider timing of the measurement following deep inspiration. In asthma it has been shown that airways are dilated by deep inspiration, but re-constrict within seconds. In addition, the bronchodilatory effect of deep inspiration holds for almost 1 minute in healthy subjects[46]. The outcome, therefore, depends greatly on the timing of the measurement following the deep inspiration.

Despite these differences many studies have been performed to examine the effects of deep inspiration on airway mechanics in healthy subjects and patients with asthma or other pulmonary diseases and have led to great insights. Deep inspirations not only have a dilatory effect on constricted airways, they also have a protective effect against airway narrowing.

2.4. Deep inspiration avoidance

In general, it has been shown that avoidance of deep inspirations for at least 20 minutes enhances subsequent airway narrowing to bronchoconstrictor stimuli in healthy subjects, whereas this is effect is not that profound in asthma[45]. However, prohibition of deep inspirations in healthy subjects makes the dose-response curves similar, but not equivalent to asthmatic subjects[51,52]. This suggests that deep inspirations have a protective effect against airway narrowing, and that this is lost in asthma[44,45,48,51,53,54].

The potentiating effect of deep inspiration avoidance on airway narrowing is subtrate-dependent. Whereas the reaction to methacholine is enhanced, the response to bradykinin is not altered by prohibition of deep inspirations in both healthy subjects and asthmatic patients[55]. The duration of deep breath avoidance also determines the subsequent bronchoconstrictor response[43], and the subsequent ability of deep inspirations to dilate the airways[44,45,48].

2.5. Deep inspiration-induced protection

Studies examining the protective effect of deep inspirations otherwise, showed that deep inspirations taken directly prior to inhalation of bronchoconstrictor stimuli prevented the subsequent airway narrowing in healthy subjects. This was not seen in patients with asthma or other disease groups with airway hyperresponsiveness[56]. The protective effect of deep inspiration is greater when more deep breaths are taken prior to spasmogen inhalation[43]. In addition, the duration of protection by deep breath seems to last longer than dilation induced by deep breath (6 vs. 1 min)[43,44,57].

In general, this led to the conclusion that deep inspirations protect against airway narrowing in healthy subjects, and that this protective effect is lost in asthma.

2.6. Deep inspiration-induced bronchodilation

Deep inspirations do not only protect against upcoming airway narrowing, they can also dilate constricted airways. This bronchodilatory effect is the effect of deep inspirations that is mostly studied[41,45,48,51,56,58,59]. When airway narrowing is induced in healthy subjects, either in absence

of deep inspirations or with high doses of bronchoconstrictor stimuli, deep inspirations can reduce the airway narrowing almost back to baseline levels[43,60].

This bronchodilatory effect is impaired in patients with asthma as well[56], and the impairment is associated with asthma severity[61], and spontaneous airways obstruction[62]. Mild asthmatics are able to dilate the airways by deep inspiration, but to a lesser extent than healthy control subjects, whereas the bronchodilatory effect is absent in severe asthma. During asthma exacerbations deep inspirations can even lead to bronchoconstriction. In other words, the response to deep inspiration in asthma is variable, ranging from bronchodilation to bronchoconstriction[56,58,63-65].

The extent of bronchodilation is directly related to the lung volume reached by the deep breath[34], and the number of deep inspirations[51]. Also the magnitude and stimulus of bronchoconstriction determines the response to deep inspiration[44,60]. For example, in asthma deep inspiration-induced bronchodilation is less during the late phase than during the early phase of the allergic asthmatic reaction[66].

Using measurements that can continuously track airway resistance during tidal breathing and deep inspiration it has been shown that renarrowing of airways following deep inspiration is faster in asthma than in healthy controls[46,48,67]. In addition, with high-resolution CT scans renarrowing of airways following deep breaths has been visualized in asthma, but was not seen in healthy subjects[59].

2.7. Deep inspiration-induced bronchoconstriction

Under baseline conditions (i.e. no induction of airways obstruction) deep inspirations can transiently induce bronchonstriction in patients with asthma[58,63,64]. This is not observed in healthy control subjects, or non-asthmatic allergic patients. During spontaneous asthma exacerbations deep inspirations result in profound bronchoconstriction related to the severity of the spontaneous obstruction[62]. Deep inspiration-induced bronchoconstriction resolved after treatment of the exacerbation with corticosteroids.

Thus, deep inspirations modulate airway responses to bronchoconstrictor agents and can therefore be considered as a very strong physiologic protective mechanism against airway narrowing. Indeed, the fact that the airways of asthmatic patients respond differently to lung inflation by deep inspiration indicates that the loss of this protective mechanism is involved in the pathophysiology of asthma. However, both the physiological mechanism responsible for lung volume-induced bronchoprotection in healthy subjects, as well as the pathophysiological mechanism of how this bronchoprotection is lost in asthma has not been elucidated yet. The next chapters resume the knowledge on these mechanisms thus far.

3. Physiological mechanism of deep inspirations

During lung inflation the diaphragm moves downwards and the thorax up-and outwards. The parenchyma follows these movements, and therefore the airways that are embedded in the parenchyma are dilated **(Figure 3)**[57,68-70].

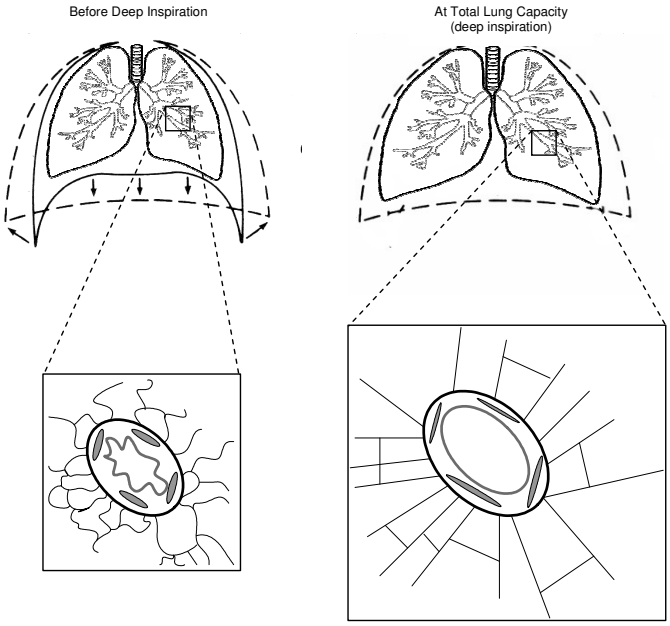


Figure 3. This figure shows that upon deep inspiration (upper figures) the airways embedded in the parenchyma are dilated as a result of parenchymal tethering (bottom figures).

Two forces potentially dilate the airways during lung inflation. One is the bulk modulus that is associated with the pressure difference acting directly across the airway wall, transmitted from the pleural space through the parenchyma, and relative to the intraluminal pressure for each airway. The other is the shear modulus, describing the force of local tethering, or pulling, by the surrounding lung parenchyma.

It is generally assumed that there is a tight coupling between lung volume changes and changes in airway caliber, as well as between airway caliber and smooth muscle length. Many of the processes described below follow this assumption.

3.1. Airway smooth muscle

Multiple investigators have linked the phenomena of deep breath-induced bronchodilation and bronchoprotection to the ability of deep inspirations to stretch the airway smooth muscle cell, thereby reducing its contractile capacities[36,45,48,71,72]. Notably, any explanation of *in vivo* phenomena, such as deep inspiration-induced bronchodilation and bronchoprotection, based

on evidence gathered from studies of isolated airway smooth muscle, can only be regarded as provisional until all interactions between ASM and other lung components are clear.

Airway smooth muscle and its contractile and cytoskeletal filaments are subjected to time-varying mechanical stress and strain associated with tidal lung inflations and spontaneous deep breaths. The forces serve to maintain airway caliber (distend or deform the cytoskeleton), but at the subcellular level also provide a mechanical stimulus to induce cellular responses, such as structural remodeling, and gene expression[73]. Consequently, the function of airway smooth muscle must be equilibrated with this dynamic environment[72].

The expected stretch of airway smooth muscle during tidal breathing is about 4%, and 12% during a sigh[72]. Stretch of airway smooth muscle of about 3% is enough to inhibit force generation by 50%, and therefore can be induced by the stretching forces of tidal breathing[72].

The effects of stretch on smooth muscle function, especially force generation, stiffness and shortening velocity, have been studied extensively. The effects are dependent on the speed[74,75], the amplitude, and the frequency[76] of the stretch imposed on the airway smooth muscle cell. However, the mechanism how stretch leads to less force generation is still under debate:

3.1.1. Bridge dynamics

The number of cross-bridges between actin and myosin determines the length change and force generation upon activation[77,78]. Stretch of the airways by lung inflation may lead to perturbation of these cross-bridges and thus in a reduction of contractility[71,72,79]. Although this theory explains deep inspiration-induced bronchodilation, it does not explain the bronchoprotective effect. This may be explained by the static and dynamic equilibrium of actin and myosin cross-bridge formation. When stretched adequately, the smooth muscle fibers are placed into disequilibrium, so that subsequent activation of smooth muscle is difficult[72].

3.1.2. Plasticity

Stretch induced changes in muscle force/tension may be caused by effects on contractile elements, as well as effects on the elastic elements in series with them. The muscle cell is able to adapt its contractile apparatus to length changes[36,80,81], which is called plasticity (rearranging the contractile properties to the new cell dimensions). This could explain bronchoprotection by deep inspirations, since the stretches would prevent adaptation to a certain length and therefore de-optimalize the length-force relationship. The structural basis and regulation mechanisms for such adaptation mechanism are still not known. A more integral approach to cell adaptation has been suggested by Fredberg[82], who considers the smooth muscle cell behavior like a soft glass. All the components of the smooth muscle cell are weakly interacting discrete elements. The ability to deform, flow and remodel is governed by nonthermal agitation (motion) energy of the cytoskeleton elements relative to the energies that constrain their motion[83,84]. A mechanical strain can act as an energy source that helps individual elements to

jump out of their energy well, so the cytoskeleton essentially 'heats up' and 'melts'. After each strain the system evolves into configurations that are more stiff and stable, and needs to be reversed by a subsequent stretch.

Although all these observations can result from direct physical effects of stretch imposed on smooth muscle cells it is also likely that stretch receptors coupled to chemical signaling pathways are involved.

3.2. Release of endogenous dilating substance (nitric oxide) from non-adrenergic, non-cholinergic nerves (Nitric Oxide, VIP)

Cholinergic and adrenergic systems control the bronchomotor tone together with the non-adrenergic non-cholinergic (NANC) system which mediates contraction (excitatoryNANC) or relaxation (inhibitoryNANC) of airway smooth muscle. Nitric oxide is the predominant neurotransmitter of the iNANC system[85]. In the respiratory tract, nitric oxide is produced by a wide variety of cell types and is generated via oxidation of L-arginine that is catalyzed by the enzyme nitric oxide synthase (NOS). Nitric oxide synthesis from endothelium is believed to be partly stretch-dependent[86], and thus may play a role in the deep inspiration dynamics since nitric oxide has bronchodilatory effects on methacholine induced bronchoconstriction[87-89]. Indeed, in anaesthetized and ventilated dogs a nitric oxide synthase inhibitor (N^G-nitro-L-arginine methyl ester) prevented bronchodilation by a large deep inspiration. These results suggest that a large inflation of the lung may normally releases of nitric oxide resulting in airway dilation[90]. Furthermore, a report only published in abstract format reported that in human subjects without asthma, inhalation of a nitric oxide synthase inhibitor (L-NAME) reduced the deep inspiration-induced bronchoprotection against methacholine-induced bronchoconstriction[91].

Whether the neuropeptide vasoactive intestinal peptide (VIP) plays a role in the beneficial effects of deep inspiration has not been investigated yet.

3.3. Neural activation

The parasympathetic nervous system mediates both cholinergic contractions and non-adrenergic, non-cholinergic (NANC) relaxations of airway smooth muscle. Activation of these pathways following chemical and/or mechanical stimulation of afferent nerves innervating the lungs can profoundly influence airway calibre and thus resistance to airflow[92]. Lung inflation induces neural activation. Increasing the volume or rate of lung inflation increases the discharge frequency of intrapulmonary stretch receptors[93]. This could explain the effects of rate and magnitude of stretch of the airways on airway tone. Namely, in healthy and asthmatic subjects fast inspirations reduce induced airway resistance more than slow inspirations[94]. Stretch receptor activation by deep inspiration may cause central inhibition of parasympathetic tone[92] leading to bronchodilation.

3.4. Release of surfactant

Surfactant reduces surface tension in the peripheral airways and help to maintain airway caliber[95]. Surfactant is produced by and secreted from alveolar type II cells, where it is stored in intracellular vesicles termed lamellar bodies. Direct distortion of the type II cell is a direct stimulus for secretion of surfactant *in vitro*[96], and thus stretch by lung inflation of the alveolar epithelium may trigger the release of surfactant. Indeed, in rats exercise (swimming) led to an increase in breathing rate and tidal volume resulting in a release of surfactant from distortion of alveolar type II cells, but also from another surfactant pool under sympathetic nerve control[97]. This suggests that lung volumes above tidal volume induces the release of surfactant by stretch and thus modulates airway caliber.

3.5. Release of autacoids

Autacoids are biological factors which act like local hormones, have a brief duration, act near the site of synthesis, and are not blood borne. Autacoids are primarily characterized by the effect they have upon smooth muscle. Prostaglandin E_2 (PGE_2) released in asthmatic airways has bronchodilator properties and inhibits allergen-induced bronchoconstriction and release of inflammatory mediators. Stretch of the airways by deep inspiration may lead to release of autacoids such as prostaglandins and atrial naturetic peptide (ANP). For example PGE_2 is released by lung inflation[98]. ANP is secreted by cardiac atria and lung tissue; it has a bronchodilator action in normal subjects[99] and patients with asthma[100], and has been shown to protect against histamine-induced bronchoconstriction in patients with asthma[101]. Also, intravenous infusion of ANP has been shown to cause bronchodilatation in patients with asthma[99], and inhaled ANP shows a strong dose-dependent protection against histamine-induced bronchoconstriction[102]. Of course, the question rises whether ANP is released upon stretch of the airways by lung inflation. Such evidence is not available, although circulating ANP levels rise upon exercise[101].

3.6. Hormonal pathway (adrenaline, epinephrine)

Circulating adrenaline is the only hormone known to influence bronchomotor tone. Adrenaline (or epinephrine) is released from the adrenal medulla, and induces bronchodilation by stimulating $beta_2$ adrenergic receptors on airway smooth muscle, and indirectly by reducing acetylcholine release[103]. Adrenaline is not released in response to allergen-induced or pharmacologically-induced bronchoconstriction. After exercise, increased adrenaline concentrations induce airway dilation. Infusion of epinephrine in normal non-atopic individuals comparable to exercise levels also results in bronchodilation[104,105]. which is probably a result of inhibition of vagal tone. There are no data on whether this is a direct result of increased volume changes during exercise or that increased adrenaline release is stretch-induced.

4. Pathophysiological mechanism

The effects of deep inspiration on airway caliber can become impaired by any factor that reduces the strain transmission from the parenchyma to the airway wall, leading to less airway dilation during lung inflation. On the other hand, even if the airways are stretched adequately during lung inflation, the airway wall, and its components, may respond differently to the stretch imposed on it (**Figure 4**).

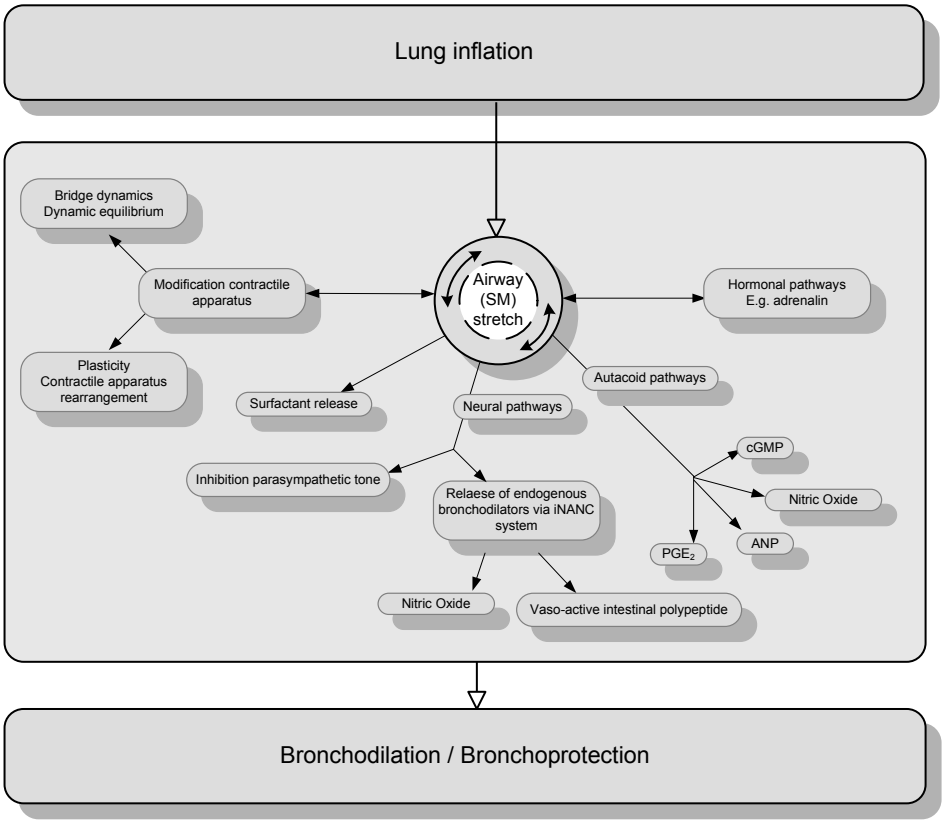


Figure 4. Possible mechanisms explaining beneficial effects of deep inspiration.
This figure shows the possible mechanisms explaining the beneficial effects of deep inspiration. First, lung inflation results in stretch of the airways and its components. This could lead to release of surfactant, activation of neural, autocoid or hormonal pathways, or modification of the contractile apparatus. On the other hand, changes in the contractile apparatus or hormonal pathways could also affect the amount of stretch induced by lung inflation. In the end, these proposed mechanisms determine the level of bronchodilation and/or bronchoprotection induced by lung inflation.

4.1. Impaired stretch

Stretch of the intrapulmonary airways, and thereby the smooth muscle, by lung inflation is caused mostly by the increase in radial traction exerted on the airways by the surrounding lung parenchyma[69,70,106]. The impairment of the beneficial effects of deep inspiration in asthma could therefore be caused by impaired coupling between the parenchyma and airways. Studies using high resolution CT (HRCT) are inconsistent with this hypothesis. These showed that the airways are stretched to the same extent in both healthy and mild asthmatic subjects. The groups were different with regard to the response following deep inspiration, resulting in bronchodilation and bronchoconstriction respectively[59]. However, in more severe asthmatic patients airway distensibility does seem to be impaired[48,61], and thus loss of airway-parenchyma interdependence may play a role in more severe asthma.

4.1.1. Increased stiffness of the airway wall

Change in airway distensibility may be due to remodeling in those proportions of the wall distinct to airway smooth muscle (ASM): adventitia, lamina propria, reticular layer under epithelium[3]. Thickening of the airway wall may have both beneficial[21,107-109] and detrimental[22,110] effects on airway mechanics during lung inflation, and airway hyperresponsiveness. Analysis of the effect of airway remodeling has to be based not only on geometry, but also on the mechanical properties of the altered airway wall components[28]. For example if deposited connective tissue in the adventitial layer is stiff, then it would attenuate the cyclical strain from the surrounding parenchyma. But if deposited matrix is highly compliant it would also prevent effective transmission of strain to the airway smooth muscle layer. Therefore, there must be an optimal coupling stiffness that allows maximal transmission of strain from the parenchyma to smooth muscle in order for the airways to receive maximal benefit of the bronchodilating effect of tidal breathing and deep inspirations. In asthma, airway wall remodeling occurs under inflammatory processes. Since it is impossible *in vivo* to examine the influence of the individual components of the airway wall on airway distensibility and the responses to stretch, airway models are needed to clearify the role of remodeling processes on airway mechanics during deep inspiration. So far, this role has not been fully elucidated.

4.1.2. Airway wall edema

Edema of the airway wall or within the peribronchial space could uncouple the interdependence between the airways and the parenchyma resulting in decreased radial forces acting on the smooth muscle during deep inspiration[55,111-113]. If so airway stretch by lung inflation would be limited. Substantial airway wall edema (up to 50% increase in airway wall area) by bradykinin or intravenous saline can be elicited in the airways. However, this potential effect of airway wall edema to decrease airway wall distention by lung inflation was not found in either dogs[114], or sheep[113,115]. On the other hand, in asthma increased transpulmonary pressure led to less bronchodilation by deep inspiration, whereas this effect was not seen in healthy subjects[116].

This may be due to fluid flux across leaky capillaries in inflamed asthmatic airways (thus not seen in healthy subjects) leading to direct loss of airway-parenchymal interdependence[116].

4.1.3. (Peri)Bronchial inflammation

(Peri)Bronchial inflammation would interfere with the distending forces of the parenchyma on the airway wall[117]. In addition it would also reduce the recoil pressures of the parenchyma, reducing the load on airway smooth muscle during contraction. Up till now no clear relationship between impairment of deep inspiration induced bronchodilation and inflammatory markers has been established. An inverse correlation between broncho-alveolar lavage (BAL) concentrations of eosinophils and deep inspiration-induced bronchodilation has been shown[118]. However, sputum inflammatory cell counts were not related to deep inspiration-induced bronchodilation[51]. Still several studies have shown that anti-inflammatory treatment improves deep-inspiration induced bronchodilation[119-121]. This could be due not only to reduction of the ongoing inflammatory process, but also to stretch-induced alterations in function of the smooth muscle itself[122,123].

4.1.4. Loss of alveolar attachments

The attachments of the parenchyma to the airway wall determine whether the transpulmonary pressures are transmitted across the airway wall. Patients who died of asthma have damaged alveolar attachments[3] which may lead to irreversible uncoupling of the expansive forces and the airways. Also in COPD loss of alveolar attachments have been described and related to less deep inspiration-induced bronchodilation[124].

4.1.5. Reduced inspiratory capacity

Any chronic change in lung volumes leading to a lower inspiratory capacity could be expected to affect airway mechanics during deep inspiration. A reduction in the magnitude of distention from FRC to total lung capacity (TLC) would lead to lower tethering forces on the airway wall by the parenchyma. Examples are obesity[125,126], supine position during bed rest[127], and hyperinflation due to chronic airways obstruction (COPD)[128]. Also, it has been suggested that prohibition of deep inspirations leads to airway closure in healthy subjects and that deep inspirations reopen these lung regions (i.e. reduce heterogeneity)[129].

4.2. Airway smooth muscle

The presence[130] and the normal function[131] of airway smooth muscle in the lung are still poorly understood. In comparison to striated muscle, we know little about how smooth muscle contracts. Therefore, it is also difficult to say whether airway smooth muscle function is altered in asthma and whether the smooth muscle responds differently to stretch. However, many hypotheses on the pathophysiological mechanism of stretch-induced modulation of airway smooth muscle function have been proposed.

4.2.1. Latch bridges

Airway remodeling may lead to reduced "force fluctuation amplitude", and thus airway smooth muscle may be subjected to diminished tidal forces. As a result the cycling of cross-bridges is reduced, and therefore the number of attached actin-myosin bridges and stiffness of the muscle is increased. Every tidal stretch has less power to perturb these slow bridges until a new static equilibrium is formed where the muscle becomes so stiff that also deep breaths are no longer able to unfreeze the muscle (=latch state)[72]. A lower amplitude (less inflation or other reasons for not transmitting force to smooth muscle cells) leads to increase in cross-bridges and thus force generation and stiffness.

4.2.2. Plasiticity

Airway smooth muscle is able to adapt its contractile apparatus to different cell lengths[132,133]. Chronic shortening[134] of airway smooth muscle (> 3 days) shifts the passive and active length-force relationships enabling it to generate the same force at a shorter length. If tidal breaths and occasional deep breaths fail to change the length of the smooth muscle cells the cell is allowed enough time to adapt itself to that length and regain force generation. In addition, this was associated to increases in passive stiffness and thus further diminishing the effects of cyclic stretch.

Rearrangement of contractile elements from a series to a parallel configuration[80,132,135] may alter airway smooth muscle respons to stretch as well. In case of a parallel configuration, stretch would not perturb the contractile filaments and force generation remains intact.

Also, abnormally long actin filaments may increase the range of sliding of contractile filaments without diminishing the overlap between myosin and actin filaments, and thus render the muscle more resistant to the relaxing effect of oscillatory stress[122,136]. Longer actin filaments were also associated with greater resistance of stimulated muscle to relax upon oscillatory strain in a computer model[137].

4.2.3. Increased airway smooth muscle tone

Several studies have shown that under both cyclic strain and increased airway smooth muscle tone (*in vitro*) cultured cells demonstrate increased stiffness, as well as increased contractility and shortening velocity. In asthma increased airway smooth muscle tone may lead to harmful uniaxial cyclic stretch, instead of harmless biaxial cyclic stretch, leading to enhanced recovery from acute large stretches[73,138].

4.2.4. Increased shortening velocity of ASM

The airways of patients with asthma reconstrict faster following deep inspiration as compared to healthy control subjects[46,48,67,139]. This suggests that the airway smooth muscle cells exhibit increased velocity of shortening, and thus contract faster after being stretched. This is in line

with *in vitro* studies demonstrating greater velocity of contraction of asthmatic or sensitized smooth muscle cells[140,141].

4.3. Alterations in neurohumoral system

4.3.1. Cholinergic system

In asthma deep inspirations may increase cholinergic tone and therefore lead to bronchoconstriction. Several studies support this hypothesis where pretreatment of asthmatic patients with anti-cholinergics attenuated subsequent deep-inspiration induced bronchoconstriction[63,142]. However, this effect can also be a result of the need for bronchotone to establish deep inspiration-induced bronchodilation. Indeed, in the presence of cholinergic blockade, but with reestablishment of bronchomotor tone with PGF2 alpha, deep inspiration-induced bronchodilation could still be demonstrated in healthy individuals[143].

4.3.2. iNANC/nitric oxide

NO synthase (NOS) catalyzes the oxidation of l-arginine to produce nitric oxide in the respiratory tract. NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS)[85,144]. NO derived from the constitutive isoforms of NOS (nNOS and eNOS) modulates airway tone. On the other hand, NO derived from iNOS seems to be a pro-inflammatory mediator with immunomodulatory effects. The concentration of this molecule in exhaled air is increased in asthma[145]. If nitric oxide is necessary for bronchodilation, and this is released by deep inspiration, then reduced availability of bronchodilatory nitric oxide under inflammatory circumstances and reduced stretch by deep inspiration may lead to less bronchodilation following deep inspiration.

The mechanism of how the beneficial effects of deep inspiration become impaired can be summarized in the same figure that shows how deep inspiration can lead to the beneficial effects (**Figure 5**). The pathways that are boxed have not yet been investigated *in vitro* or *in vivo* by intervention studies. The other pathways have been investigated but have not yet resulted in a definitive pathophysiological mechanism. Therefore major research questions and hypotheses remain to be examined regarding the (patho)physiological mechanism of deep inspiration dynamics.

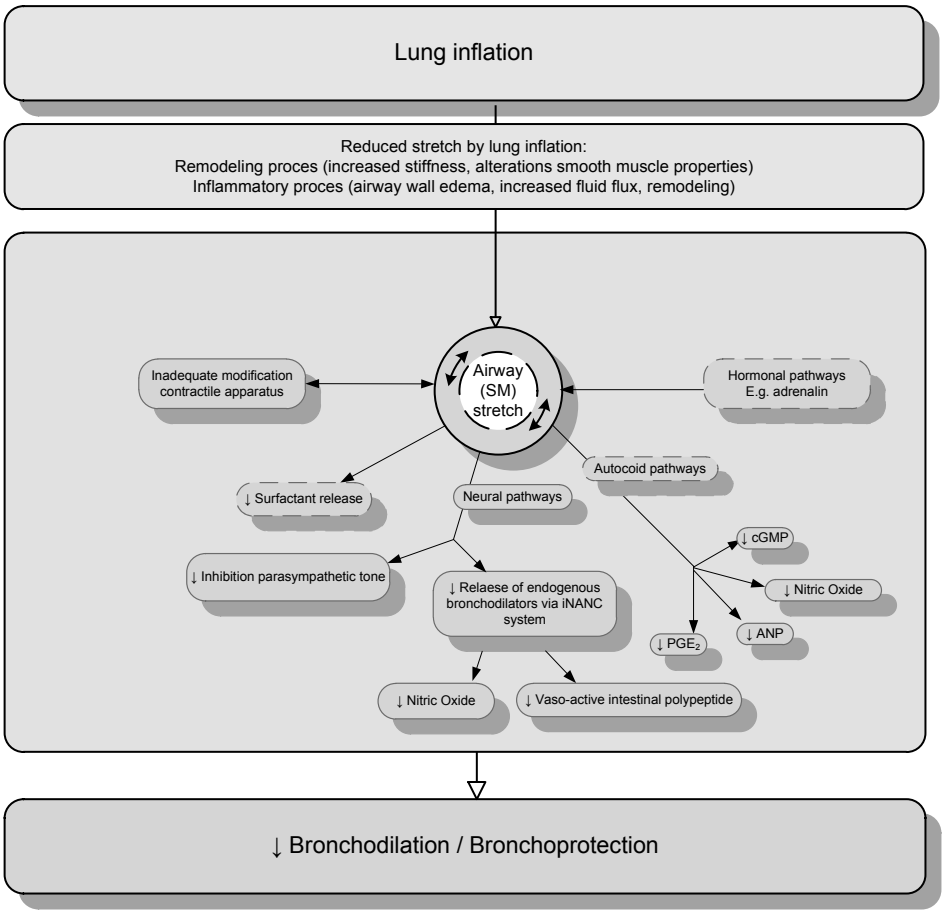


Figure 5. Possible mechanisms explaining impaired beneficial effects of deep inspiration.
This figure shows the possible mechanisms explaining impaired beneficial effects of deep inspiration. First, stretch of the airways and its components by lung inflation is reduced by the remodeling or inflammatory process within the airways. This could lead to reduced release of surfactant, changed activation of neural, atuocoid or hormonal pathways, or inadequate modification of the contractile apparatus. On the other hand, changes in the contractile apparatus or hormonal pathway could also affect the amount of stretch induced by lung inflation. In the end, these proposed mechanisms determine the level of bronchodilation and/or bronchoprotection induced by lung inflation. The pathways that are boxed have not yet been investigated *in vitro* or *in vivo* by intervention studies.

5. Rationale of the current thesis

In summary, deep inspirations provide the strongest endogenous protection against airway narrowing in healthy subjects, whereas this protective mechanism is lost in asthma. The physiological mechanism underlying the prevention and reduction of airways obstruction by deep inspirations in healthy subjects has not been elucidated. In addition, the pathophysiological

mechanism how this mechanism is impaired in asthma is unclear. In this thesis we addressed the following issues concerning the (patho)physiological mechanism of deep inspiration-induced bronchodilation and attempts to restore this mechanism.

Chronic airway inflammation in asthma can influence airway mechanics through the remodeling processes and thus impair deep inspiration-induced bronchodilation. The improvement of deep inspiration-induced bronchodilation by anti-inflammatory treatment in asthma is in line with this. As described above, this relationship has only been addressed by examining inflammatory cells in bronchoalveolar lavage fluid or sputum, but not in bronchial biopsies. In this thesis we aimed to further investigate the relationship between airway inflammation and airway responses to deep inspiration. We, therefore, investigated the relationship between the response of the airways to deep inspiration and airway inflammation measured in bronchial biopsies of patients with asthma. In addition, to examine whether impaired deep inspiration-induced bronchodilation is asthma-specific we compared these results with those from patients with chronic obstructive pulmonary disease (COPD) (chapter 2).

The airway smooth muscle cell has frequently been the center of the debate on deep inspiration-induced bronchodilation. It is thought that lung inflation stretches the smooth muscle cells within the airways and thereby changes the contractile apparatus of the cell, but the exact mechanism how the smooth muscle cell and/or the contractile apparatus is affected by stretch is unknown. Smooth muscle cells express several contractile and structural proteins. The level of expression and the type of proteins is depending on the functional phenotype of the cell, and is related to lung function in severe asthma. We examined the expression of several smooth muscle proteins that are likely to play a role in airway responsiveness in relation to deep inspiration-induced bronchodilation in asthma. In addition, we measured the expression of several components of the extracellular matrix, since the smooth muscle cell is likely to have a functional interaction with the surrounding extracellular matrix (chapter 3).

In asthma, during an exacerbation deep inspirations lead to further bronchoconstriction instead of relieving it. Edema of the airway wall has been suggested as one of the mechanisms leading to deep inspiration-induced bronchoconstriction. It is unclear whether airway wall edema *perse* leads to bronchoconstriction following deep breath or that the presence of airway inflammation, such as during an exacerbation, must be present as well Therefore, we aimed to study the airway responses to deep inspiration in patients with peribronchial edema due to mitral valve regurgitation in the absence of airway wall inflammation. In addition, we studied whether reduction of pulmonary congestion after mitral valve repair would alter airway responses to deep inspiration (chapter 4).

Restoring the physiological mechanism to prevent airway narrowing by lung inflation may provide more sustained asthma control, and reduce the need of current asthma treatment. We performed several intervention studies to investigate whether this protective mechanism could be restored in asthma. First, chronic ongoing inflammation and airway wall remodeling processes as seen in asthma can lead to reduced strain transmission from the parenchyma

to the airway wall or to an altered respons of the airway wall to the stretch imposed on it. Therefore, we aimed to maximally reduce airway inflammation in asthma by systemic corticosteroid therepy on top of inhaled corticosteroids in order to improve airway responses to deep inspiration by reducing airway wall thickness and therefore restoring the airway-parenchymal interdependence (chapter 5).

Also, anticholinergic drugs have been shown to protect against airway wall remodeling in animal models of allergic inflammation. It inhibited both airway smooth muscle proliferations, as well as smooth muscle contractility. Since the airway smooth muscle cell and its altered response to stretch has been frequently postulated as pathophysiological mechanism for impaired deep inspiration-induced bronchodilation, we hypothesized that treatment with a long-acting anticholinergic agent, tiotropium, in allergic asthma would improve bronchodilation by lung inflation (chapter 6).

Passive inflation instead of an active deep inspiration would induce stretch of the airways without large intrathoracic pressures and could therefore prevent extravasation of fluid in inflamed airways. In addition, the inflated volume may open closed airways, thereby redistributing tethering forces of the parenchyma on the airway wall leading to increased stretching forces. In the last intervention study we examined whether passive inflation could reverse airways obstruction in asthma, and thus restore this strong physiological protective mechanism against airway narrowing (chapter 7).

Finally, we have used time series of the respiratory system impedance data from the study shown in chapter 2 and 3 to study the respiratory system with high temporal resolution. Fluctuations in time series of respiratory system impedance measurements by forced oscillation technique exist in the healthy lung, and the variability of these fluctuations differs from an asthmatic lung. We hypothesized that the temporal course of respiratory system impedance is differentially affected by respiratory disease. In addition, we considered the impedance signal to arise from a dynamic system, and assumed that this system contains a deterministic component, that changes in distinct ways in respiratory diseases. In other words, a specific respiratory disease corresponds to changes in the control parameters that modify the dynamic behaviour of the system (chapter 8).

In the following chapters these studies are fully described and discussed separately. In the last chapter, the major results are summarized followed by a general discussion (chapter 9).

Study aims of this thesis

- In chapter 2, we compared the bronchodilatory and bronchoprotective effects of deep inspiration using the forced oscillation technique in patients with asthma, patients with COPD, and healthy control subjects. In addition, we investigated the relationship between the response of the airways to deep inspiration and bronchial wall inflammation in patients with asthma and COPD.
- We analyzed the bronchial biopsies from the study presented in chapter 2, and examined the relationship between airway responses to deep inspiration and the expression of structural and contractile markers of airway smooth muscle cells in patients with asthma, which is shown in chapter 3.
- In chapter 4, we examined the effect of airway wall edema on airway mechanics during deep inspiration in patients with mitral valve disease as compared to healthy subjects. In addition we explored whether this would change in absence of airway wall congestion following mitral valve repair.
- In chapter 5, we aimed to investigate the effects of high dose systemic corticosteroids on top of inhaled corticosteroids on deep inspiration-induced bronchodilation at a given level of airway narrowing in patients with asthma.
- In chapter 6, the results of anti-cholinergic treatment are shown on multiple parameters of lung function, including deep inspiration-induced bronchodilation and airway hyperresponsiveness.
- Chapter 7 presents the intervention study using passive inflation of the lungs to investigate whether this would improve bronchodilation as compared to an active deep inspiration in patients with asthma.
- Finally, in chapter 8 we evaluated fluctuations in time series of respiratory system impedance measurements by forced oscillation technique in the healthy and asthmatic lung to observe whether the temporal course of respiratory system impedance is differentially affected by respiratory disease, and whether the impedance signal arises from a dynamic system

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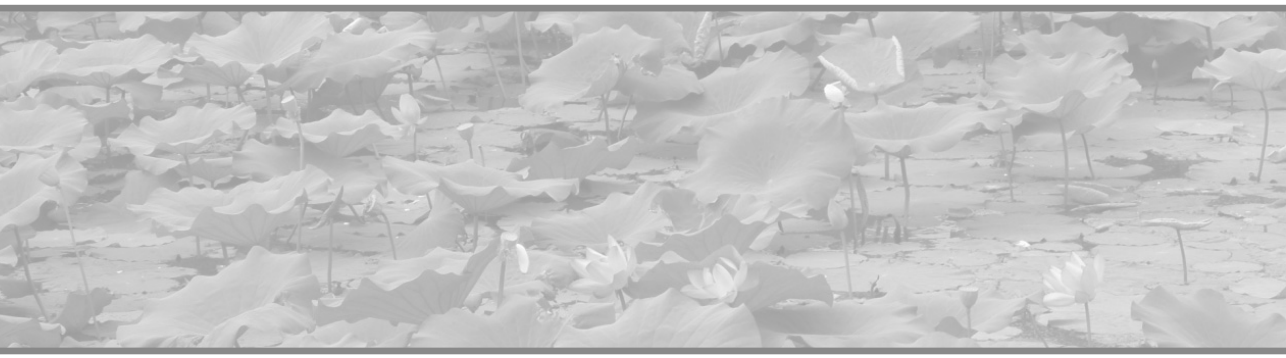

Chapter 2

Bronchial inflammation and airway responses to deep inspiration in asthma and chronic obstructive pulmonary disease

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Abstract

Rationale: Deep inspirations provide physiologic protection against airway narrowing in healthy subjects, which is impaired in asthma and chronic obstructive pulmonary disease (COPD). Airway inflammation has been suggested to alter airway mechanics during deep inspiration.

Objectives: We tested the hypothesis that the number of bronchial inflammatory cells is related to deep inspiration–induced bronchodilation in asthma and COPD.

Methods: In a cross-sectional study, three modified methacholine challenges were performed in 13 patients with mild, persistent asthma, 12 patients with mild to moderate COPD, and 12 healthy control subjects.

Measurements and Main Results: After a 20-minute period of deep inspiration avoidance, inhalation of methacholine was followed by either one or five deep inspirations, or preceded by five deep inspirations. The response to deep inspiration was measured by forced oscillation technique. Inflammatory cells were counted within the lamina propria and airway smooth muscle area in bronchial biopsies of patients with asthma and COPD. The reduction in expiratory resistance by one and five deep inspirations was significantly less in asthma (mean change ± SD: −0.5 ± 0.8 and −0.9 ± 1.0 cm H_2O/L/s, respectively) and COPD (+0.2 ± 1.1 and +0.4 ± 1.0 cm H_2O/L/s, respectively) as compared with healthy subjects (−1.5 ± 1.3 and −2.0 ± 1.2 cm H_2O/L/s, respectively; $p = 0.05$ and $p = 0.001$, respectively). In asthma, this was related to an increase in mast cell numbers within the airway smooth muscle area ($r = 0.73$; $p = 0.03$), and in CD4+ lymphocytes in the lamina propria ($r = 0.61$; $p = 0.04$).

Conclusions: Inflammation in the airway smooth muscle bundles and submucosa of bronchial biopsies is positively associated with impaired airway mechanics during deep inspiration in asthma, but not in COPD. Clinical trial registered with www.clinicaltrials.gov (NCT OO279136).

Introduction

Airway hyperresponsiveness is a key feature of asthma[1] and is also frequently present in patients with chronic obstructive pulmonary disease (COPD)[2]. Deep breaths play a major role in modulating airway responsiveness. In healthy subjects, deep breaths reduce the level of pharmacologically induced airways obstruction (bronchodilation)[3], whereas prohibition of taking deep breaths enhances the reaction to a bronchoconstrictor agent[4]. Furthermore, deep breaths taken before bronchial challenge reduce the consequent airways obstruction (bronchoprotection)[5,6]. Thus, deep inspirations provide physiologic protection against airway narrowing.

In asthma, it has been shown that these beneficial effects of deep inspiration are impaired[5,7,8], and that deep inspirations may even enhance obstruction during exacerbations[9]. Several studies have demonstrated that the bronchodilatory effect of a deep inspiration is also reduced in COPD[10,11], which may be related to parenchymal damage[12,13]. Understanding of the pathologic processes that lead to impairment of this protective mechanism against airway narrowing is required for attempts to restore it, and thereby advancing treatment in asthma and COPD.

Both asthma and COPD are characterized by airway inflammation, although the predominant inflammatory cell profiles are different[14,15]. Indeed, inflammation of the airways has been suggested to influence airway mechanics by inducing airway remodeling and thereby increasing airway wall thickness[16]. This could result in reduced strain transmission from the parenchyma to the airways during deep inspiration or altered responses of the airway wall to the stretch imposed on it[17]. Anti-inflammatory treatment improves deep inspiration–induced bronchodilation in asthma, suggesting a role of airway inflammation as contributive to this mechanism[18-20]. Although a relationship between airway responses to deep inspiration, without pharmacologically induced airway narrowing, and inflammatory cell counts in sputum has been shown twice[21,22], this has not yet been shown for inflammatory cells within bronchial biopsies. We hypothesized that the number of inflammatory cells in the airway smooth muscle bundles and lamina propria of bronchial biopsies of patients with asthma and COPD is related to impaired airway responses to deep inspiration.

The aim of the present study was to examine airway responses to deep inspiration in relation to the number of inflammatory cells in the airway smooth muscle bundles and bronchial submucosa in patients with asthma and COPD. Because a difference has been found in the response of the airways to either one or five deep inspirations[6,23], we aimed to examine this relationship under both circumstances. We used the forced oscillation technique to examine the resistance of the respiratory system (respiratory resistance), as this technique allows the continuous recording of deep inspiration–induced changes, and for a longer period of time after deep inspiration as compared to spirometry. Some of the results of this study have been previously reported in the form of an abstract[24,25].

Methods

Subjects

The complete methods are provided in the online supplement of this article: http://ajrccm.atsjournals.org/cgi/data/176/2/121/DC1/1. For this study, we enrolled 13 nonsmoking atopic patients with intermittent and mild persistent asthma (Global Initiative for Asthma [GINA] steps 1 and 2; provocative concentration of methacholine producing a 20% fall in FEV_1 [PC_{20} methacholine] < 8 mg/ml)[1], 12 patients with mild to moderate COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD] I and II [26]; > 10 pack years; FEV_1 reversibility to salbutamol < 12% of predicted), and 12 nonsmoking, healthy subjects (< 2 pack years; PC_{20} methacholine > 16 mg/ml). All patients were clinically stable, and had not used inhaled or oral corticosteroids within 3 months before the study. The institutional review board for human studies approved the protocol, and the subjects gave their written, informed consent before entering the study.

Study design

The study had a cross-sectional design. Baseline clinical and functional assessments were performed, divided over 2 days, including medical history taking, skin prick test, spirometry with reversibility testing, and a standard methacholine challenge. In the second phase, three modified (single-dose) methacholine challenges were performed[5] (see the online supplement). During the first challenge, the single dose of methacholine capable of producing a 20% reduction in FEV_1 was established while the bronchodilator response to one deep inspiration (slow inspiration to total lung capacity followed by a passive exhalation) was measured (**Figure 1A**). During the following single-dose challenges, the inhalation of this dose of methacholine was either preceded by (bronchoprotection; **Figure 1B**) or followed by (bronchodilation; **Figure 1C**) five consequent deep breaths in randomized order. The resistance of the respiratory system (respiratory resistance) was measured continuously during the breathing maneuvers using a forced oscillation device (Woolcock Institute, Sydney, Australia)[8] with an applied oscillation frequency of 8 Hz and an amplitude of ±1 cm H_2O (see the online supplement). Within 1 week, a bronchoscopy was performed and six bronchial biopsies were taken in the patients with asthma and COPD. The healthy subjects were not included in the biopsy study, because we aimed to examine the relationship between inflammation and the impaired effect of a deep inspiration within these disease groups.

Bronchoscopy, immunohistochemistry, and image analysis

Bronchoscopy was performed according to a standardized and validated protocol in our laboratory[27]. Disposable forceps (radial edge; Boston Scientific, Boston, MA) were used to take six biopsy specimens at the (sub)segmental level. A total of 4 biopsies were fixed for 24 hours in 4% neutral-buffered formaldehyde, processed, and embedded in paraffin. From paraffin-embedded tissues, 4-μm-thick sections were cut, and hematoxylin and eosin staining was used to evaluate overall bronchial architecture. Sections of two biopsies per subject, selected

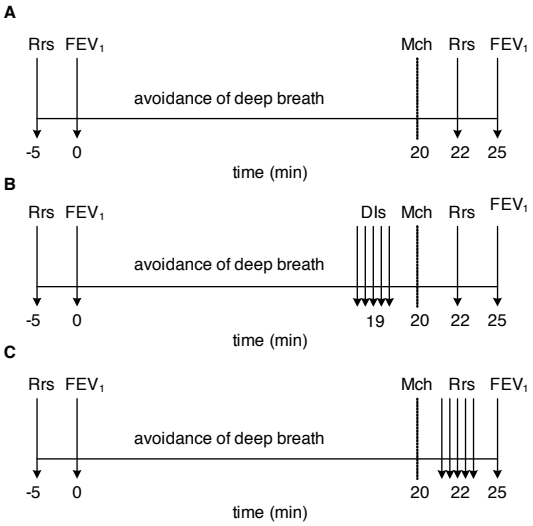

Figure 1. Single-dose challenge measurements.
This figure describes the three different single-dose methacholine (Mch) challenges. The line shows the time in minutes, and the arrows show the number of deep breaths taken. Baseline measurements of respiratory resistance (Rrs) and FEV_1 were followed by a period of 20 minutes with deep-breath avoidance. **(A)** Mch inhalation was followed by Rrs measurement with one deep breath and FEV_1 measurement to determine whether this dose could reduce FEV_1 by at least 20%. **(B)** Mch inhalation preceded by five deep inspirations (DIs), followed by Rrs measurement with one deep breath. **(C)** Mch inhalation followed by Rrs measurement with five deep breaths.

on morphologic quality criteria (intact reticular basal membrane and submucosa without crushing artifacts, large blood clots, or only epithelial scrapings), were stained and analyzed. Sections were incubated at room temperature with monoclonal antibodies directed against CD3, CD4, CD8 (T lymphocytes), EG2 (eosinophils), AA1 (tryptase-positive mast cells), CD68 (macrophages), and NE (neutrophils). Digital images from the stained sections were obtained, and fully automated cell counts (KS400; Carl Zeiss B.V., Sliedrecht, The Netherlands) were performed in the lamina propria (at least 0.125 mm^2) by a validated method[28]. The number of tryptase-positive mast cells in the airway smooth muscle bundles were automatically counted in a manually selected airway smooth muscle area (at least 0.1 mm^2)[29] using serial sections stained for -smooth muscle actin and myosin to identify the airway smooth muscle area. Positively stained cells were expressed as the number of cells per 0.1 mm^2.

Analysis

Respiratory resistance was measured during 60 seconds of tidal breathing, followed by one or five slow, deep breaths to total lung capacity, and another minute of tidal breathing. Deep inspiration–induced bronchodilation was expressed as the difference between the mean resistance of all data points of three tidal breaths after and of three tidal breaths before the deep inspiration[30,31], which was calculated separately for inspiratory resistance and expiratory

resistance. The latter was done because respiratory resistance fluctuates during tidal breathing due to volume and flow differences between inspiration and expiration, and may be affected differently by deep inspiration maneuvers[32]. Bronchoprotection by deep inspirations was expressed as the difference in the increase in resistance by methacholine when either five or no deep inspirations were taken before methacholine inhalation. The outcome parameters were log transformed to obtain a normal distribution. The differences between the three groups were analyzed using analysis of variance, with Tukey's honestly significant difference test as post hoc analysis or Kruskal-Wallis test. Within-group differences were analyzed by two-tailed paired t tests or Wilcoxon ranks test. Spearman's rank correlation coefficient was used to explore associations between inflammatory cell counts and deep inspiration–induced changes in respiratory resistance. We used SPSS version 12.01 (SPSS, Inc., Chicago, IL) for all analyses. Statistical significance was associated with p values less than 0.05. Sample size estimation and details on the analysis are given in the online supplement.

Results

Functional parameters

The patient characteristics are given in Table 1. PC_{20} methacholine (geometric mean ± SD in doubling dose) was significantly lower in patients with asthma (1.0 ± 1.5 mg/ml) and COPD (2.15 ± 1.8 mg/ml) as compared with that in healthy control subjects (50.1 ± 1.3 mg/ml; $p < 0.001$). Patients with COPD were significantly older, had smoked, and their lung function was significantly more impaired than the patients with asthma and healthy control subjects (**Table 1**).

Table 1. Patient characteristics

Variable	Patients with Asthma	Patients with COPD	Healthy Control Subjects
Sex, male/female	5/8	8/4	2/10
Age, yr	23.8 ± 5.7	57.9 ± 7.5*†	32.8 ± 13.8
BMI, kg/m^2)	22.9 ± 2.1	26.3 ± 3.3*†	22.2 ± 3.4
Pack years	0.04 ± 0.1	38.9 ± 15.6*†	0.33 ± 0.8
Post-salb FEV_1% pred	103.9 ± 11.1	78.6 ± 13.9*†	107.4 ± 12.6
Post-salb FEV_1/FVC, %	87.0 ± 6.4	60.9 ± 7.6*†	85.6 ± 8.3
PC_{20} methacholine, mg/ml	1.0 ± 1.5	2.2 ± 1.8	50.7 ± 1.3*‡
Single-dose methacholine, mg/ml	3.3 ± 1.4	10.0 ± 1.9†	72.5 ± 1.5*‡
Fall in FEV_1, % (single-dose methacholine challenge)	29.7 ± 8.0	23.5 ± 2.8	26.8 ± 7.6

Data are expressed as mean ± SD, except for sex (number), PC_{20} methacholine (geometric mean ± SD in doubling doses). Analysis of variance, *post hoc* Tukey's honestly significant difference test. * $p < 0.05$ Healthy control subjects vs. patients with COPD. † $p < 0.05$ Patients with asthma vs. those with COPD. ‡ $p < 0.05$ Healthy control subjects vs. patients with asthma.

Single-dose methacholine challenges

FEV_1 dropped more than 20% from baseline in all subjects by the single dose of methacholine (mean % fall in FEV_1 ± SD: 29.7 ± 8.0%, 23.5 ± 2.8%, and 26.8 ± 7.6% for asthma, COPD, and healthy control subjects, respectively), which was not significantly different between the groups

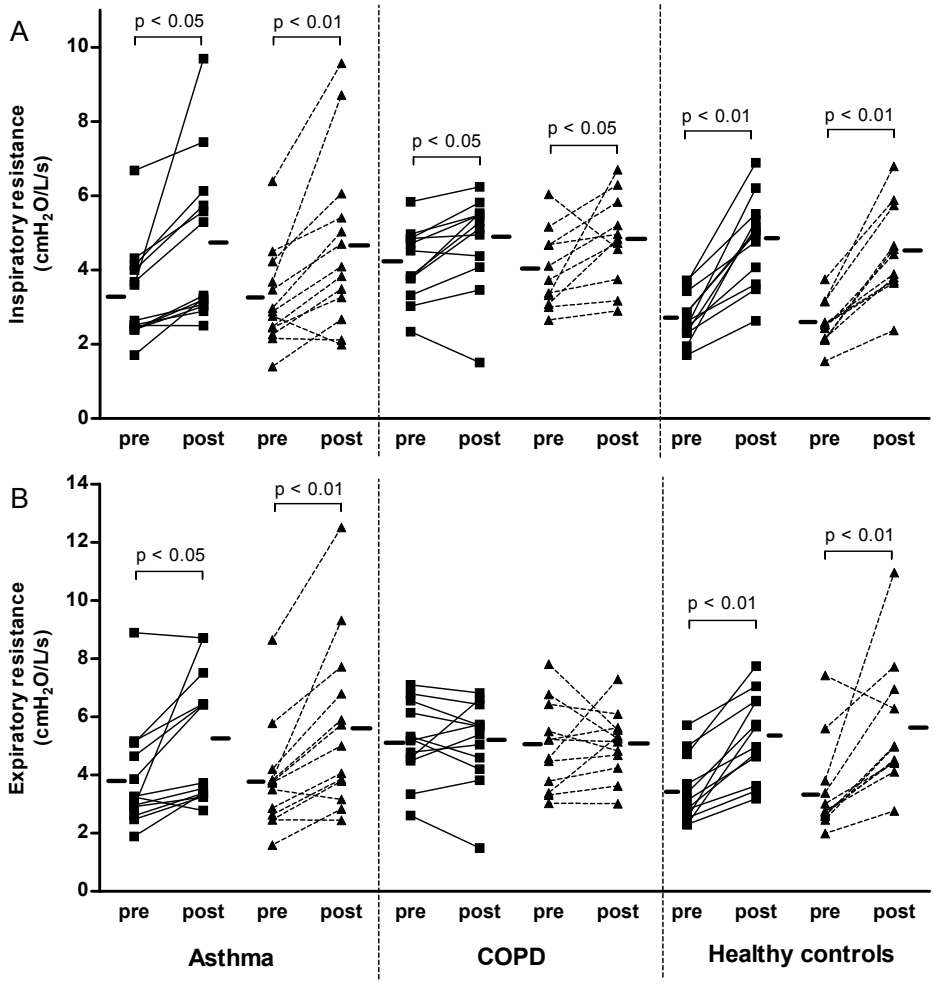

Figure 2. Changes in respiratory resistance by methacholine.
This figure shows the individual data points per group before (pre) and after (post) Mch inhalation for inspiratory **(A)** and expiratory **(B)** resistance. Data are expressed in cm H_2O/L/second, and the horizontal lines represent the mean. Squares connected by solid lines represent the data with no deep inspirations taken before Mch inhalation (**Figure 1A**); triangles connected by dashed lines represent the challenge when five deep inspirations were taken before Mch inhalation (**Figure 1B**). Mch significantly increased inspiratory resistance in all three groups, and expiratory resistance in only the asthma group and healthy control subjects, and not in the COPD group. Five deep inspirations did not protect against the increase in inspiratory and expiratory resistance in any of the three groups.

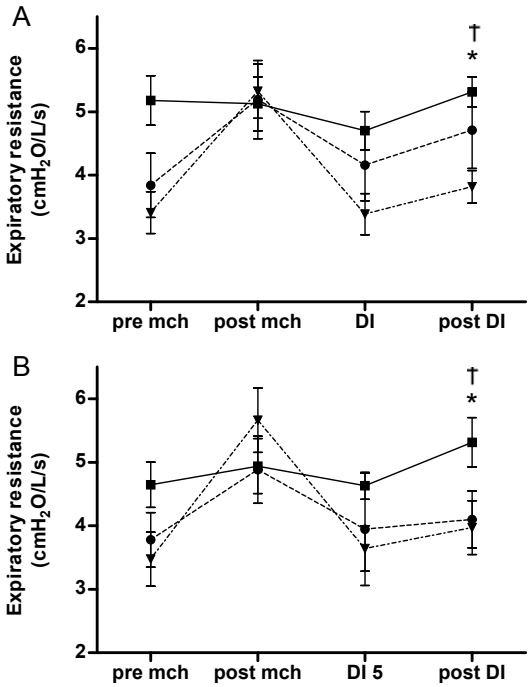

Figure 3. Changes in expiratory resistance by deep breath.
In this figure, the paired data (mean ± SEM) for patients with asthma (circles, dashed line), those with COPD (squares, solid lines), and healthy control subjects (inverted triangles, dashed-dotted lines) are depicted. The data are expressed as the mean expiratory resistance during three tidal expirations before Mch inhalation (pre mch), three tidal expirations after Mch inhalation (post mch), the passive expiration of the deep inspiration (DI), and three tidal expirations after deep inspiration (post DI). **(A)** Data of the measurement when one deep inspiration was taken after Mch inhalation (**Figure 1A**). **(B)** Data of the measurement when five deep inspirations were taken (**Figure 1C**), where data point "DI 5" represents the mean of the resistance during the five passive expirations of the five deep breaths. The reduction in expiratory resistance during tidal breathing by one and by five deep breaths was significantly larger in healthy subjects as compared with patients with asthma ($^{\dagger}p < 0.05$) and those with COPD ($^{*}p < 0.05$). Furthermore, the reduction in expiratory resistance during tidal breathing by five deep breaths was significantly larger in patients with asthma than in those with COPD ($p < 0.05$).

($p = 0.08$). Tidal volume before and after methacholine inhalation was not significantly different between the groups ($p > 0.7$), nor was the inspiratory volume of either one (mean ± SD: asthma, 1.6 L ± 0.5; COPD, 1.6 L ± 0.5; healthy control subjects, 1.5 L ± 0.5; $p = 0.9$) or the mean of five deep inspirations (asthma, 2.2 L ± 0.7; COPD, 2.0 L ± 0.4; healthy control subjects, 2.1 L ± 0.6; $p = 0.6$). In the three groups, inspiratory resistance was significantly increased by the single dose of methacholine (**Figure 2A**; mean change ± SD: asthma, +1.4 ± 1.5 cm H_2O/L/s; COPD, +0.6 ± 0.7 cm H_2O/L/s; healthy control subjects, +2.1 ± 1.0 cm H_2O/L/s). Expiratory resistance was also significantly increased in asthma and healthy control subjects (**Figure 2B**; +1.4 ± 1.6 and +1.9 ± 1.1 cm H_2O/L/s, respectively), but not in patients with COPD (–0.05 ± 0.9 cm H_2O/L/s). The increase in resistance by a single dose of methacholine was not significantly reduced when

Table 2. Changes in inspiratory and expiratory resistance by one and five deep inspirations

Change in Resistance (cm H_2O/L/s)						
No. of Deep Inspirations	Patients with Asthma		Patients with COPD		Healthy Control Subjects	
	Insp	Exp	Insp	Exp	Insp	Exp
One	−0.6 ± 0.3	−0.5 ± 0.2	−0.2 ± 0.3	0.2 ± 0.3	−1.5 ± 0.3†	−1.5 ± 0.4†‡
Five	−1.1 ± 0.4	−0.9 ± 0.3*	0.0 ± 0.4	0.4 ± 0.3	−1.9 ± 0.4	−2.0 ± 0.4†‡

Data are expressed as mean ± SEM. Analysis of variance, *post hoc* Tukey's honestly significant difference test. *$p < 0.05$ Patients with asthma vs. those with COPD. $^{\dagger}p < 0.05$ Healthy control subjects vs. patients with COPD. $^{\ddagger}p < 0.05$ Healthy control subjects vs. patients with asthma.

five deep inspirations were taken before methacholine (bronchoprotection) in any of the three groups (**Figure 2**).

In patients with asthma and healthy subjects, after inhalation of methacholine, both one and five deep inspirations significantly reduced inspiratory and expiratory resistance (**Table 2; Figures 3A and 3B** for one and five deep inspirations, respectively). In COPD, no significant reduction in expiratory resistance was observed by either one or five deep inspirations, and only inspiratory resistance was significantly reduced by five deep inspirations. The reduction in expiratory resistance induced by both one and five deep breaths was significantly larger in healthy control subjects than in patients with asthma and COPD (Table 2; $p < 0.05$ and 0.01, respectively). Furthermore, the reduction in expiratory resistance during tidal breathing by five deep breaths was significantly larger in asthma than in COPD (**Table 2**; $p < 0.05$). The absolute change induced in expiratory resistance by five deep inspirations is a mean (± SD) percent reduction of 67(±4.4)% in healthy control subjects and 22(±2.3)% in patients with asthma, and a percent increase of 13(±3.3)% in patients with COPD.

Bronchial inflammation

The numbers of inflammatory cells in the lamina propria of bronchial biopsies per cell type are shown in **Table 3**. Patients with asthma had significantly more eosinophils (EG2+ cells) in the

Table 3. Inflammatory cell counts in bronchial biopsies

Cell Type	Asthma	COPD
$CD3^+$ cells	53.3 (14.0–134.0)	25.5 (3.0–160.5)
$CD4^+$ cells	24.8 (9.5–86.0)	12.0 (1.5–83.0)
$CD8^+$ cells	25.8 (7.0–62.0)	15.0 (5.0–93.0)
$CD4^+/CD8^+$ cells	1.7 (0.6–4.4)	0.6 (0.1–4.4)
$EG2^+$ cells	1.5 (0.0–8.0)*	0.3 (0.0–3.0)
$AA1^+$ cells	10.0 (1.0–24.0)	16.0 (2.0–56.0)
$AA1^+$ cells in airway smooth muscle bundles	2 (0.0–7.0)	1.5 (1.0–3.0)
$CD68^+$ cells	19.0 (8.0–53.0)	9.3 (3.0–100.0)
NE^+ cells	1.8 (0.0–14.0)	2.0 (0.0–41.0)

The numbers of cells are expressed as median (range) per 0.1 mm^2. *$p < 0.05$ between groups.

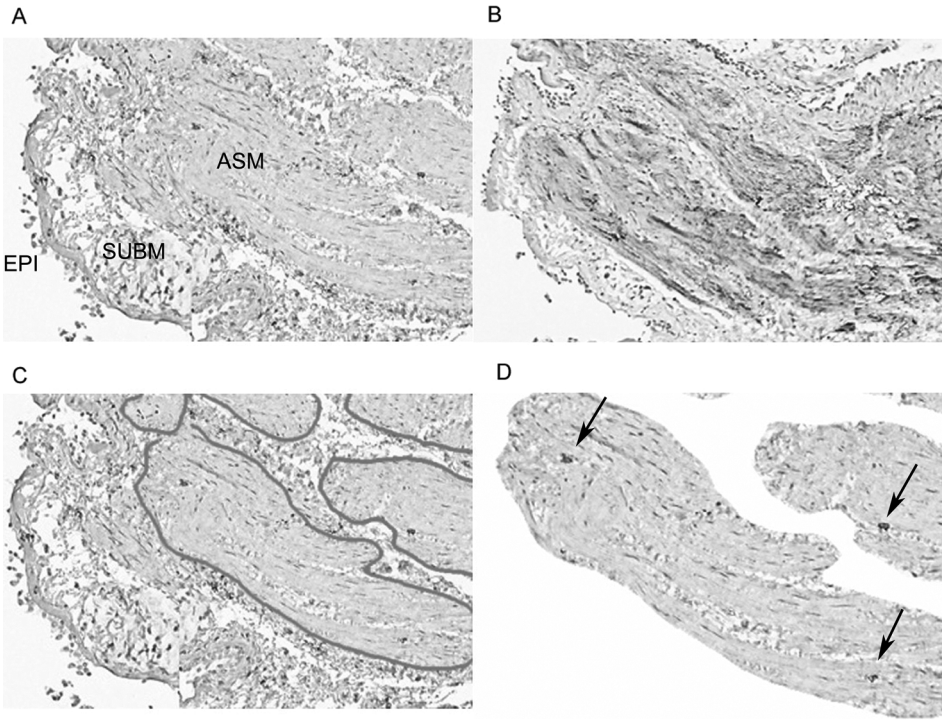

Figure 4. Photomicrographs of mast cell and myosin staining.
Example of a bronchial biopsy section immunohistochemically stained for **(A)** tryptase-positive mast cells (ASM = airway smooth muscle; EPI = epithelium; SUBM = submucosa), and **(B)** a serial section of the same biopsy stained for myosin. The smooth muscle area was manually selected **(C)** in the mast cell staining by myosin staining. In the selected area **(D)**, mast cells (arrows) were automatically counted. Original magnification: x200.

lamina propria as compared with patients with COPD. Also, the number of CD4+ lymphocytes and the CD4+/CD8+ lymphocyte ratio tended to be higher in asthma than in COPD, but this did not reach significance ($p = 0.09$ and 0.06, respectively). Among the inflammatory cell types analyzed, predominantly mast cells were observed in the airway smooth muscle bundles (**Figure 4**). In asthma, 74%, and in COPD, 76% of the biopsies contained sufficient (> 0.1 mm^2) airway smooth muscle area. The mean area analyzed in asthma was 0.24 (± 0.11) mm^2 and, in COPD, 0.36 (± 0.20) mm^2 ($p = 0.11$).

In asthma, the reduction in resistance by one deep breath was positively associated with the number of CD4+ cells per 0.1 mm^2 ($r = 0.61$; $p = 0.04$; **Figure 5A**). In addition, the number of mast cells in the airway smooth muscle bundles correlated positively with the reduction in resistance by five deep breaths ($r = 0.72$; $p = 0.03$, **Figure 5B**). In COPD, there were no significant correlations between the changes in resistance by deep inspirations and inflammatory cell counts within the lamina propria, or the number of mast cells in the airway smooth muscle bundles.

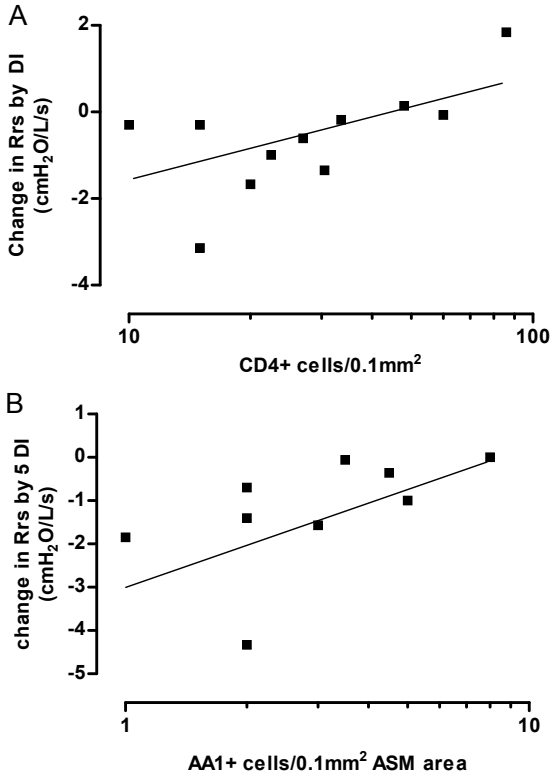

Figure 5. Relationship between inflammatory cell counts and deep inspiration–induced bronchodilation in asthma.
This figure shows the relationship between the change in inspiratory resistance (Rrs) by **(A)** one deep inspiration (DI) and the number of CD4+ lymphocytes/0.1 mm2 in the lamina propria ($r = 0.61$; $p = 0.04$), and by **(B)** five deep inspirations and the number of tryptase-positive (AA1+) mast cells/0.1 mm2 in the airway smooth muscle (ASM) bundles ($r = 0.73$; $p = 0.03$).

Discussion

The results of this study demonstrate that the bronchodilatory effect of deep inspiration is impaired in intermittent and mild persistent asthma as compared with that in healthy subjects, and even more markedly impaired in patients with mild to moderate COPD. Interestingly, in asthma, the reduced bronchodilatory effect of a deep inspiration was associated with increased numbers of mast cells within the airway smooth muscle bundles and increased CD4+ lymphocyte counts in the bronchial lamina propria. These findings suggest that the impairment of deep inspiration–induced bronchodilation in asthma is a result of inflammatory mechanisms within the airway smooth muscle area and bronchial wall, possibly resulting in altered airway mechanics by influencing airway smooth muscle characteristics or increasing airway wall thickness. To our knowledge, this is the first study showing a relationship of inflammatory

cell counts in the airway smooth muscle area and lamina propria of bronchial biopsies with airway responses to deep inspiration in asthma. In general, our physiologic results are in line with previous studies showing reduced bronchodilation after deep inspiration in asthma and COPD as compared with that in healthy control subjects[23,33]. Although, we did find a significant reduction in respiratory resistance by both one and five deep inspirations in the patients with asthma, this was significantly less than in the healthy subjects. This partly preserved deep inspiration–induced bronchodilation in asthma differs from other studies showing almost no reduction in airways obstruction by deep inspiration in patients with asthma[34]. This might be explained by differences in disease severity and the level of airway hyperresponsiveness of the participating subjects. The patients with asthma in our study had intermittent or mild persistent asthma, needing no other medication than bronchodilators on demand. Furthermore, the method of measuring airway responses to deep inspiration differs among studies, and may influence the outcome parameters as well[35].

Notably, we did not find a bronchoprotective effect of deep inspirations in the healthy control group, whereas this has been shown by several studies in the past[3,5]. This seems to be explained by the methods used to assess airways obstruction. Bronchoprotection by deep inspiration has predominantly been observed by using measurements implicitly including a deep breath, such as FEV_1, whereas it could not be established by parameters without a deep breath during the measurement[36]. We purposely chose the latter to examine the unaffected protective effect of deep inspirations against the dynamics of airway narrowing and, therefore, may have missed bronchoprotection as reported when using FEV_1. Taken together, these findings suggest that deep inspirations taken before methacholine inhalations improve subsequent bronchodilatory effects of deep breaths in healthy subjects, and thus prevent a fall in FEV_1, but may not necessarily prevent the obstruction itself.

We aimed to look at relationships between bronchial inflammation and deep-breath effects within a group of patients with asthma and those with COPD, and therefore selected the patients that matched the key features of these two distinct disease groups. As expected, this resulted in significant differences between the groups with regard to age and lung function. However, neither in COPD nor in healthy control subjects was a relationship found between deep breath–induced reduction in respiratory resistance and age or lung function ($r < 0.4$; $p > 0.2$). Therefore, the differences between COPD and healthy control subjects are most likely a result of pathophysiologic changes in COPD.

We used a modified single-dose methacholine challenge to induce a given level of airways obstruction in all subjects to measure both the bronchoprotective and the bronchodilatory effect of deep inspirations. During the first challenge, we established the dose that induced a reduction in FEV_1 of at least 20%, and used that dose for the other two challenges. We could not determine whether the subsequent single-dose challenges induced the same fall in FEV_1 in absence of performing spirometry. However, because there was no significant difference within the groups between the three challenges with regard to respiratory resistance

after methacholine inhalation, we presume that the level of obstruction was approximately the same as in the dose-finding challenge. Interestingly, in the patients with COPD, the fall in FEV_1 induced by methacholine was not accompanied by a significant increase in respiratory resistance, a finding that we cannot fully explain. This may have limited the possibility of reducing respiratory resistance by deep inspiration in this group. However, there was no direct relationship between the increase in respiratory resistance by methacholine and the reduction in respiratory resistance by deep inspirations, suggesting that the absence of the bronchodilatory effect of deep inspirations in COPD was not necessarily dependent on the absence of an increase in respiratory resistance. Hence, this finding may provide new information on the functionally relevant pathophysiology of the airways in patients with mild to moderate COPD, which requires further investigation. In this study protocol, we have used the forced oscillation technique to measure airway responses to deep inspiration. The limitation of this method is that the results represent resistance of the complete respiratory system, including the upper airways, and thus the site of the obstruction or deep inspiration–induced bronchodilation is difficult to determine. However, this technique enabled us to monitor respiratory resistance continuously, and, therefore, we were able to measure the effect of deep breaths on the dynamics of airway obstruction during both the deep breaths and tidal breathing. How can we interpret these results? During a deep inspiration, the airways are dilated, as shown on computed tomographic scan[37], both in healthy adults and those with asthma, presumably as a result of the airway–parenchymal coupling.

However, it appeared that, in asthma, deep breaths could not reduce respiratory resistance to the same extent as in healthy subjects. We found that increased numbers of CD4+ lymphocytes in the lamina propria of bronchial biopsies were associated with impaired bronchodilation after a deep breath in asthma. It is likely that these cells indirectly reflect the inflammatory changes within the bronchial wall that prevent adequate stretch of the airways and airway smooth muscle layer. CD4+ lymphocytes are involved in eosinophilic inflammation, and are associated with vasodilation and microvascular leakage[38]. These inflammatory changes may narrow the internal airway diameter, and, at the same time, increase the outer wall perimeter, thereby decreasing the force applied to the airways by the parenchyma during deep inspiration[17,39]. In addition, a similar relationship with CD4+ cells was not found at slightly larger changes in resistance induced by five deep inspirations. This may indicate that inflammation, as reflected by CD4+ lymphocytes within the lamina propria, indeed decreases deep inspiration–induced stretch of the airways, but does not fully prevent it, which may be overcome by multiple stretching maneuvers. Another hypothesis regarding the role of inflammation in the impairment of the bronchodilatory effect of deep inspiration is the reduction in the stretch-induced release of inhibiting factors, such as nitric oxide. The CD4+ lymphocytes within the bronchial wall may counteract these active bronchodilating mechanisms. Most strikingly, we found a correlation between the number of mast cells within the smooth muscle bundles and deep inspiration–induced bronchodilation in asthma. Mast cells can promote airway smooth

muscle contraction by releasing histamine, prostaglandin D_2, and tumor necrosis factor-α[29,40]. We speculate that the localization of the mast cells within the smooth muscle cells could result in a physiologically altered intrinsic contractile function, leading to an increased formation of actin and myosin cross bridges, more difficult to disrupt by deep inspiration–induced stretch of the airways, which has been referred to as the latch state[41]. These data further extend the results obtained by Brightling and colleagues[29], showing increased numbers of mast cells in the airway smooth muscle bundles in bronchial biopsies of patients with asthma as compared with healthy control subjects or patients with eosinophilic bronchitis, which was related to airway hyperresponsiveness. Interestingly, in COPD, there was no significant reduction in respiratory resistance by deep breaths. An absolute loss of alveolar attachments might explain this observation, as this would result in uncoupling of the airway–parenchyma interdependence, leading to less strain imposed on the airways by the parenchyma during deep inspiration[42]. Indeed, it has been shown that the loss of alveolar attachments was related to less bronchodilation by deep breaths in patients with mild to moderate COPD[13]. Because we did not find a direct relationship between inflammatory cells within the bronchial wall and deep breath–induced bronchodilation in COPD, we speculate that the marked loss in the ability to reduce respiratory resistance by deep inspiration is predominantly due to structural damage of the airways or lung parenchyma in this disease.

What could be the clinical implication of our study? The correlation of inflammatory cells within the submucosa and airway smooth muscle bundles with the bronchodilatory effect of a deep inspiration in asthma indicates that the impaired airway mechanics may, at least partially, be restored by treatment. Indeed, it has been shown that airway responses to deep inspirations can be improved by treatment with (inhaled) corticosteroids[18-20]. Furthermore, because deep inspirations are likely to play a role in airway hyperresponsiveness[3], perceived symptoms[43], and excaberations[9] in asthma, measurement of airway responses to deep inspiration may give additional information on current disease status. In COPD, our findings indicate that airway inflammation plays a less prominent role in the pathophysiologic mechanism of deep breath–induced bronchodilation, which limits the options for intervention. However, deep-breath responses may be a sensitive parameter for finding early lung damage caused by smoking.

We conclude that deep inspiration–induced bronchodilation is reduced in patients with intermittent and mild persistent asthma as compared with healthy subjects, and absent in patients with mild to moderate COPD. In asthma, the bronchodilatory effect of deep inspirations is related to inflammatory cell counts within airway smooth muscle bundles and bronchial wall, whereas in moderate COPD, this relationship could not be found. These results indicate that the physiologic protection against airway narrowing by deep inspiration is impaired in both asthma and COPD, but this may be due to different pathophysiologic mechanisms.

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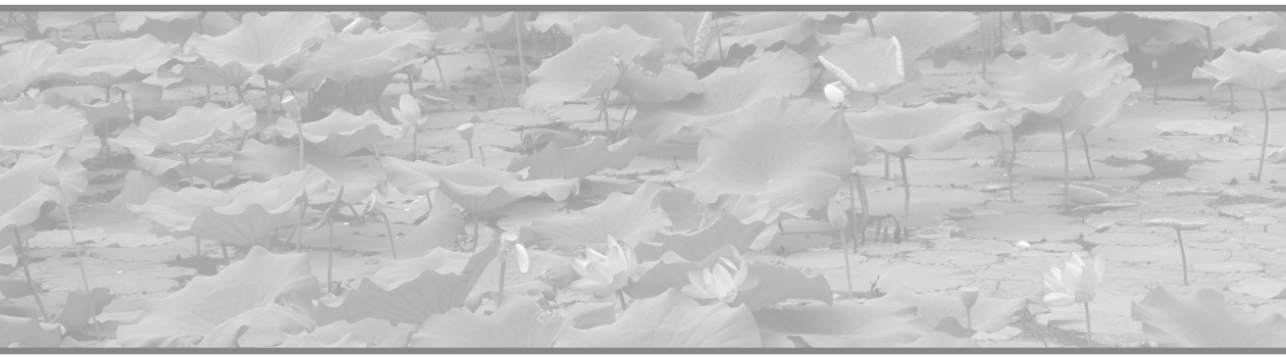

Chapter 3

Expression of smooth muscle and extracellular matrix proteins in relation to airway function in asthma

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Abstract

Background: Smooth muscle content is increased within the airway wall in patients with asthma and is likely to play a role in airway hyperresponsiveness. However, smooth muscle cells express several contractile and structural proteins, and each of these proteins may influence airway function distinctly.

Objective: We examined the expression of contractile and structural proteins of smooth muscle cells, as well as extracellular matrix proteins, in bronchial biopsies of patients with asthma, and related these to lung function, airway hyperresponsiveness, and responses to deep inspiration.

Methods: Thirteen patients with asthma (mild persistent, atopic, nonsmoking) participated in this cross-sectional study. FEV_1% predicted, PC_{20} methacholine, and resistance of the respiratory system by the forced oscillation technique during tidal breathing and deep breath were measured. Within 1 week, a bronchoscopy was performed to obtain 6 bronchial biopsies that were immunohistochemically stained for α-SM-actin, desmin, myosin light chain kinase (MLCK), myosin, calponin, vimentin, elastin, type III collagen, and fibronectin. The level of expression was determined by automated densitometry.

Results: PC_{20} methacholine was inversely related to the expression of α-smooth muscle actin ($r = -0.62$), desmin ($r = -0.56$), and elastin ($r = -0.78$). In addition, FEV_1% predicted was positively related and deep inspiration-induced bronchodilation inversely related to desmin ($r = -0.60$), MLCK ($r = -0.60$), and calponin ($r = -0.54$) expression.

Conclusion: Airway hyperresponsiveness, FEV_1% predicted, and airway responses to deep inspiration are associated with selective expression of airway smooth muscle proteins and components of the extracellular matrix.

Introduction

Asthma is characterized by chronic airway inflammation, which is presumed to contribute to variable airways obstruction and bronchial hyperresponsiveness[1]. However, recent studies have led to a reappraisal of the role of airway smooth muscle in asthma pathophysiology[2]. Because smooth muscle contraction leads to airway narrowing, abnormalities in airway smooth muscle size, mass, or function could easily lead to exaggerated airway narrowing. In addition, mast cells within the airway smooth muscle bundles have been associated with airway hyperresponsiveness[3] and more recently, we observed a similar association with impaired deep inspiration-induced bronchodilation in patients with asthma[4].

Increased smooth muscle mass has been demonstrated in bronchial biopsies[5-7] as well as in resected lung tissue[8,9] from patients with asthma compared with healthy subjects. Mathematical models have shown that increased smooth muscle mass can explain exaggerated airway narrowing to contractile stimuli in patients with asthma[10], especially at high lung volumes[11]. Interestingly, although increased smooth muscle area in bronchial biopsies has been associated with impaired lung function[5,7], no relationship was found with airway hyperresponsiveness. Nevertheless, in vitro studies have shown that smooth muscle cells obtained from bronchial biopsies of patients with asthma exhibit an increase in isotonic shortening[12] and shortening velocity compared with controls without asthma[13].

Smooth muscle cells express several contractile and structural proteins[14,15]. Cultured airway smooth muscle cells with a contractile phenotype are relatively rich in smooth muscle myosin heavy chain (sm-MHC), α-smooth muscle actin (α-SM-actin), calponin, desmin, and myosin light chain kinase (MLCK), whereas when proliferating they express less sm-MHC, calponin, α-SM-actin, and desmin, and significantly more vimentin. Benayoun et al[5] examined the expression of some of these contractile proteins in bronchial biopsies in relation to asthma severity. MLCK expression correlated inversely with lung function, but this was not the case for the proteins α-SM-actin or myosin. This suggests that the level of expression of these proteins may have different functional consequences. We selected several contractile and structural proteins that may influence airway responsiveness and function, namely α-SM-actin, myosin, desmin, vimentin, calponin, and MLCK. Furthermore, it has been shown that stretch of smooth muscle cells can increase the expression of contractile proteins[16,17]. Because smooth muscle cells are most likely stretched during deep inspiration, we analyzed the relationship between protein expression and airway responses to deep inspiration.

In addition, the smooth muscle bundles are embedded in and also contain extracellular matrix. The amount and the composition of the matrix may have functional consequences by altering the physical properties of the airway wall[18-20] and can also influence the proliferation of smooth muscle cells[21-23]. Therefore, we analyzed the expression of different extracellular matrix proteins (type III collagen, fibronectin, and elastin) within and surrounding the smooth muscle bundles.

We hypothesized that a higher level of expression of the selected contractile and structural proteins of smooth muscle cells, as well as components of the extracellular matrix, in bronchial biopsies are associated with increased airway hyperresponsiveness and impaired deep inspiration–induced bronchodilation in asthma. The aim of this study was to relate FEV_1% predicted, airway hyperresponsiveness, and deep inspiration–induced changes in resistance of the respiratory system as measured by forced oscillation technique to the level of expression of α-SM-actin, myosin, desmin, vimentin, calponin, and MLCK, as well as type III collagen, fibronectin and elastin, in bronchial biopsies of patients with asthma.

Methods

Subjects

This study was performed in the framework of a previously published project[4]. Thirteen patients with mild persistent asthma (Global Initiative for Asthma steps 1 and 2)[24] were recruited for this study. All patients had a history of episodic chest tightness or wheezing. Their baseline FEV_1 was more than 70% of predicted[25]. The PC_{20} methacholine was less than 8 mg/mL[26]. All patients were atopic, as determined by a positive skin prick test result (≥3mm wheal) to 1 or more of 10 common aeroallergen extracts (ALK-Abelló, Nieuwegein, The Netherlands). The patients were clinically stable, nonsmokers or exsmokers with less than 2 pack-years, and did not have a recent (≤2 weeks) upper respiratory tract infection or other relevant diseases. None of the patients had used inhaled or oral corticosteroids within 3 months before or during the study. The protocol was approved by the institutional review board for human studies, and before entering the study, the patients gave their written informed consent.

Study design

In this cross-sectional study, measurements were performed on 4 separate days within 3 to 4 weeks. On the first visit, medical history was taken, atopy was determined, and FEV_1% predicted and resistance of the respiratory system (Rrs) were measured before and after 400 µg salbutamol. On 2 additional visits, the patients returned for a methacholine challenge to determine airway hyperresponsiveness and deep inspiration–induced bronchodilation after a single dose of methacholine that induced a 20% fall in FEV_1[4]. Within 1 week of the last visit, a bronchoscopy was performed to obtain 6 bronchial biopsies.

Airway hyperresponsiveness

Methacholine bromide in normal saline was used for the bronchial challenges that were performed by standardized methodology[26]. At 5-minute intervals, aerosolized serial doubling concentrations of methacholine (0.15-40 µmol/L) were inhaled by tidal breathing (DeVilbiss, Somerset, Pa) for 2 minutes with the nose clipped. The challenge was stopped when FEV_1

dropped by more than 20% from baseline, and the response was expressed as the provocative concentration causing a 20% fall in FEV_1 PC_{20}.

Airway responses to deep inspiration

Deep inspiration–induced bronchodilation was measured using a single-dose methacholine challenge to induce a fall in FEV_1 of 20% in the absence of deep inspirations before methacholine inhalation. Baseline measurements of FEV_1 and Rrs were followed by a period of 20 minutes without deep inspirations. A single dose of methacholine (approximately the cumulative dose of the PC_{20} of the previous challenge) was inhaled, and 2 minutes later, Rrs was measured during tidal breathing, a deep inspiration to total lung capacity, a passive expiration, and again tidal breathing. This was directly followed by spirometry to measure the fall in FEV_1. The forced oscillation technique with an applied oscillation frequency of 8 Hz and an amplitude of ±1 cmH_2O was used to measure Rrs continuously during tidal breathing and a deep inspiration (Woolcock Institute, Sydney, Australia)[4]. Deep inspiration–induced bronchodilation was expressed as the reduction in Rrs during tidal breathing induced by the deep inspiration.

Bronchoscopy, immunohistochemistry, and image analysis

Bronchoscopy was performed by experienced pulmonologists according to a standardized and validated protocol[27]. All patients received 400 µg salbutamol 30 minutes before bronchoscopy. Six biopsies were taken at the (sub)segmental level using disposable forceps (radial edge; Boston Scientific, Boston, Mass).

The biopsies were fixed for 24 hours in 4% neutral buffered formaldehyde, processed, and embedded in paraffin. Sections 4 µm thick were cut, and hematoxylin-eosin staining was used to evaluate morphologic quality (intact reticular basal membrane and submucosa without crushing artifacts, blood clots, or only epithelial scrapings). Two sections per subject were selected on the quality of the submucosa, and not on the quantity of smooth muscle area. This was done to avoid a selection bias with regard to the main outcome parameter. In addition, the observers were blinded with regard to the subject number and their disease. The latter was chosen to avoid a selection bias with regard to the main outcome parameter. Antigen retrieval was performed on paraffin-embedded sections with citrate (desmin, myosin, and MLCK) or trypsin (type III collagen). α-SM-actin, calponin, vimentin, and fibronectin did not need antigen retrieval. The sections were incubated with mouse mAbs directed against α-SM-actin (1:50,000, clone 1A; Santa Cruz Biotechnology, Santa Cruz, Calif), myosin (1:40, clone 1A4; Sigma-Aldrich, St Louis, Mo), desmin (1:200, clone D33; Dako UK Ltd, Cambridgeshire, United Kingdom [UK]), vimentin (1:1000, clone V9; Dako), calponin (1:10,000, clone hCP; Sigma), MLCK (1:4000, clone k36; Sigma), type III collagen (1:2000, clone III-53; Merck Calbiochem, Darmstadt, Germany), and fibronectin (1:100, clone 568; Novocastra, Newcastle upon Tyne, UK). As a secondary antibody, Envision-HRP (Dako), was used. Positive cells stained red after development with NovaRed (Vector Laboratories, Burlingame, Vt). Sections were counterstained with Mayer hematoxylin. As a

negative control, the primary antibody was omitted from this procedure. For elastin expression, we used Weigert staining with Oxone (Klinipath BV, Duiven, The Netherlands)[28].

Morphometry was performed by means of digital image analysis[29]. The expression of the smooth muscle proteins (α-SM-actin, myosin, desmin, vimentin, MLCK) was determined in the total biopsy area (including the epithelial layer and glands). Type III collagen, fibronectin, and elastin were measured within the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. We used the desmin-stained adjacent biopsy sections to detect manually the positive stained area that appeared in bundles . Protein expression was quantified by fully automated densitometry (KS400; Zeiss, Oberkochen, Germany)[27,29]. This was performed by using a linear combination of red-filtered and blue-filtered grayscale images to derive a grayscale image (range, 0-255) in which the brown-red staining of interest is highlighted above a uniform background (white = gray value 255). This resulted in a narrow and peaked gray value distribution of background pixels with a longer tail on the left, which represented the positive stained pixels. The distribution was normalized toward the background peak, and subsequently inversed to obtain a zero value for the white background peak (white = gray value 0).

Data and statistical analyses

Airway hyperresponsiveness was expressed as PC_{20}. Reversibility was defined as the change in FEV_1% predicted or Rrs by 400 µg salbutamol. Rrs was calculated from all the data points, within the 95% CI, during 3 tidal inspirations (Rrs_{Insp}) and during 3 tidal expirations (Rrs_{Exp}) before and after deep inspiration. Deep inspiration–induced bronchodilation was expressed as the difference between Rrs after deep inspiration and Rrs before deep inspiration[4]; thus, a negative value indicates bronchodilation. This was performed for Rrs_{Insp} and Rrs_{Exp} separately because the airways may behave differently during inspiration and expiration[30].

Positive staining intensity was expressed as mean density (gray value). The outcome parameters were (log)transformed if necessary to obtain a normal distribution. Within-group differences were analyzed by 2-tailed paired t tests or Wilcoxon ranks test. Spearman rank correlation coefficient was used to explore associations between the expression of the proteins and the functional parameters. P values <.05 were considered statistically significant.

Results

Smooth muscle protein expression

The density of the smooth muscle protein staining was determined in the whole biopsy section. The mean density (gray value) for each marker is given in **Table 1**. All available sections (2 per patient) were used in the analysis. **Figure 1** presents examples of the immunohistochemical staining in the same biopsy section of 1 subject. All markers, except for vimentin, strongly

Table 1. Mean density of the expression the smooth muscle proteins

Smooth muscle protein	Mean density
α-SM-actin	20.8 (11.9-38.9)
Myosin	14.3 (5.9-18.1)
Desmin	14.4 (6.1-26.7)
Vimentin	13.2 (9.7-19.0)
Calponin	20.2 (9.8-40.4)
MLCK	15.9 (6.5-26.0)

The mean density (gray value) of each marker as measured in the bronchial biopsies. The smooth muscle proteins were measured in the total biopsy area. The data are expressed as median (range).

Table 2. Mean density of the expression the extracellular matrix proteins

	Within SM bundles	Outside SM bundles
Type III collagen	20.6 (13.1-35.6)	34.3 (25.9-46.2)*
Fibronectin	42.1 (15.7-64.6)	30.8 (16.5-42.3)†
Elastin	13.1 (9.2-15.2)	10.4 (7.2-16.2)

The mean density (gray value) of each marker as measured in the bronchial biopsies. The data are expressed as median (range). The extracellular matrix proteins were measured in the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. The mean density of type III collagen staining was significantly higher in the area surrounding the smooth muscle bundles compared with within the bundles (*$p = .003$), whereas this was opposite for mean density of fibronectin (†$p = .003$). Elastin expression was not significantly different between the compartments.

stained the airway smooth muscle cells. Vimentin was negative or weakly expressed in the smooth muscle cells. Outside the smooth muscle bundles, there was staining of the contractile markers, mainly around vessels, around submucosal glands (myoepithelial cells), and in scattered mesenchymal cells (fibroblasts and myofibroblasts).

Extracellular matrix expression

The density of the extracellular matrix protein expression of type III collagen, fibronectin, and elastin was determined in the smooth muscle bundles and the area surrounding the smooth muscle bundles separately. The proteins were expressed both in the lamina propria as well as within the airway smooth muscle bundles, with a fibrillar pattern for type III collagen and elastin and more diffuse pattern for fibronectin. Fibronectin and type III collagen stained the subepithlial basal membrane as well. Airway smooth muscle cells stained negatively for these markers. There were significant differences in density of type III collagen and fibronectin between the smooth muscle bundles and the area surrounding the muscle (**Table 2**).

Airways obstruction

FEV_1% predicted correlated positively with the mean density of calponin ($r = 0.58$), desmin ($r = 0.61$), and MLCK ($r = 0.55$; $p < 0.05$; **Fig 2, A and B**). There was a borderline significant correlation

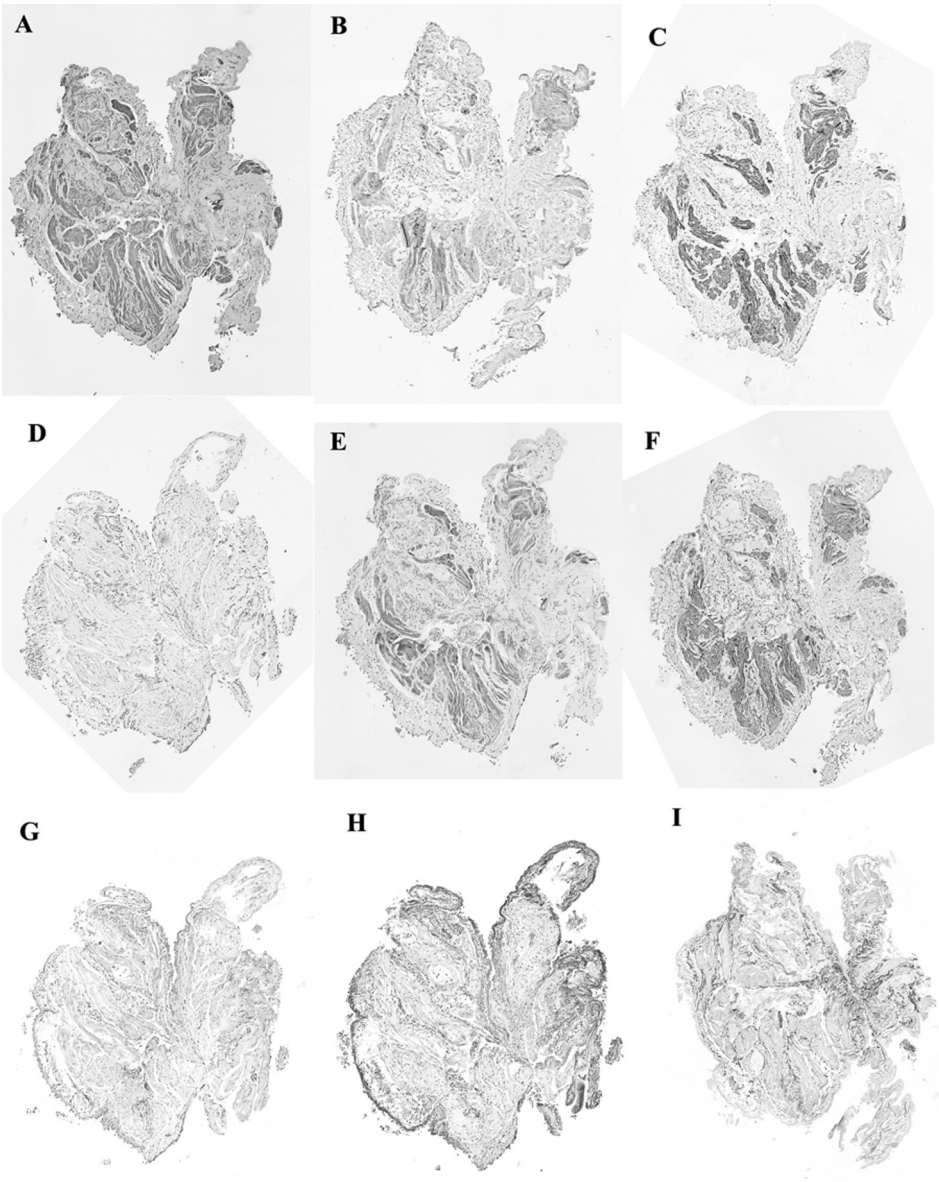

Figure 1. Examples of the immunohistochemical stainings. The same biopsy section is shown for the immunohistochemical staining for α-SM-actin **(A)**, myosin **(B)**, desmin **(C)**, vimentin **(D)**, calponin **(E)**, MLCK **(F)**, type III collagen **(G)**, fibronectin **(H)**, and elastin **(I)**. Original magnification ×200 for all graphs.

between FEV_1% predicted and the expression of α-SM-actin (r = 0.58), myosin (r = 0.53), and fibronectin (within the smooth muscle bundles; r = 0.56; p = 0.06). None of these correlations was seen for FEV_1% predicted after salbutamol. In addition, mean density of both α-SM-actin and calponin correlated inversely with FEV_1 reversibility (r = –0.54 and r = –0.61, respectively;

$p < 0.05$). Also, the change in Rrs_{Insp} and Rrs_{exp} by salbutamol correlated positively with mean density of α-SM-actin and calponin ($r > 0.70$; $p < 0.01$).

Dynamics of airway narrowing

PC_{20} methacholine was inversely related to the expression of the contractile smooth muscle protein α-SM-actin ($r = -0.62$; $P < .05$; **Fig 2, D**), the structural smooth muscle protein desmin ($r = -0.56$; $P < .05$; **Fig 2, C**), and the extracellular matrix protein elastin outside the smooth

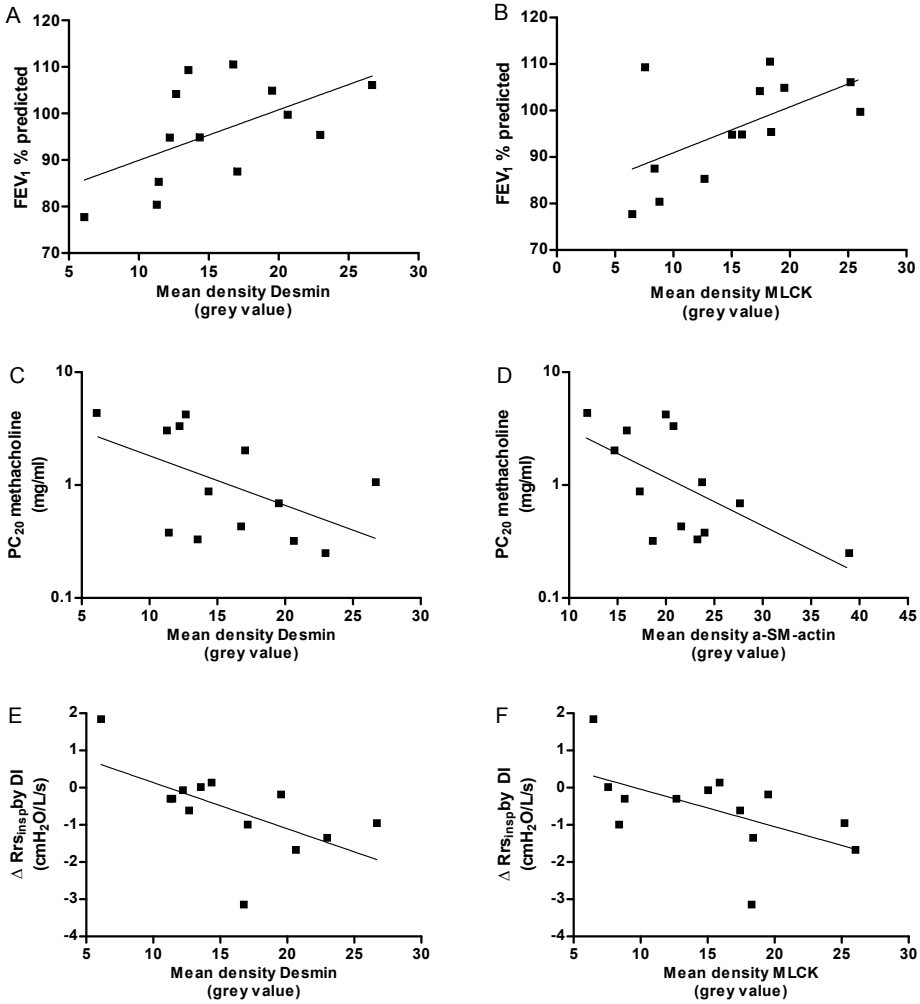

Figure 2. Associations between smooth muscle markers and lung function parameters.
The mean density of desmin ($r = 0.61$; $p = 0.02$) **(A)** and MLCK ($r = 0.55$; $p = 0.05$) **(B)** in relationship with FEV_1% of predicted; desmin ($r = -0.56$; $p = 0.04$) **(C)** and actin ($r = -0.62$; $p = 0.02$) **(D)** in relationship with PC_{20} methacholine; and desmin ($r = -0.60$; $p = 0.03$) **(E)** and MLCK ($r = -0.60$; $p = 0.03$) **(F)** with the change in Rrs_{Insp} by deep breath..

muscle bundles ($r = -0.78$; $P < .01$). PC_{20} methacholine was not significantly related to expression of elastin within the smooth muscle bundles.

Airway responses to deep inspiration

The reduction in Rrs_{insp} by deep inspiration was inversely related to the expression of desmin ($r = -0.60$; **Fig 2, E**), MLCK ($r = -0.60$; **Fig 2, F**), and calponin ($r = -0.54$) in the bronchial biopsies.

Discussion

Our results demonstrate an inverse association between PC_{20} methacholine and the level of expression of α-SM-actin, desmin, and elastin in bronchial biopsies in patients with asthma. Also, we showed that FEV_1% predicted was positively related, and deep inspiration–induced reduction in respiratory resistance inversely related to calponin, desmin, and MLCK expression. Thus, airway hyperresponsiveness, lung function, and airway responses to deep inspiration are associated with the level of expression of some, but not all, of the smooth muscle contractile and structural proteins, as well as the composition of the extracellular matrix within the airway wall. This suggests that the dynamics of airway function are influenced by the expression of several distinct smooth muscle and extracellular matrix proteins.

To our knowledge, this is the first study showing an association between airway hyperresponsiveness and smooth muscle cell protein expression in patients with asthma. We used markers of different functional components of smooth muscle cells and densitometry to analyze the expression of these proteins in the total biopsy area. An association between airway hyperresponsiveness and smooth muscle area has been shown in bronchial biopsies of both healthy control subjects and patients with asthma. However, no significant association was found in the subanalysis of the patients with asthma only[6]. We found a relationship between PC_{20} methacholine and the level of α-SM-actin and desmin expression in bronchial biopsies of patients with asthma. This suggests that airway hyperresponsiveness is associated with the expression of smooth muscle contractile and structural proteins within the airway wall, and not with smooth muscle area per se.

In addition, we found a positive relation between FEV_1% predicted and the expression of desmin, myosin, and calponin. Associations between FEV_1% predicted and MLCK expression[5] or smooth muscle area[7] have been shown, but with opposite results. This may be due to differences in asthma severity of the selected patients, because we included only patients with mild disease who were steroid-naive, whereas patients with more severe disease on steroid treatment were included in the other studies.

In our study, we used densitometry of the immunohistochemically stained sections to quantify the expression of different contractile and extracellular matrix proteins in or surrounding the smooth muscle bundles. Stereological methods have been used to analyze numbers and

size of airway smooth muscle cells in bronchial biopsies[6]. However, densitometry is a reliable and reproducible method[27], and it may also be a valuable tool to examine airway smooth muscle protein expression in bronchial biopsies.

None of the patients included in this study used inhaled or oral steroids within 3 months before or during the study. Therefore, the results were not affected by the effects of glucocorticosteroids. However, as part of the bronchoscopy procedure, all patients received 400 µg salbutamol 30 minutes before the bronchoscopy. We cannot exclude that this may have altered smooth muscle and extracellular matrix protein expression as measured in the biopsy sections. On the other hand, it is feasible to speculate that all patients would be equally affected by the use of salbutamol[31,32].

How can we interpret these results? We found an inverse correlation between airway hyperresponsiveness and the level of α-SM-actin, desmin, and elastin expression in asthma, but not with the other smooth muscle contractile proteins or extracellular matrix components. A higher level of α-SM-actin expression may indicate more actin monomers that can form longer actin filaments by polymerization in the asthmatic inflammatory environment[33]. Smooth muscle cells with longer actin filaments show a more elastic behavior, which can generate force even after being stretched[34,35], and may therefore increase airway hyperresponsiveness. Desmin, on the other hand, is an intermediate filament, present in dense bodies, and stabilizes the contractile units and participates in stress transmission between contractile units and anchorage sites linking to the extracellular matrix[36-38]. An increase in desmin could enhance force transmission between contractile units, which may increase total force generation, and therefore airway narrowing on stimulation. Indeed, in mice lacking desmin, lung stiffness and airway hyperresponsiveness were decreased compared with mice with desmin expression[39,40]. Increased presence of elastin has been shown in central airways of patients who died of asthma[28], but also a paucity of elastic fibers just underneath the basal membrane was demonstrated in these patients. The mechanical consequences of extracellular matrix, both within and surrounding smooth muscle bundles, is still under debate and most likely depends on the load it provides to airway smooth muscle[41-43], and can either enhance airway narrowing or oppose it. Overall, our data indicate that expression of α-SM-actin and desmin in the airway wall contribute to airway hyperresponsiveness, further augmented by an increase in elastin expression outside the smooth muscle bundles.

Interestingly, we found a positive relationship between FEV_1% predicted and the positive staining intensity for the smooth muscle proteins calponin, desmin, and MLCK, as well as a negative relationship between these markers and deep breath–induced reduction in respiratory resistance. These proteins are associated with a contractile phenotype of smooth muscle cells[14]. However, a study using ovalbumin-sensitized rats showed a reduction of 50% to 60% in the smooth muscle proteins α-SM-actin, smooth muscle 1 smooth muscle-myosin heavy chain, and smooth muscle-MLCK 24 hours after allergen (ovalbumin) challenge[44,45]. Expression of smooth muscle-(myosin heavy chain)1, calponin, and sm-MLCK was also reduced after 35 days

of allergen challenge[44]. This suggests that allergen exposure may lead to a change in smooth muscle phenotype from contractile to proliferative, in parallel with impaired lung function. When extrapolating these results, our findings may indicate that the patients, who were all atopic, with lower lung function had more smooth muscle cells of the proliferative phenotype, and thus less expression of contractile proteins. On the other hand, it has been shown that cultured smooth muscle cells with increased tone produce enhanced levels of contractile proteins, such as myosin, MLCK and desmin, when cultured under cyclic stretch conditions[16,17,46]. The positive correlations between FEV_1% predicted and deep breath–induced bronchodilation could therefore also reflect the effect of stretch on contractile protein production in these patients with asthma, rather than the influence of increased expression of these contractile markers on lung function. However, both suggestions are purely speculative and require further investigation.

What is the clinical implication of our study? Our data show that an increased expression of different components of the contractile unit of the smooth muscle cell, as well as of elastin in the surrounding extracellular matrix, may lead to an increase in the response of the airways to methacholine. Symptoms in mild persistent asthma are most likely the result of airway smooth muscle stimulation by direct or indirect stimuli. Even though it is controversial whether selective destruction of smooth muscle by thermoplasty can improve hyperresponsiveness[47,48], it has been shown to reduce symptoms, asthma control, prebronchodilator FEV_1, and exacerbations, and improve quality of life for as long as 12 months[48,49]. Also, long-acting anticholinergic treatment in a guinea pig model of ongoing asthma prohibited the allergen induced increase in airway smooth muscle cell proliferation and contractility[50]. In addition, glucocorticosteroids have been shown to influence airway smooth muscle function as well, in particular actin-filament dynamics[51,52]. Understanding the structure and function of airway smooth muscle cells in asthma could therefore lead to new targeted therapeutic strategies.

We conclude that airway hyperresponsiveness is associated with the level of expression of α-SM-actin, desmin, and elastin within the bronchial wall, but not with myosin, calponin, vimentin, type III collagen, or fibronectin. This suggests that expression of each of the contractile and structural smooth muscle proteins, as well as components of the extracellular matrix, influences dynamic airway function distinctly.

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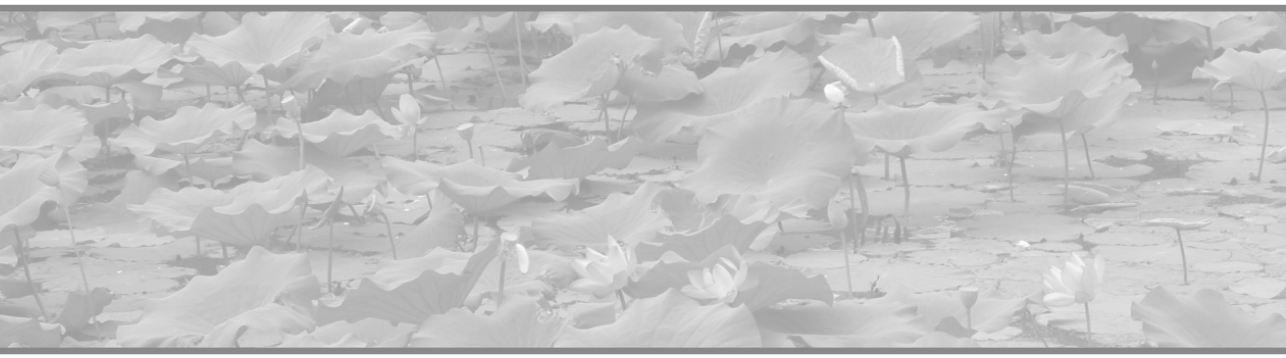

Chapter 4

Influence of pulmonary congestion in mitral valve disease on airway responses to deep inspiration

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Abstract

Rationale: In healthy subjects deep inspirations can reverse bronchoconstriction. In asthmatic patients bronchodilation by deep inspiration appears to be impaired or even result in bronchoconstriction. The latter has been presumed to be due to fluid-flux within the inflamed airway wall or across leaky capillaries. Therefore, we postulated that pulmonary congestion secondary to mitral valve disease leads to deep inspiration induced-bronchoconstriction.

Methods: Spirometry, lung volumes, resistance(Rrs) and reactance(Xrs) of the respiratory system were measured during tidal breathing and following a single deep breath using forced oscillation technique at 8 Hz before, and 12 weeks after surgery in 12 patients with mitral valve disease(MVD) and in 11 control patients with coronary artery disease(CAD). A healthy control group(HC) was added to the baseline measurements.

Results: A deep inspiration did not significantly increase respiratory resistance in patients with mitral valve disease, nor was this changed by mitral valve repair. As compared to the healthy control subjects, both patients with mitral valve disease as well as patients with coronary artery disease had significantly lower vital capacity (mean±SD(% predicted): MVD 96±17, CAD 95±18, HC 114±12, p=0.02), forced vital capacity (MVD 89±19, CAD 90±11, HC 108±18, p=0.033), and FEV_1 (MVD 85±20, CAD 92±16, HC 105±15, p=0.036) values. Interestingly, respiratory reactance was significantly increased in the mitral valve disease group as compared to coronary artery disease patients (Xrs_{insp}±SD ($cmH_2O/L/s$): MVD 0.15±0.19, CAD −0.03±0.27, p=0.044) and reversed to comparable levels following mitral valve repair.

Conclusion: Pulmonary congestion in patients with mitral valve disease does not lead to deep inspiration-induced bronchoconstriction. Our data suggest that edema within the airways as such may not be the key factor that leads to deep inspiration-induced bronchoconstriction as observed in asthma, but the site and the cause of the fluid accumulation within the airways and/or parenchyma may be of influence.

Introduction

Deep inspirations play an important role in regulating airway caliber. Stretch of the airways by deep inspiration reduces pharmacologically induced airways obstruction in healthy subjects[1]. In asthma on the other hand, this bronchodilatory effect of deep inspiration is impaired[2,3]. Furthermore, during spontaneous asthma exacerbations deep inspirations can even lead to bronchoconstriction[4]. Edema of the airway wall has been suggested as one of the mechanisms leading to bronchoconstriction following deep inspiration in asthma[5,6].

The chronic inflammatory changes in asthma, such as increased vascularity, increased mucosal blood flow, leaky capillaries, or inflammatory exudates may lead to edema of the airway wall[7]. The large-sub atmospheric pressures during deep inspiration could further enhance airway wall edema by extravasation of fluid from leaky capillaries into the inflamed airway wall[5]. This would increase airway wall thickness and thus decrease the luminal area and at the same time uncouple the interdependence of the airway and parenchyma[8,9]. Also, edema could stiffen the airway wall, which thereby becomes less distensible in response to lung inflation. However, Brown et al demonstrated using high resolution computed tomography that induction of airway wall edema by infusion of saline or bradykinine did not lead to decreased distensibility of airways in dogs[10] and sheep[11]. Also, in healthy volunteers rapid saline infusion, although it led to a small increase in respiratory resistance, had no effect on airway distensibility or deep inspiration-induced bronchodilation[12]. Airway wall edema can be present secondary to chronic diseases, for instance as a result of chronic pulmonary congestion secondary to mitral valve regurgitation. It is possible that this has different effects on airway mechanics as opposed to rapid infusion of fluids by either the cause or the site of pulmonary congestion within the airways[13-15]. Indeed, it has been shown that lung function (FVC, FEV_1, MEF) is diminished in patients with mitral valve disease and is, at least partially, reversed after mitral valve repair[16-18]. Airway wall edema in patients with mitral valve disease may also change airway dynamics during deep inspiration[19], which may be in keeping with the clinical diagnosis of "asthma cardiale".

We hypothesized that in patients with mitral valve disease, in contrast with healthy control subjects, deep inspirations would lead to bronchoconstriction. In addition mitral valve repair would lead to a reduction in pulmonary congestion, and therefore restore the coupling between airway and lung parenchyma, and thus improve airway mechanics during deep inspiration. The aim of this study was to measure resistance (Rrs) and reactance (Xrs) of the respiratory system during deep inspiration in addition to several parameters of lung function in patients with mitral valve disease who require surgery to control symptoms, and compare these findings to healthy subjects. Moreover, we aimed to compare these measurements before and 12 weeks after mitral valve repair. A group of patients with coronary artery disease was added in order to control for the effects of cardiopulmonary bypass during mitral valve repair.

Methods

Subjects

We recruited 12 patients with mitral valve disease and 11 patients with coronary artery disease who were referred to the outpatient clinic of the department of cardio-thoracic surgery for mitral valve repair or coronary artery bypass graft (CABG) surgery respectively. Mitral valve regurgitation grade and pressure difference were estimated by an experienced cardiologist by echocardiography using international guidelines[20,21]. All patients had grade 3-4 mitral valve regurgitation on echocardiography, whereas the patients with coronary artery disease did not have clinically significant mitral regurgitation. Patients were excluded if they had primary pulmonary, neurologic, or myopathic disease. Lung function and general condition were sufficient for operation, and surgery was planned within 3 months of the echocardiographic examination. None of the patients had a history of asthma, or had had a recent upper or lower respiratory tract infection. In addition, 11 healthy control subjects were recruited within the same age range. They were non-smokers or ex-smokers, did not have a history of cardiac or pulmonary disease, and were not hyperresponsive to methacholine.

Study design

Medical history and symptoms were assessed at visit 1 in all patients and healthy control subjects. They performed spirometry to measure FEV_1 and FVC % predicted as the highest of three technically satisfying flow-volume curves[22]. The diffusion capacity for carbon monoxide corrected for hemoglobin level ($DLCO_c$) was measured using the single breath holding method with a rolling seal closed system[23]. Total lung capacity (TLC), functional residual capacity (FRC) and residual volume (RV) were determined by re-breathing helium dilution technique[24]. Resistance (Rrs) and reactance (Xrs) of the respiratory system were measured by forced oscillation technique[25] at 8 Hz during one minute of tidal breathing, a single deep inspiration to TLC, passive expiration to FRC, and another minute of tidal breathing. All the measurements were repeated at a follow-up visit 12 weeks after surgery in the patients with mitral valve disease and coronary artery disease.

Echocardiography

In the patients with mitral valve disease and coronary artery disease a echocardiography was performed within 12 weeks prior to surgery and 12 weeks following surgery. Patients were imaged in the left lateral decubitus position using a commercially available system (Vivid 7, General Electric Vingmed, Milwaukee, Wisconsin, USA) equipped with a 3.5-MHz transducer. Standard gray-scale two-dimensional images were obtained in the parasternal (standard long- and short-axis) and apical views (2- and 4-chamber and long-apical axis views). Color Doppler echocardiography was performed in all views after optimizing gain and Nyquist limit. Standard continuous-wave and pulse-wave Doppler examinations were performed. The severity of mitral

valve regurgitation was determined on a qualitative scale according to the ACC/AHA guidelines for the management of patients with valvular heart disease: mild (grade 1), moderate (grade 2) and severe (grades 3-4)[21,26].

Forced oscillation technique

Rrs and Xrs were measured continuously during the breathing maneuvers using a forced oscillation device (Woolcock institute, Australia)[27] with an applied oscillation frequency of 8 Hz and an amplitude of ± 1 cmH_2O. This method has been fully described in a previous study[28;29]. Flow was measured using a 50-mm diameter Fleisch Pneumotachograph (Vitalograph Ltd, Maids Moreton, UK), and differential pressure was measured using a ± 2.5 cm H_2O solid-state transducer (Sursense DCAL4; Honeywell Sensing and Control, Milpitas, CA, USA). Mouth pressure was measured using a similar transducer with a higher range (±12.5 cmH_2O). Analog pressure and flow signals were digitized at 400 Hz. The time- and frequency-dependent respiratory impedance Zrs was estimated based on the hypothesis that random errors occur in both pressure and flow. This yields a Total Least Squares (TLS) estimate of respiratory impedance as a function of time and frequency and allows an estimation of confidence intervals in the course of time.

Analysis

Mean Rrs and Xrs were calculated from all data points within the 95% confidence interval during 3 tidal inspirations (Rrs_{insp} and Xrs_{Insp}) and 3 tidal expirations (Rrs_{Exp} and Xrs_{Exp}) separately. The response of the airways was calculated as the difference between Rrs and Xrs following and preceding the deep inspiration. The sample size of 12 patients per group was based on our data with regard to Rrs measurements[28,29], allowing the detection of a 1 $cmH_2O/l/s$ difference within and between the groups, if $\alpha=0.05$ and $1-\beta=0.80$. Between group differences were analyzed using Kruskal Wallis with Mann-Whitney post-hoc analysis. Within group differences were explored using Wilcoxon signed-rank tests. We used SPSS version 12.01 for all analyses (SPSS Inc, Chicago). P values < 0.05 were considered statistically significant.

Results

12 patients with mitral valve disease, 11 with coronary artery disease, and 11 healthy control subjects were included in the study. 9 patients with mitral valve disease and 8 patients with coronary artery disease performed visit 2 at 12 weeks following surgery. Patients were lost to follow up because of non-compliance (n=4) and death (n=1). The 3 groups were within the same age range (p=0.5). However, the number of pack years was significantly lower and presence of allergies was significantly less in the healthy control group as compared to the other two groups. The patient characteristics are summarized in **table 1**.

Table 1. Patient characteristics

	Mitral valve disease (MVD)	Coronary artery disease (CAD)	Healthy controls (HC)	P value
Age (yrs)	58 ± 12.6	61 ± 9.6	58 ±8.2	0.518
Sex (M/F)	8/4	10/1	4/7	0.027
Pack years	23.5 ± 24.9	18.4 ± 15.3	0.1 ± 0.3$^{\$\#}$	0.002
Allergy (yes/no)	3/9	5/6	0/11	0.042
MI grade	III-IV	0-I	N/A	0.000

Data are expressed as mean ± SD, or numbers (sex, allergy). Sex and allergy was analyzed by chi-square. Mitral valve regurgitation (MI) grade was analyzed by Mann-Whitney Test. Age and pack years was analyzed by Kruskal-Wallis and Mann-Whitney ($^{*}p < 0.05$ MVD vs. CAD, $^{\$}$ $p < 0.05$ MVD vs. HC, $^{\#}$ $p < 0.05$ CAD vs. HC)

Echocardiography

All patients had grade 3-4 mitral regurgitation on echocardiography; after mitral valve repair, no significant regurgitation remained (grade 0-1) (p=0.002). Patients with coronary artery disease had no significant mitral regurgitation at baseline (grade 0-1), which was not altered by coronary artery bypass graft surgery.

Respiratory resistance and reactance

Mean Rrs during 3 tidal inspirations or expirations was not significantly different between the groups at baseline (**Table 2**). A deep inspiration significantly decreased mean Rrs during inspiration, but not expiration, in the mitral valve disease group (p=0.034), but this did not result in significant differences between the groups following deep inspiration (**Figure 1**).

Mean Xrs during 3 tidal in- or expirations was significantly increased in the mitral valve disease group (p = 0.044 and p = 0.019 respectively) as compared to the coronary artery disease group at baseline (**Table 2**). A deep inspiration did not significantly change Xrs during in- or expirations in all 3 groups. Following surgery, however, Xrs was significantly lower in the mitral valve disease group as compared to before surgery (Xrs_{insp} p = 0.025, Xrs_{exp} p = 0.05). Also, following surgery Xrs values in the mitral valve disease group were comparable to the coronary artery disease group (p = 0.2 and p = 0.5 respectively) (**Figure 2**).

Table 2. Baseline Rrs and Xrs

$CmH_2O/L/s$	Mitral valve disease (MVD)	Coronary artery disease (CAD)	Healthy controls (HC)	p value
Rrs Insp	2.53 ± 0.56	2.96 ± 0.98	2.78 ± 0.58	0.633
Rrs Exp	2.79 ± 0.65	3.72 ± 1.26	3.34 ± 0.93	0.127
Xrs Insp	0.15 ± 0.19	−0.14 ± 0.37	−0.03 ± 0.27	0.079
Xrs Exp	0.47 ± 0.31	−0.21 ± 0.85	0.30 ± 0.37	0.065

Data are expressed as mean ± SD, analyzed by Kruskal-Wallis (p values showed) and Mann-Whitney as post-hoc testing ($^{*}p < 0.05$ MVD vs. CAD, $^{\$}$ $p < 0.05$ MVD vs. HC, $^{\#}$ $p < 0.05$ CAD vs. HC).

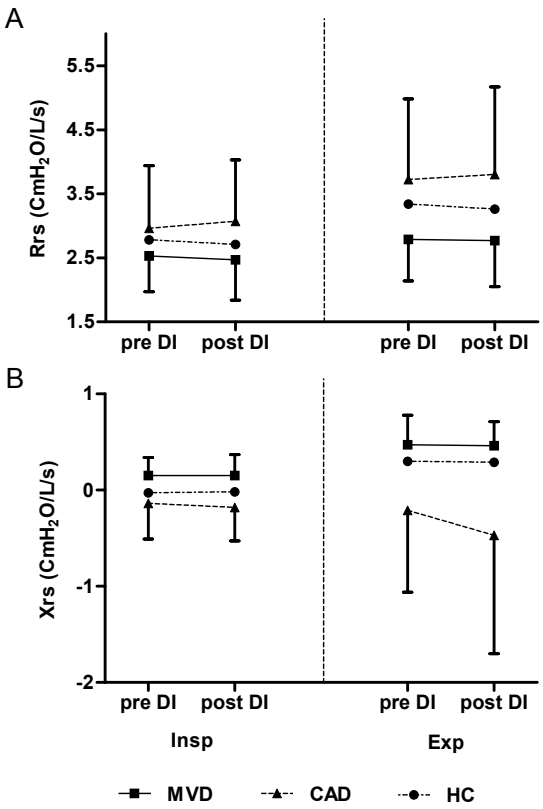

Figure 1. Change in Rrs and Xrs by deep inspiration.
Rrs **(A)** and Xrs **(B)** during tidal inspirations (left side) and expirations (right side) before (pre DI) and after (post DI) deep inspiration. Squares with solid lines represent the mitral valve disease group, triangles with dashed lines represent the coronary artery disease group, and closed circles with interrupted lines represent the healthy control subjects. Only Rrs during tidal inspirations was significantly reduced by deep inspiration in the mitral valve disease group ($p = 0.034$, Mann-Whitney Test). No significant change was observed in the other groups, nor was Xrs significantly changed by deep inspiration.

Spirometry

Both patients with mitral valve disease as well as the patients with coronary artery disease had significantly lower VC % predicted, FVC % predicted, and FEV_1 % predicted values as compared to the healthy control subjects (**Table 3**). None of these parameters improved following surgery in both groups. In contrast, in the mitral valve disease group VC % predicted tended to be significantly lower 12 weeks after surgery (mean ± SEM 95.3 ± 6.2% pre surgery vs. 89.4 ± 5.9% post surgery, $p = 0.053$), and in the coronary artery disease group FEV_1 % predicted was significantly lower following surgery (91.4 ± 5.9% vs. 83.9 ± 7.6%, $p = 0.047$).

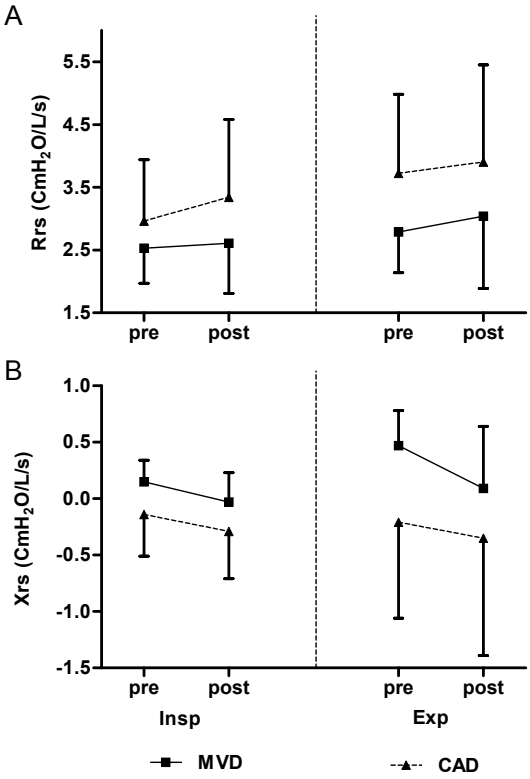

Figure 2. Change in Rrs and Xrs by surgery.
Rrs **(A)** and Xrs **(B)** during tidal inspirations (left side) and expirations (right side) before (pre) and after (post) surgery. Squares with solid lines represent the mitral valve disease group, and triangles with dashed lines represent the coronary artery disease group. In the mitral valve disease group Xrs, both during tidal inspirations (p = 0.025) and tidal expirations (p = 0.05), was significantly reduced by the surgery. This resulted in comparable values between the 2 groups post-surgery, whereas Xrs was significantly higher in the mitral valve disease group pre-surgery (p= 0.044 and p = 0.019 for in- and expiration respectively).

Table 3. Spirometry values

	Mitral valve disease (MVD)	Coronary artery disease (CAD)	Healthy controls (HC)	P value
VC (%pred)	96 ± 17$^{\$}$	95 ± 18$^{\#}$	114 ± 12	0.020
FVC (%pred)	89 ± 19$^{\$}$	90 ± 11$^{\#}$	108 ± 18	0.033
FEV_1 (%pred)	85 ± 20$^{\$}$	92 ± 16	105 ± 15	0.036
FEV1/FVC ratio	78 ± 10	81 ± 12	84 ± 12	0.680
TLCOc (%pred)	76 ± 19	78 ± 18	85 ± 6	0.520
FRC (%pred)	106 ± 22	82 ± 24	90 ± 22	0.109
RV (%pred)	96 ± 26	87 ± 29	84 ± 11	0.716
TLC(%pred)	92 ± 17	92 ± 12	100 ± 9	0.161

Data are expressed as mean ± SD, analyzed by Kruskal-Wallis (p values showed) and Mann-Whitney as post-hoc testing ($^{*}p < 0.05$ MVD vs. CAD, $^{\$}$ $p < 0.05$ MVD vs. HC, $^{\#}$ $p < 0.05$ CAD vs. HC).

CO diffusion

$DLCO_c$ % predicted was not significantly different between the groups (p = 0.36 and p = 0.47 respectively). Also, $DLCO_c$ % predicted did not significantly change in both mitral valve disease and coronary artery disease following surgery.

Lung volumes

FRC level tended to be higher in the mitral valve disease group as compared to coronary artery disease (p = 0.07), but not to healthy control subjects. In addition, RV and TLC were not significantly different between the groups. Both FRC and TLC tended to be lower 12 weeks following surgery in the mitral valve disease group (p = 0.07) as compared to before surgery. The same was shown for TLC in the coronary artery disease group (p=0.053).

Discussion

The results of this study show that a deep inspiration in patients with mitral valve disease does not lead to bronchoconstriction, although lung function was diminished as compared to healthy subjects. This suggests that airway wall edema per se may not lead to bronchoconstriction following deep inspiration. In addition, we showed that respiratory reactance was increased in the mitral valve disease group as compared to coronary artery disease patients, and tended to be higher as compared to healthy controls. Following mitral valve repair respiratory reactance was decreased, which resulted in comparable values between the groups.

In this study we aimed to evaluate the effect of airway wall edema on airway mechanics during deep inspiration in absence of allergic inflammatory-induced changes as noted in asthmatic patients. Our data extend previous findings by showing that chronic pulmonary congestion does not further reduce the airway lumen during deep inspirations. Earlier studies examined the effect of airway wall edema on airway mechanics during deep inspiration by inducing edema in healthy subjects[12], sheep[11,30], and dogs[10]. These authors showed that induced-airway wall edema reduces airway lumen, and increases airway wall thickness. In line with our results, airway wall edema did not reduce airway wall distensibility or impair deep inspiration-induced bronchodilation. In contrast, Burns et al[5] showed that enhanced negative intrathoracic pressure associated with deep inspiration temporarily increases airway resistance. They suggested that this may occur in the context of increased leakiness of the airway vasculature, and may temporarily increase airway edema and thus reduce luminal diameter in subjects with asthma. A difference in site and cause of fluid accumulation within the airways and/or parenchyma may explain these contrasting results.

In the current study we measured lung function parameters in addition to resistance measurements of the respiratory system during deep inspiration. Ideally, we would have liked to add physiological parameters of heart function as well. However, we considered right

heart catheterization an inappropriate risk for the patients. Accordingly, the severity of mitral regurgitation was assessed by echocardiography, and revealed grade 3-4 mitral regurgitation, which improved to grade 0-1 following mitral valve repair. Also, we do not have objective measurements of the level of pulmonary congestion. However, the need for surgery (rather than medical therapy), to relieve symptoms implies that pulmonary congestion was present at the time of the baseline measurements.

How do we interpret our results? Pulmonary congestion in patients with mitral valve disease did not lead to increased respiratory resistance following deep inspiration. Possibly, the deep inspiration did not result in increased fluid flux into the airway wall. In chronic heart failure, airway wall edema is a result of increased intra-vascular pressure by increased intravascular volume load. Enhanced negative pressures may therefore not lead to fluid flux across capillaries such as may occur in more permeable capillaries due to chronic airway inflammation as suggested by Burns et al[5]. Studies that examined increased airway responsiveness in patients with pulmonary congestion also suggested a synergistic effect of bronchial vascular hemodynamics, cholinergic reflexes, and permeability changes, instead of local effects of edema alone[31,32] This may also explain the association between asthma severity and reduced bronchodilation by deep inspiration[3,33], and the beneficial effects of anti-inflammatory treatment on deep inspiration-induced bronchodilation[34,35]. In addition, the site of the airway wall edema within the airway wall tree, due to either chronic heart failure or asthmatic airway wall inflammation, may be different. One would expect airway wall edema due to chronic heart failure in the pulmonary circulation reaching from the bronchioli to the alveoli[13], whereas asthmatic airway wall inflammation would influence the airways of the bronchial circulation from the larger airways up to the bronchioli[14]. However, it has been shown that also in asthma there is remodeling of both the bronchial as well as the pulmonary vasculature[36-38], and that in chronic heart failure there may be engorgement of the bronchial circulatory bed that may play a role in pulmonary function abnormalities[15]. Therefore the site of the airway wall edema in both diseases shows some overlap and may not explain the absence of deep inspiration-induced bronchoconstricion in patients with mitral valve disease.

A secondary finding is the increased level of reactance in the mitral valve disease group as compared to healthy controls and coronary artery disease patients. Notably, this difference was dissolved following surgery. Many studies[17,39,40] have shown that airways obstruction, including small airways obstruction, leads to a negative slope of resistance at low frequencies associated with a more negative reactance. In addition, Depeursinge et al[41], showed a low reactance in combination with a frequency dependent Rrs in patients with acute left-sided heart failure following myocardial infarction. They suggested that mechanical inhomogeneities and increased resistance of the small airways, as a result of mechanical compression of the small airways by adjacent distended vessels, narrowing of the small airways by interstitial edema and/or vagally mediated active bronchoconstriction are responsible for this finding. The reversible increase in Xrs at 8 Hz in the patients with mitral valve disease as compared to patients with

coronary artery disease is in contrast to these findings. This may be due to increased stiffness of the airway wall, making the small airways more resistant to airway closure that impedes small airways obstruction.

What is the clinical relevance of our results? First, the findings suggest that airway wall edema alone is not responsible for deep inspiration-induced bronchoconstriction or diminished deep inspiration-induced bronchodilation in asthma. Research addressing asthma-treatment should therefore focus on the interaction between airway dynamics and inflammatory changes, and not inflammation alone. In addition, measurements of respiratory resistance may be useful in detecting pulmonary congestion. Usually, heart failure is detected too late to reverse the remodeling changes of the heart muscle. Our study shows the potential that a change in reactance may reveal early signs of heart failure. Naturally, this needs further investigation prospectively in larger groups of patients.

We conclude that pulmonary congestion as a result of chronic left-sided heart failure in patients with mitral valve disease did not lead to deep inspiration-induced bronchoconstriction. This suggests that airway wall edema alone may not be responsible for altered airway responses to deep inspiration. Further studies examining the effects of deep inspiration induced bronchodilation in asthma should therefore focus on the interaction between airway wall thickening, inflammation and altered smooth muscle responses.

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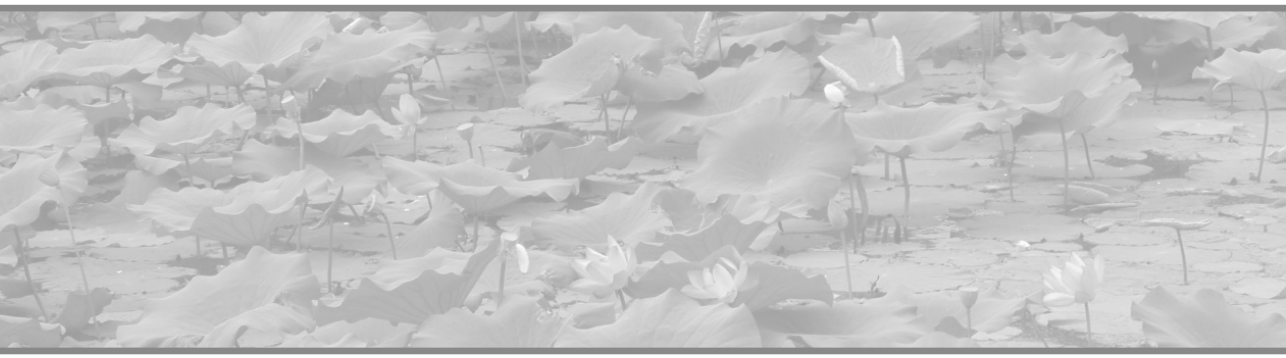

Chapter 5

Improvement in bronchodilation following deep Inspiration after a course of high-dose oral prednisone in asthma

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Chest 2006; 130 (1): 58-65

Abstract

Background: Bronchodilation following deep inspiration is usually impaired in patients with asthma. This might be due to changes in airway mechanics in the presence of inflammation or structural changes within the airways. Although inhaled corticosteroid treatment has been shown to improve airway responses to deep inspiration in patients with asthma, airway inflammation can persist despite inhaled corticosteroid treatment, and thus could still influence the airway mechanics during deep breaths. We hypothesized that oral steroid treatment further optimizes deep inspiration-induced bronchodilation in clinically stable asthmatic patients who are receiving therapy with inhaled corticosteroids.

Methods: Twenty-four atopic patients with mild-to-moderate persistent asthma (FEV_1, > 70% predicted; provocative concentration of methacholine causing a 20% fall in FEV_1 [PC_{20}], < 8 mg/mL), who were treated with 250 to 2,000 µg of beclomethasone-dipropionate or equivalent, participated in a parallel-design, double-blind study. Before and after treatment with 0.5 mg/kg/d prednisone or placebo for 14 days, a methacholine challenge was performed. Deep inspiration-induced bronchodilation was measured by the ratio of flow at 40% of FVC on the flow-volume curve after maximal inspiration/flow at 40% of FVC on the flow-volume curve after partial (60% of FVC) inspiration (M/P ratio).

Results: The M/P ratio significantly increased from a mean of 1.31 (range, 1.0 to 1.7) to 1.49 (range, 1.1 to 2.3) in the prednisone group. Interestingly, the improvement in the M/P ratio did not correlate with an accompanying significant increase in PC_{20} for methacholine (mean change, 1.02; SD doubling dose, 0.97) and a decrease in exhaled nitric oxide (mean change, 14 parts per billion [ppb]; SD, 33.4 ppb).

Conclusions: Systemic anti-inflammatory treatment in addition to maintenance therapy with inhaled corticosteroids increases bronchodilation by deep inspiration in patients with mild-to-moderate persistent asthma. This suggests that residual inflammation impairs airway mechanics in asthma patients.

Introduction

Asthma is a chronic inflammatory disorder of the airways, which is associated with excessive airway narrowing in response to stimuli that have no or little effect on healthy subjects. Interestingly, the degree of airway narrowing appears to be related to the response of the airways to deep inspiration[1]. In healthy subjects deep inspirations can protect against airway narrowing (bronchoprotection) and also can reverse induced bronchoconstriction (bronchodilation)[2,3,4]. These effects of deep inspiration are impaired or even absent in patients with asthma[5,6]. The mechanisms that are responsible for the ineffective response of the airways to deep inspiration in asthma patients have yet to be unraveled, but seem to be a key for understanding the pathophysiology of the disease and possibly for the development of truly effective therapy.

The uncoupling of the mechanical airway-parenchyma interdependence has been hypothesized as one of the mechanisms that are responsible for the impaired responses of the airways to deep inspiration in asthma patients[7,8,9]. Uncoupling can be a result of inflammatory changes within the airways, such as edema, thereby thickening the peribronchial airway wall and thus decreasing the radial forces acting on the airways during a deep inspiration[7,10].

Indeed, some studies have shown beneficial effects of anti-inflammatory treatment on the bronchodilatory effect of a deep inspiration. Systemic corticosteroid treatment of spontaneous asthma exacerbations improved the airway responses to deep inspiration as lung function recovered[11]. Treatment with inhaled corticosteroids, on the other hand, improved deep inspiration-induced bronchodilation in steroid-naïve patients with mild asthma in two studies[12,13] but had no effect on bronchodilation following deep inspiration in a recent article[14]. It has been previously shown[15,16] that airways inflammation can persist in asthmatic patients despite treatment with inhaled corticosteroids. It may therefore not be surprising that deep inspiration-induced bronchodilation is still deficient in some asthmatic patients whose conditions are being clinically well-controlled with inhaled steroid therapy[1].

We hypothesized that systemic anti-inflammatory therapy, when added to regular treatment with inhaled corticosteroids, reduces any ongoing inflammation in clinically stable patients with persistent asthma, and therefore optimizes the bronchodilatory effect of deep inspiration. The aim of this study was to examine the effect of a course of high-dose oral prednisone on the degree of deep inspiration-induced bronchodilation at a given level of airway narrowing in patients with mild-to-moderate asthma who were already treated with inhaled steroids. In order to estimate the effect of the treatment on airway inflammation, which is a noninvasive marker of airway inflammation, exhaled nitric oxide (NO) was added as a secondary outcome parameter[17].

Materials and Methods

Subjects

Twenty-four nonsmoking, atopic subjects with mild-to-moderate persistent asthma, according to Global Initiative for Asthma guidelines[18], participated in this study. All subjects had experienced symptoms of episodic chest tightness or wheezing within the previous 12 months, had a baseline FEV_1 of > 70% of predicted[19], and had a provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) of < 8 mg/mL[20]. All patients were atopic, which was determined by a positive skin-prick test (wheal size, ≥3mm) to ≥1 of 10 common airborne allergen extracts (ALK; Abelló; Nieuwegein, the Netherlands), and all patients were asked to avoid overt allergen exposure during the study. The patients were clinically stable, indicating that there had been no change in their clinical condition or medication use within the previous 6 weeks. They were all receiving inhaled corticosteroid treatment (*ie*, beclomethasone-dipropionate, 500 to 2,000 µg daily or equivalent) in combination with short-acting or long-acting β_2-agonists and had no history of a recent (*ie*, ≤2 weeks) upper respiratory tract infection or other relevant diseases. None of the subjects had used oral corticosteroids within 3 months prior to the study. The patient characteristics are shown in **Table 1** . The study was approved by the institutional review board, and the subjects gave their written consent before entering the study.

Study design

The study had a placebo-controlled, double-blind, parallel design. The 24 patients were randomly assigned to receive either prednisone treatment or placebo. The patients received therapy with prednisone, 0.5 mg/kg/d (rounded to the nearest tenth) once daily for 14 days, in addition to their regular inhaled corticosteroid treatment. The study consisted of the following two visits: visit 1 at day 0; and visit 2 at day 14 or 15. On both visits, the Juniper Asthma Control Questionnaire[21], exhaled NO level, FEV_1, partial and maximal flow-volume curves, and airway responsiveness to methacholine were measured. The degree of bronchodilation following deep inspiration was measured at baseline and at a given level of airway narrowing in response to methacholine. All tests were performed after adequately stopping bronchodilator therapy (for > 8 h for short-acting β_2-agonists and > 24 h for long-acting β_2-agonists).

Maximal and partial flow-volume curves

The effect of a deep inspiration on airways obstruction was measured by partial and maximal expiratory flow-volume curves[22]. First, baseline FVC was measured. The mean of three technically satisfactory FVC measurements was used to calculate the starting point of the partial flow-volume curve throughout all measurements during that visit, including the methacholine challenge. Since total lung capacity (TLC) does not change during a methacholine challenge[23], this point was used to calculate 60% or 40% of FVC above the baseline residual volume. Each measurement was therefore preceded by an inhalation to TLC. Forty-five seconds after inhaling

Table 1. Patient characteristics and posttreatment values of FEV_1, airway hyperresponsiveness, M/P ratio, and exhaled NO Levels

Characteristics	Baseline	Post-treatment	p value
Prednisone group			
Male	4		
Female	8		
Age, yr	27.6 (7.3)		
Asthma Control Questionnaire score	1.1 (0.6)	0.8 (0.5)	0.14
FEV_1, % predicted	90.1 (13.7)	91.8 (14.0)	0.36
PC_{20},† mg/mL	0.64 (1.76)	1.29 (1.74)	0.004
$PC_{40}V'_{40}P$,† mg/mL	0.24 (1.04)	0.43 (1.53)	0.07
M/P ratio			
At baseline‡	1.12 (0.9–1.6)	1.14 (0.8–1.7)	0.71
At $PC_{40}V'_{40}P$‡	1.31 (1.0–1.7)	1.49 (1.1–2.3)	0.006
At PC_{20}‡	1.71 (1.2–2.9)	1.81 (1.1–3.0)	0.30
MPslope	0.89 (0.23)	0.87 (0.30)	0.94
Exhaled NO,§ ppb	31.5 (3.5–89.8)	17.5 (10.7–63.5)	0.21
Placebo group			
Male	5		
Female	7		
Age, yr	31.6 (11.6)		
Asthma Control Questionnaire score	1.0 (0.6)	1.0 (0.6)	0.7
FEV_1, % predicted	85.6 (11.2)	87.6 (8.7)	0.20
PC_{20},† mg/mL	1.75 (1.78)	1.29 (1.29)	0.10
$PC_{40}V'_{40}P$,† mg/mL	0.74 (1.91)	0.61 (1.15)	0.43
M/P ratio			
At baseline‡	1.19 (1.0–1.6)	1.15 (1.0–1.3)	0.33
At $PC_{40}V'_{40}P$‡	1.45 (1.1–1.9)	1.41 (1.1–1.9)	0.46
At PC_{20}‡	1.59 (1.2–3.0)	1.57 (1.2–2.9)	0.80
MPslope	0.97 (0.23)	0.94 (0.23)	0.57
Exhaled NO,§ ppb	22.9 (7.7–45.6)	34.7 (12.7–48.3)	0.10

Values are given as the mean (SD), unless otherwise indicated. †Values are given as the geometric mean (SD in doubling doses). ‡Values are given as the geometric mean (range). §Values are given as the median (range).

to TLC, the patients performed a forced expiratory maneuver starting at 60% of baseline FVC (partial flow-volume curve), marked off from TLC, directly followed by a forced expiratory maneuver starting from TLC (maximal flow-volume curve). Expiratory flow was measured at 40% of the baseline FVC (marked off from TLC) on both the maximal flow-volume curve ($V'_{40}M$) and partial flow-volume curve ($V'_{40}P$). The effect of a deep inspiration on airways obstruction was expressed as the ratio between $V'_{40}M$ and $V'_{40}P$ (M/P ratio)[13]. An M/P ratio of > 1 indicates

bronchodilation following deep inspiration, as the flow on the curve after inspiration to TLC is higher than the flow on the curve after a partial inspiration, whereas an M/P ratio of < 1 indicates bronchoconstriction. The values of $V'_{40}M$ and $V'_{40}P$ at a 40% fall in $V'_{40}P$ were obtained by linear interpolation between the values before and after a 40% fall in $V'_{40}P$, and the M/P ratio was calculated from these interpolated values. MPslope was determined as slope of the linear regression line of all values of flow at 40% of FVC on the flow-volume curve after partial (60% of FVC) inspiration against all values of flow at 40% of FVC on the flow-volume curve after maximal inspiration during the challenge test[3].

Airway hyperresponsiveness

Methacholine challenge was performed by a standardized methodology[20], using methacholine bromide in a normal saline solution. Serial double concentrations of methacholine (0.15 to 40 µmol/L) were aerosolized (DeVilbiss; Somerset, PA) and were inhaled by tidal breathing for 2 min at 5-min intervals with the nose clipped until FEV_1 dropped > 20% from baseline. The response was expressed as the PC_{20} and as the provocative concentration of methacholine causing a 40% fall in flow of the partial flow-volume curve at 40% of FVC ($PC_{40}V'_{40}P$).

Exhaled NO measurements

Exhaled NO was measured online with an expiratory flow rate of 50 mL/s according to American Thoracic Society guidelines and European Respiratory Society[24], and was analyzed with a chemiluminescence analyzer (NIOX; Aerocrine AB; Solna, Sweden). NO concentrations were determined at a 3-s plateau and were expressed as parts per billion (ppb). During the measurements the subject inspired "NO-free" air (*ie*, NO level, < 2 ppb). Three successive recordings were made, and the mean value was used in the analysis.

Statistical analysis

The sample size of 12 patients per group was based on previous data from our laboratory with regard to partial and maximal flow-volume curves, allowing the detection of a change in the M/P ratio of 0.2 within and between groups, if $\alpha = 0.05$ and $1 - \beta = 0.80$[25]. M/P ratios and PC_{20} values were log-transformed before analysis. Within-group and between-group differences were analyzed using Student paired and unpaired *t* tests. NO values were tested by the Wilcoxon signed rank test and the Mann-Whitney *U* test. Pearson correlation was used to examine the relationship between the changes in M/P ratio and PC_{20} and $PC_{40}V'_{40}P$. Spearman rank correlation was used to examine the correlation between the changes in exhaled NO and M/P ratio, PC_{20}, and $PC_{40}V'_{40}P$. A p value of < 0.05 was considered to be statistically significant.

Results

All patients completed the study. There were no baseline differences between the two treatment groups with respect to age, sex, medication use, FEV_1, exhaled NO levels, and M/P ratio (**Table 1**). However, there was a significant difference in the $PC_{40}V'_{40}P$ for methacholine at baseline between the two groups ($p = 0.016$), and a trend toward a difference in the PC_{20} for methacholine ($p = 0.057$). Furthermore, there was no significant difference in the Asthma Control Questionnaire score between the groups, which ranged from 0.3 to 2. According to the Global Initiative for Asthma classification, there were three patients with mild persistent asthma, six patients with moderate persistent asthma, and three patients with severe persistent asthma in the prednisone group, and there were five patients with mild persistent asthma, four patients with moderate persistent asthma, and three patients with severe persistent asthma in the placebo group.

The M/P ratio at a 40% fall in $V'_{40}P$ improved significantly in the prednisone group (geometric mean at baseline, 1.31; range, 1.0 to 1.7) after treatment (geometric mean, 1.49; range, 1.1 to 2.3; $p = 0.006$). No significant change was observed in the placebo group (geometric mean at baseline, 1.45; range, 1.1 to 1.9) after treatment (geometric mean, 1.41; range, 1.1 to 1.9; $p = 0.46$). Moreover, the change in M/P ratio at a 40% fall in $V'_{40}P$ in the prednisone group was significantly different from the change in M/P ratio in the placebo group ($p = 0.006$) (**Figure 1 , Table 2**).

There were no significant differences in FEV_1 (in liters or percent predicted) or FVC (in liters) within or between the groups after 2 weeks of treatment. The PC_{20} increased significantly in the prednisone group compared to baseline (mean change in doubling dose, 1.02; SD, 0.97; $p = 0.004$) (**Figure 2** , ***left***, ***A***), whereas no significant changes were found in the placebo group. Furthermore, the $PC_{40}V'_{40}P$ increased in the prednisone group (mean change in doubling dose, 0.84; SD, 1.21), but this did not reach significance ($p = 0.07$) (**Figure 2**, ***right***, ***B***). Moreover, the changes in hyperresponsiveness, both the PC_{20} and $PC_{40}V'_{40}P$, were significantly different between the treatment groups (**Table 2**).

Table 2. Changes in airway hyperresponsiveness, deep inspiration-induced bronchodilation, and exhaled NO values between the prednisone-treated group and the placebo-treated group

	Prednisone	Placebo	p value
ΔPC_{20},†	1.02 (0.97)	−0.44 (0.83)	0.001
$\Delta PC_{40}V'_{40}P$,†	0.84 (1.21)	−0.29 (1.21)	0.05
ΔM/P ratio			
At $PC_{40}V'_{40}P$‡	1.14 (0.95–1.45)	0.97 (0.74–1.17)	0.006
At PC_{20}‡	1.06 (0.85–1.58)	0.99 (0.74–1.12)	0.31
ΔExhaled NO, ppb	−14.0 (33.4)	9.7 (11.7)	0.03

Values are given as the mean change (SD), unless otherwise indicated. †Values are given as the mean change (SD) in doubling dose. ‡Values are given as the fold change (range).

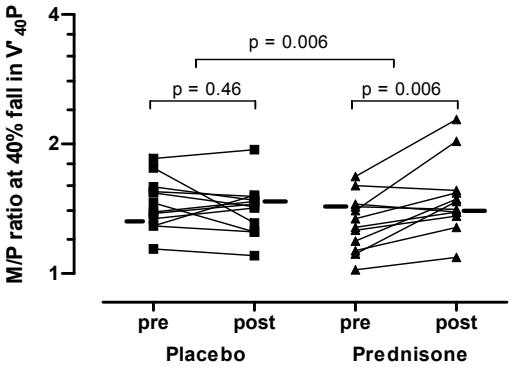

Figure 1. Individual values of M/P ratio measured at a 40% fall in $V'_{40}P$ to methacholine before (pre) and after (post) 2 weeks of treatment in the placebo-treated and prednisone-treated group. The data are expressed as the ratio between the flow on the maximal and partial flow-volume curves at 40% of FVC. The geometric mean is depicted as a horizontal bar. At baseline, there was no difference in the M/P ratio between the treatment groups. In the prednisone group, the M/P ratio improved significantly ($p = 0.006$). Furthermore, the change in M/P ratio was significantly different between the prednisone-treated and the placebo-treated groups ($p = 0.006$).

Two weeks of treatment did not significantly change the level of exhaled NO either within the prednisone group (mean change, −14.0 ppb; SD, 33.4 ppb) or within the placebo-treated group (mean change, 9.7 ppb; SD, 12.8 ppb) (**Table 2**). However, the changes in exhaled NO were significantly different between the two treatment groups ($p = 0.03$, **Figure 3**). Notably, within the prednisone group there were no significant correlations between the changes in M/P ratio at 40% fall in $V'_{40}P$, and those in $PC_{40}V'_{40}P$ and exhaled NO ($p > 0.15$) **(Figure 4**).

Discussion

The results of this study demonstrate that a course of high-dose oral prednisone therapy improves the degree of deep inspiration-induced bronchodilation at a given level of airways obstruction in stable patients with asthma who are receiving regular treatment with inhaled corticosteroids. It appears that this improvement is not related to concurrent reductions in airway hyperresponsiveness or to changes in the level of exhaled NO. These findings indicate that the degree of bronchodilation following a deep breath in clinically stable asthmatic patients is still impaired, presumably based on the level of residual airway inflammation while receiving regular treatment with inhaled corticosteroids. Our data suggest that the optimizing of deep inspiration-induced bronchodilation by the use of systemic steroids occurs partially independent of improvements in hyperresponsiveness and the level of exhaled NO.

To our knowledge, the improvement in M/P ratio in asthma patients by the use of systemic steroid treatment on top of maintenance therapy with inhaled steroids is a novel finding. The present results extend the previous findings by Corsico et al[12] and Bel et al[13], who observed an

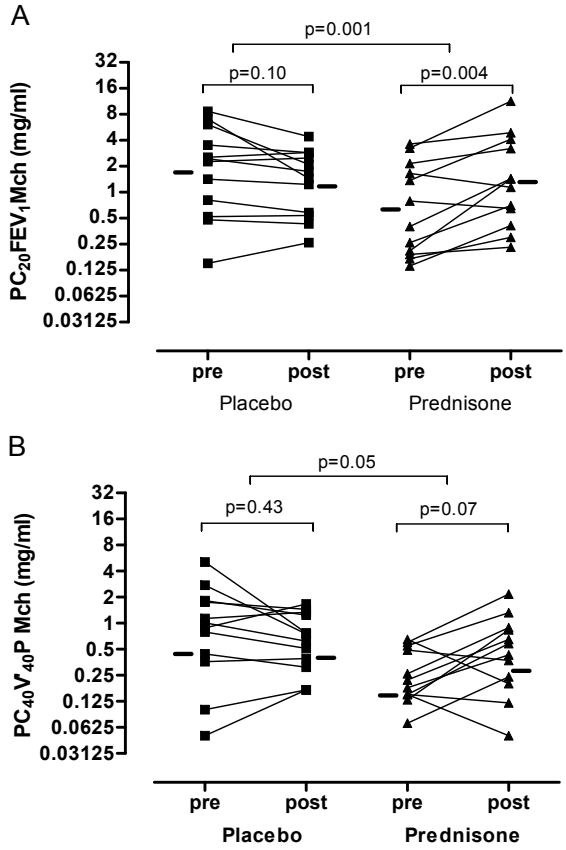

Figure 2. Individual values of PC_{20} (left, A) and $PC_{40}V'_{40}P$ (right, B) for methacholine before and after 2 weeks of treatment in the placebo-treated and prednisone-treated groups. The data are expressed in milligrams per milliliter, and the geometric mean is depicted as a horizontal bar. The PC_{20} significantly increased in the prednisone group ($p = 0.004$), and the changes in PC_{20} were significantly different between the groups ($p = 0.001$). The $PC_{40}V'_{40}P$ increased in the prednisone group after 2 weeks of treatment, which was not significant ($p = 0.07$). However, the change in $PC_{40}V'_{40}P$ after treatment was significantly different between the groups ($p = 0.05$). See the legend of Fig 1 for abbreviations not used in the text.

increase in deep inspiration-induced bronchodilation after 4 weeks of treatment with inhaled corticosteroids in steroid-naïve asthmatic patients. However, recently Scichilone et al[14] found no effect of inhaled corticosteroids on the bronchodilatory effect of a deep inspiration in a study with asthmatic patients with mild-to-severe airway hyperresponsiveness. The methods of assessing the airway responses to deep inspiration differed among these three studies, as well as the dose and type of inhaled steroids used. In a *post hoc* analysis, we have also calculated the slope of the linear regression line of all values of flow at 40% of FVC on the flow-volume curve after partial (60% of FVC) inspiration against all values of flow at 40% of FVC on the flow-volume curve after maximal inspiration during the challenge test (MPslope), as introduced

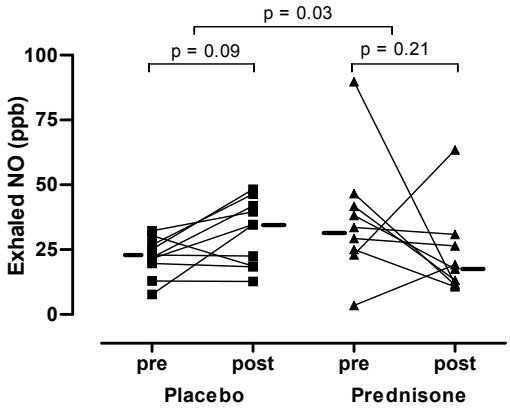

Figure 3. Individual values of the changes in the concentration of exhaled NO before and after 2 weeks of treatment in the placebo and prednisone groups. The data are expressed as parts per billion. The change in the concentration of exhaled NO was significantly different between the two groups (p = 0.03). See the legend of Fig 1 for abbreviations not used in the text.

by Pellegrino et al[3], but found no significant differences within or between the groups after 2 weeks of treatment. This discrepancy might be explained by the fact that M/P ratio and MPslope represent different features, representing the level and relative change in deep breath responses, respectively.

Apparently, there is still significant room for improvement in the response of asthmatic airways to a deep breath by adding systemic steroids to their regular controller medication therapy. This is also discernable from the changes in our secondary outcome parameters, which are in line with previous observations. The decrease in hyperresponsiveness achieved by prednisone therapy confirms the findings by Meijer et al[26] in a group of asthmatic patients two thirds of whom were receiving therapy with inhaled steroids. In their study, this was accompanied by reductions in both the sputum and serum levels of eosinophils. Similarly, the reduction in exhaled NO by adding prednisone to maintenance therapy with inhaled steroids extends the findings by Payne et al[27] in children with difficult asthma. Therefore, it appears that the deep inspiration-induced bronchodilation follows other functional characteristics in asthmatic patients in not being optimized by the currently recommended regular therapy.

In this study, we recruited patients with mild-to-moderate persistent asthma using inhaled corticosteroids as maintenance treatment[18]. These patients are representative of a large group of patients who can reach adequate clinical control by using the currently recommended controller medication. The patients were selected based on the use of inhaled corticosteroids as part of their regular asthma treatment, and based on the fact that no change in clinical condition or medication use had occurred during the previous 6 weeks. As a result, the maintenance treatment in some patients included in the study may not have been optimal. However, at baseline there was no significant difference between the two groups in terms of Asthma Control Questionnaire score. Furthermore, there was a significant difference in airway

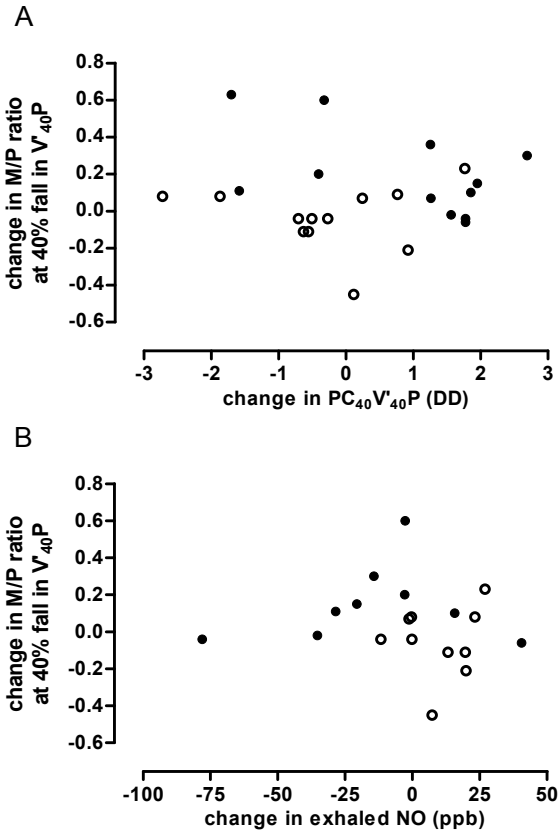

Figure 4. Relationship between the change in M/P ratio at a 40% fall in $V'_{40}P$ and (***A***) the change in $PC_{40}V'_{40}P$ (prednisone group, $r = -0.44$; p = 0.15; placebo group, $r = -0.04$; p = 0.91) and (***B***) the change in exhaled NO (prednisone group, $r = 0.13$; p = 0.73; placebo group, $r = 0.07$; p = 0.86). The absence of a relationship between the change in the bronchodilatory effect of a deep inspiration and airway hyperresponsiveness or exhaled NO suggests that these changes occur independently. ○ = placebo group; ● = prednisone group.

hyperresponsiveness between the two treatment groups at baseline. However, the baseline PC_{20} and $PC_{40}V'_{40}P$ were not related to the change in the M/P ratio, indicating that the change in deep inspiration-induced bronchodilation was not influenced by the difference in baseline airway hyperresponsiveness.

We chose to use a course of high-dose oral prednisone in addition to the regular treatment with inhaled steroids in order to reduce any residual inflammation in the airways[15,16]. The systemic administration was meant to provide access through the circulation to the peripheral airways and the peribronchial area, which may not be reached optimally by inhaling corticosteroids[28]. The dose and duration of prednisone therapy was based on the recommended dose regimen for asthma exacerbations[18]. Therefore, we believe that the current prednisone therapy increased the bioavailability of steroids throughout the bronchial tree in these asthmatic

patients, who were already being treated with conventional doses of inhaled steroids. In order to avoid carryover effects due to a variable washout period of prednisone, the study was performed with a parallel design rather than a crossover design to ensure that all measurements were performed under comparable clinical conditions.

The M/P ratio at a 40% fall in $V'_{40}P$ changed significantly after 2 weeks of treatment with prednisone, but not the M/P ratio at a 20% fall in FEV_1. The difference between these two parameters is that the latter was based on a measurement of airway responsiveness that implicitly includes a deep breath. Inducing a fall in FEV_1 implies that the deep inspiration itself can no longer prevent the airways obstruction. Apparently, prednisone only increased deep inspiration-induced bronchodilation for a measure that was not directly preceded by a deep inspiration.

How can we interpret these results? Inflammation is thought to play a role in deep inspiration-induced bronchodilation by uncoupling the airways and the parenchyma as a result of airway wall thickening and/or peribronchial edema[7,29]. Tidal breathing, and occasional deep inspirations, provide load fluctuations on airway smooth muscle that are necessary to keep the airway smooth muscle in a flexible and less contractile state[30,31]. Glucocorticosteroids exert anti-inflammatory effects, resulting in decreased inflammatory cell counts in sputum[32], BAL fluid[33], and bronchial biopsy specimens[34], and the inhibition of cytokine expression and production[33.34]. Furthermore, steroids can decrease bronchial blood flow, particularly under inflammatory conditions[35.36], and they inhibit vascular permeability and edema in the capillaries and postcapillary venules[36.37]. Therefore, the improvement in deep inspiration-induced bronchodilation observed in the present study could be a result of the effects of glucocorticosteroids on peribronchial inflammation and edema, thereby reducing airway wall thickness and restoring airway-parenchyma interdependence.

In the presence of inflammation, changes in the airway smooth muscle function itself may also play a role in the reduced airway response to deep inspiration in asthma. First, adjustment of the contractile apparatus of the airway smooth muscle cell to length changes (plasticity) enables the cell to optimize its contractility to the mechanical conditions under which it is activated[38,39,40]. Second, an increase in shortening velocity enables the airway smooth cell to reshorten much faster after being stretched[41]. *In vitro* studies[42] have shown direct effects of corticosteroids on airway smooth muscle contractility. Airway smooth muscle cells exposed to tumor necrosis factor-α and interleukin-1β show an increased asthma-like contractility to a constrictor agent, which can be prevented by pretreatment with dexamethasone[43]. Improvements in deep inspiration-induced bronchodilation could therefore also be a result of changes in airway smooth muscle function by corticosteroid treatment, reducing the force generation and recontraction after being stretched. However, since the change in deep inspiration-induced bronchodilation was not related to the change in airway responsiveness (*ie*, $PC_{40}V'_{40}P$ or PC_{20}), one could speculate that prednisone therapy predominantly influenced the ability to stretch

the airways or smooth muscle rather than restoring the effect of stretch on the contractility of the airway smooth muscle cell *per se*.

What are the clinical implications of this study? Patients with asthma, who are regularly treated with inhaled corticosteroids, can have residual airway inflammation[15,16] that impairs the mechanical properties of the airways even during clinically stable episodes. This may have implications during exacerbations when physiologic protective mechanisms, such as deep inspiration-induced bronchodilation, become of vital importance[11]. Patients with impaired airway responses to deep inspiration, although being clinically stable, may therefore be more at risk for the development of exacerbations. As airway responses to deep inspiration tend to be related to asthma severity[44] and the severity of breathlessness[22], long-term studies are required in order to address the prognostic implications of impaired responses of the airways to deep inspiration in asthma patients.

We conclude that adding systemic anti-inflammatory treatment to regular therapy with inhaled corticosteroids improves the dilation of preconstricted airways by deep inspiration in patients with mild-to-moderate persistent asthma. Since it is obvious that prednisone cannot be recommended as maintenance therapy in clinically stable asthma patients, other interventions specifically targeting the mechanisms of impaired deep-breath responses in asthma patients need to be explored.

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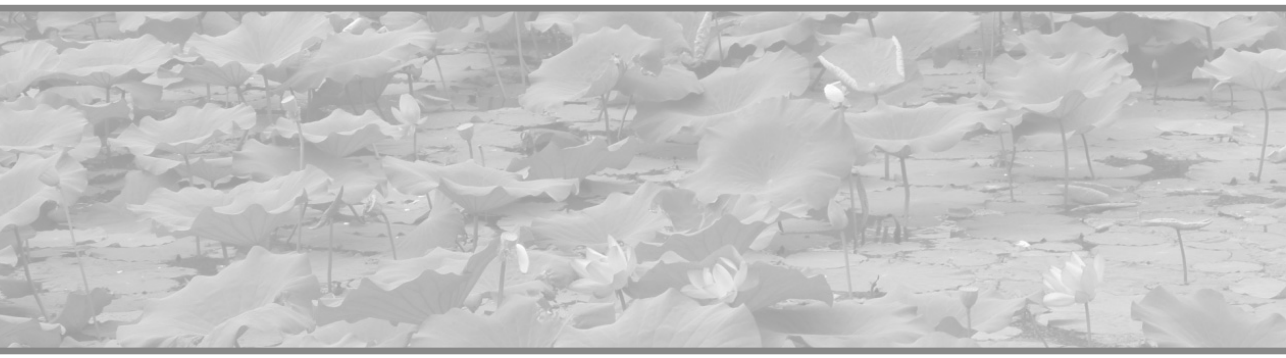

Chapter 6

Treatment with tiotropium improves airways obstruction, but not airway hyperresponsiveness, in asthma

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Submitted for publication

Abstract

Rationale: In asthma anticholinergics exhibit beneficial effects in terms of daytime dyspnea and daily peak flow measurements with relatively low side effects. Anticholinergic drugs provide acute bronchodilation, but have also shown to inhibit remodeling in models of allergic inflammation, including airway smooth muscle proliferation. Therefore, we hypothesized that 3 weeks daily usage of tiotropium would improve lung function and airway hyperresponsiveness in mild asthma as compared to placebo treatment.

Methods: 24 patients with mild asthma (atopic, FEV_1 >70 %pred, PC_{20} histamine <8 mg/ml) participated in a double blind placebo controlled parallel study. Spirometry (FEV_1, FVC) and respiratory resistance were measured before and 180 min following inhalation of 18 µg tiotroprium. Then airway responsiveness to histamine was assessed. These measurements were performed before and 21 days after treatment with tiotropium 18 µg once daily or placebo.

Results: A single dose of tiotropium significantly improved FEV_1 %predicted (median change [range] in all patients at visit 1: ΔFEV_1 % predicted +7.4 [−10 – 15], $p < 0.001$), reduced respiratory resistance (ΔRrs at 8 Hz ($cmH_2O/L/s$): −0.7 [−1.9 – 1.1], $p < 0.001$) and airway responsiveness to histamine (ΔPC_{20} mg/ml: +1.2 [−1.9 – 11.2], $p < 0.001$]. After 21 days of daily usage of tiotropium FEV_1 improved non-significantly (median change [range]: ΔFEV_1 % predicted: +3.7 [−9 – 11], p=0.13), but a significant increase was found in FEV_1/FVC ratio as compared to placebo (ΔFEV_1/FVC: +0.04 [−0.1 – 0.1], p=0.04). Airway responsiveness to histamine normalized to screening levels in both groups (p>0.5).

Conclusions: Sustained blockage of muscarinic receptors by tiotropium improves airways obstruction. Our findings also suggest that pre-treatment with tiotropium to histamine challenge temporarily alters airway responsiveness to histamine regardless of maintenance therapy.

Introduction

Asthma is characterized by episodic symptoms, variable airways obstruction, and airway inflammation[1]. Beta-agonists are used as reliever therapy (short-acting) or as maintenance therapy in combination with inhaled corticosteroids. Notably, a systematic review showed a beneficial effect of anticholinergics as compared to placebo in terms of daytime dyspnea, daily peak flow measurements with relatively low side effects. However, the addition of anticholinergics to regular inhaled bronchodilators (i.e. beta-agonists) has not been shown to confer significant benefits[2]. Since beta-agonists have proven to be successful in most patients with asthma, anticholinergics have not been broadly used as reliever medication or long-acting bronchodilators.

Recent evidence suggesting that usage of long-acting beta-agonists without concomitant inhaled corticosteroids increases the risk of asthma-mortality has resulted in an official recommendation by the Food & Drug Administration (FDA) not to prescribe long-acting beta agonists without the combination of inhaled corticosteroids and to discontinue long-acting beta-agonists when possible[3,4]. This development underlines the need for studies on the short- and long-term effects of other long-acting bronchodilators, such as tiotropium, on lung function in asthma.

Anticholinergic drugs provide acute bronchodilation by blocking the muscarinic receptors on airway smooth muscle cells[5]. Tiotropium is a long-acting selective antagonist of M1 and M3 receptors involved in bronchoconstriction, whereas M2 receptors whose stimulation provides feedback inhibition of further acetylcholine release is not antagonized[6].

Interestingly, anticholinergics have also been shown to protect against remodeling in models of allergic inflammation. In an ovalbumin guinea pig asthma model 12 weeks of inhaled tiotropium considerably inhibited the allergen-induced increase in airway smooth muscle mass of non-cartilaginous airways, as well as myosin expression and smooth muscle contractability[7]. An additional study showed that tiotropium prevented an increase in smooth muscle cell thickening, contractile protein expression, tracheal hypercontractility and mucous gland hyperthrophy[8]. This suggests that tiotropium modulates lung function in ways other than direct bronchodilation only. Therefore, we hypothesized that in allergic asthma daily use of tiotropium reduces smooth muscle contractility, thereby improving lung function and airway hyperresponsiveness.

A major feature of normal airway function is the ability to dilate constricted airways by a deep inspiration. In asthma such bronchodilatory effect by deep inspirations is impaired as compared to healthy subjects[9]. Altered response of airway smooth muscle to inspiration-induced stretch of the airways has been hypothesized as pathophysiological mechanism for this impairment. Our second hypothesis was that inhibition of smooth muscle proliferation and contractility by daily use of tiotropium improves deep inspiration-induced bronchodilation as well.

The aim of this study was to examine the effect of 3 weeks of 18µg tiotropium-bromide (tiotropium) once daily on lung function (spirometry (FEV_1, FVC) and respiratory resistance), airway hyperresponsiveness (maximal dose-response curve), and deep inspiration-induced bronchodilation in patients with mild asthma.

Methods

Subjects

24 patients with mild persistent asthma (GINA step 2)[1] were recruited for this study. Inclusion criteria were: a history of episodic chest tightness or wheezing, baseline forced expiratory volume in 1 s (FEV_1) more than 70% of predicted[10], provocative concentration of histamine causing a 20% fall in FEV_1 (PC_{20} histamine) less than 8 mg/ml[11], atopic (determined by a positive skin prick test (≥ 3mm wheal) to one or more of 10 common aeroallergen extracts (ALK, Abelló, Nieuwegein, the Netherlands)), clinically stable, non-smokers or ex-smokers with less than 5 pack years, and no recent (≤ 2 weeks) upper respiratory tract infection or other relevant diseases. None of the patients had used inhaled corticosteroids within 4 weeks or oral corticosteroids within 3 months prior to or during the study. The protocol was approved by the institutional review board for human studies, and before entering the study the patients gave their written informed consent.

Study design

This study was a placebo-controlled, double-blind, parallel study (**Figure 1A**). After the baseline measurements all patients were randomly assigned to receive either 18 µg tiotropium or placebo once daily for 21 days. The primary end-point was the maximal response to histamine and the change in respiratory resistance by tiotropium. The secondary end-points were FEV_1, FVC, FEV_1/FVC ratio, and the provocative concentration the provocative concentration causing a 20% fall in FEV_1 ($PC_{20}FEV_1$).

Measurements were performed at baseline (visit 1, day 0) and after 21 days of treatment with either tiotropium (Handihaler) 18 µg o.d. or placebo (visit 2). All tests were performed after adequately stopping bronchodilator therapy (short-acting β_2-agonists for > 8 h and long-acting β_2-agonists or tiotropium> 24 tiotropium). Figure 1b shows the order of measurements at both visits. First, respiratory resistance (Rrs), reactance (Xrs) as measured by forced oscillation technique, and spirometry (FEV_1 and FVC were assessed before and 180 minutes after inhalation of 18 µg tiotropium. Subsequently, airway hyperresponsiveness to histamine was determined by a maximal dose response curve to histamine with maximal and partial flow-volume curves measured as described below. After the last dose of histamine Rrs and Xrs were measured during 1 minute of tidal breathing followed by a slow deep inspiration to total lung capacity (TLC) and another minute of tidal breathing (**Figure 1B**).

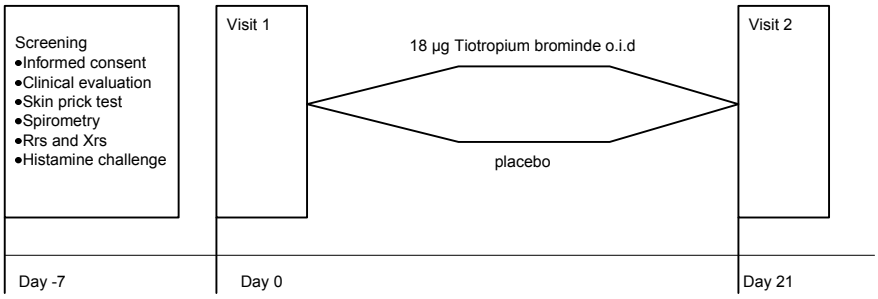

Figure 1A. Study design.
During a screenings visit in- and exclusion criteria were checked using a clinical evaluation, skin prick test, and histamine challenge. Visit 1 followed within one week of the screenings visit. Following visit 1 patients were randomized to either tiotropium 18 mcg once daily or placebo, and were re-evaluated at day 21 (visit 2).

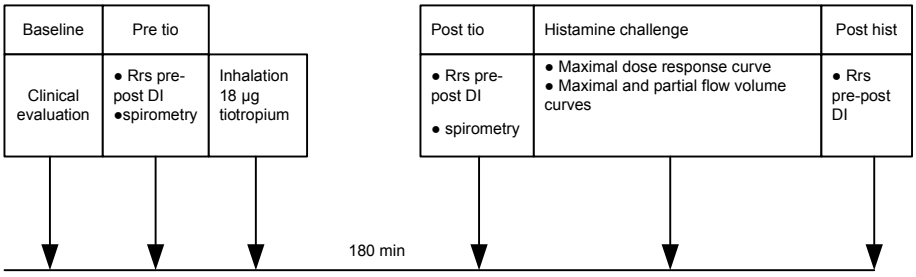

Figure 1B. Visit design.
The measurements were identical at visit 1 and 2, and performed in both groups. At baseline a clinical evaluation was performed to check for detoriation of asthma symptoms and/or acute infections. Following baseline FOT measurements and spirometry patients inhaled 18mcg of tiotropium. 180 Minutes later spirometry and FOT measurements were repeated. Then a maximal dose response curve to histamine was performed until FEV_1 dropped by more than 40% or the maximal dose was reached. M/P ratio was measured after each dose. Following the last dose of histamine another FOT measurement was performed to measure the change in Rrs and Xrs by deep inspiration.

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Maximal dose response curve to histamine

A histamine challenge with partial and maximal flow volume curves was performed by standardized methodology[12,13], using histamine in normal saline. Before the challenge test, FVC was measured three times by normal spirometry. The mean of the two highest technically satisfied values (within 5%) was considered as baseline FVC (Morgan spiroflow). Subsequently, after inhalation of saline, three partial and maximal flow volume curves were performed to measure baseline FEV_1, partial expiratory flow ($V'_{40}P$), and maximal expiratory flow ($V'_{40}M$). The highest value (% FEV_1 predicted within 5%) was used as baseline value. At 5 minutes intervals aerosolized serial doubling doses of histamine (0.03-32 mg/ml) were inhaled by tidal breathing (DeVilbiss, Somerset, PA) for 2 minutes with the nose clipped. The challenge was stopped when FEV_1 dropped > 40% from baseline or when the last dose was reached (32 mg/ml). At 90 seconds after each dose, the response was measured by partial and maximal expiratory flow-volume curves that were standardized for volume history effects. First, a maximal inhalation to total lung capacity (TLC) was carried out, followed by tidal breathing for 45 seconds. Then a partial flow-volume curve started at 60% of baseline FVC, marked off from TLC, directly followed by a maximal flow-volume curve. Expiratory flow was measured at 40% of baseline FVC (marked of from TLC) on both the maximal ($V'_{40}M$) and partial ($V'_{40}P$) flow volume curve. The response to histamine was expressed as the provocative concentration causing a 20% fall in FEV_1 ($PC_{20}FEV_1$), a 40% fall in $V'_{40}P$ ($PC_{40}V'_{40}P$), and as the maximal dose response (cumulative dose/% fall in FEV_1).

Forced Oscillation Technique (FOT)

Respiratory resistance (Rrs) and reactance (Xrs) were measured continuously using the Forced Oscillation Technique (FOT)[14] with an applied oscillation frequency of 8, 12 and 16 Hz with an amplitude of ± 1 cmH_2O(Woolcock institute, Australia). Flow was measured using a 50-mm diameter Fleisch Pneumotachograph (Vitalograph Ltd, Maids Moreton, UK), and differential pressure was measured using a ± 2.5 cm H_2O solid-state transducer (Sursense DCAL4; Honeywell Sensing and Control, Milpitas, CA, USA). Mouth pressure was measured using a similar transducer with a higher range (±12.5 cmH_2O). Analogue pressure and flow signals were digitized at 400 Hz. The time- and frequency-dependent respiratory impedance (*Z*rs) was estimated based on the hypothesis that random errors occur in both pressure and flow. This yields a Total Least Squares (TLS) estimate of respiratory impedance as a function of time and frequency and allows an estimation of confidence intervals in the course of time[9].

Rrs and Xrs were measured prior to spirometry measurements before and 180 minutes after tiotropium inhalation, and following the last dose of histamine during the challenge. The measurement contained 30 seconds of tidal breathing, followed by a slow DI to total lung capacity (TLC) and a passive exhalation back to tidal breathing for another 30 seconds.

Analysis

Lung function and airway hyperresponsiveness

The sample size of 12 patients per group was sufficient to detect an improvement of 5% in FEV_1 % predicted within the tiotropium treatment group, if α=0.05 and 1-β=0.80. The data on airway hyperresponsivness were logtransformed. Within-group and between-group differences were analyzed with 2-tailed paired t-tests and 2 tailed unpaired t-tests respectively or with non-parametric equivalents.

Effect of deep inspiration

Mean Rrs and Xrs were calculated from all datapoints during 3 tidal expirations[15]. The effect of deep inspiration on the airways was calculated as the change in Rrs and Xrs by the deep inspiration[15]. The effect of deep inspiration was also calculated from maximal and partial flow volume curves, as the ratio of $V'_{40}M$ and $V'_{40}P$ (M/P ratio) at 40% of FVC at different levels of airways obstruction[12,16,17]. An M/P ratio > 1 indicates bronchodilation.

Results

Patient characteristics

Tiotropium administration for 21 days was well tolerated and all patients completed the study. The treatment groups were not significantly different with regard to age, lung function (FVC % predicted, FEV_1 % predicted and FEV_1/FVC ratio) or hyperresponsiveness. The patient's characteristics are given in **Table 1**.

Table 1. Patient characteristics

	Tiotropium	Placebo
Age (years)	23.2 ± 3.2	23.4 ± 2.8
Body mass index	23.0 ± 3.8	24.8 ± 6.8
FVC (% predicted)	103.8 ± 15.8	102.8 ± 10.2
FEV_1 (% predicted)	93.3 16.8	92.3 10.8
FEV_1/FVC	0.78 ± 0.11	0.78 ± 0.07
PC_{20} (mg/ml)	0.84 ± 1.5	1.64 ± 1.6

Data are expressed as mean ± standard deviation, except PC_{20} is expressed as geometric mean ± standard deviation in doubling doses. Groups were not significantly different with regard to these baseline characteristics (p values > 0.14).

Effect of a single dose of tiotropium

Spirometry. FEV_1 % predicted was significantly increased by a single dose of tiotropium in both groups at visit 1 (**Figure 3**, median change [range]: tiotropium +7.7 [−10 – 13]; placebo +7.7 [3.0 – 15], ($p < 0.05$) and at visit 2 (tiotropium +1.3 [−17 – 6.0]; placebo +7.1 [0.0 – 14], $p < 0.05$). Likewise, FEV_1/FVC ratio was significantly improved by tiotropium at both visits (tiotropium

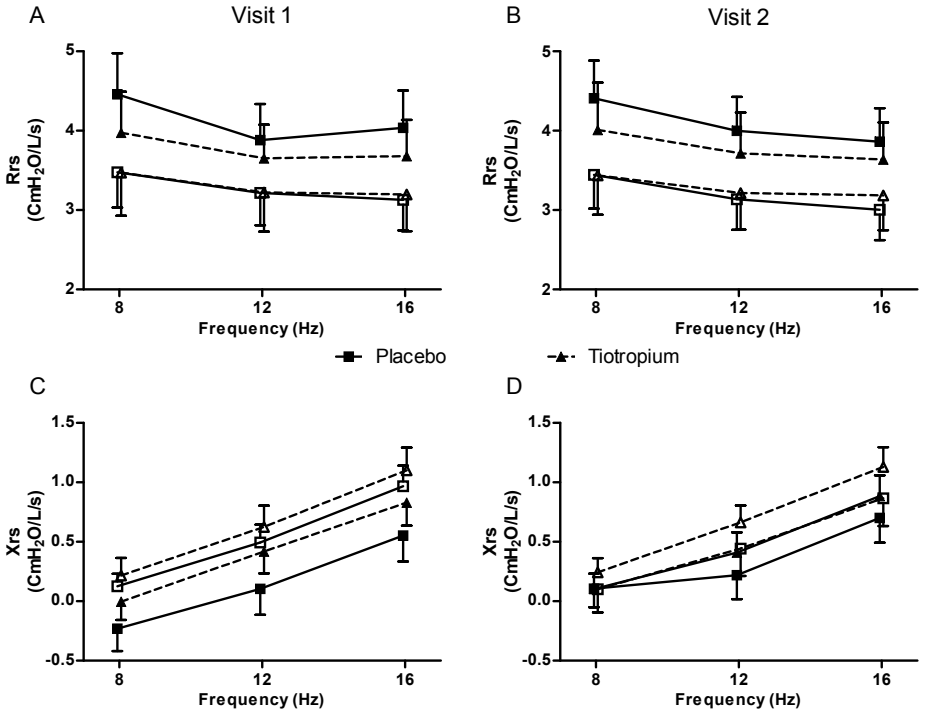

Figure 2. Rrs and Xrs values at visit 1 and visit 2.
These figures show the mean for Rrs and Xrs at 8, 12 and 16 Hz, before (closed symbols) and 180 minutes after (open symbols) tiotropium inhalation. Figure **2A** and **2C** show the results from visit 1 (before treatment) and figure **2B** and **2D** show the data of visit 2 (after treatment for 21 days) for the tiotropium treatment group (triangles) and the placebo group (squares). Both at visit 1 and at visit 2 Rrs was significantly reduced after inhalation of tiotropium in both groups at all 3 frequencies ($p< 0.05$). Xrs was significantly increased at all 3 frequencies at visit 1 ($p < 0.05$). At visit 2 (21 days of treatment) Xrs was only significantly increased in the placebo treated group ($p < 0.05$).

visit 1: +0.07 [−0.03 – 0.10], visit 2: +0.02 [−0.04 – 0.06]; placebo visit 1: +0.06 [0.03 – 0.1], visit 2: +0.06 [0.02 – 0.1], $p < 0.03$). At visit 2, but not visit 1, the improvement in FEV_1 % predicted and FEV_1/FVC was significantly larger in the placebo group as compared to tiotropium ($p < 0.01$).

*FOT***. Table 2** shows median Rrs and Xrs values before (pre) and 180 minutes after (post) inhalation of a single dose of tiotropium at both visits. Both at visit 1 and 2 Rrs was significantly reduced by a single dose of 18 µg tiotropium in both groups at all 3 frequencies (**Figure 2A and 2B**, within groups $p< 0.05$). In addition, a single dose of tiotropium significantly increased Xrs values at all 3 frequencies at visit 1, but not visit 2, in both groups (**Figure 2C**, $p < 0.05$). The improvement in Rrs and Xrs by a single dose of 18mcg tiotropium was not significantly different between the maintenance treatment groups both at visit 1 and visit 2 at all 3 frequencies ($p > 0.1$ and $p > 0.3$ respectively).

Tabel 2. Rrs and Xrs values before and after tiotropium

Median [range]		Visit 1 (before treatment)				Visit 2 (after treatment)			
		tiotropium		placebo		tiotropium		placebo	
		pre	post	pre	post	pre	post	pre	post
Rrs ($cmH_2O/L/s$)	8Hz	3.6 [2.2–8.9]	3.2* [1.9–8.9]	4.2 [2.2–8.0]	3.1* [2.0–7.3]	3.7 [2.0–9.4]	2.8* [1.8–7.7]	3.9 [2.7–7.2]	2.8* [1.8–6.6]
	12Hz	3.4 [2.1–7.9]	3.0* [1.8–8.1]	4.0 [2.0–7.3]	2.9* [1.8–6.6]	3.5 [1.8–8.2]	2.6* [1.7–7.1]	3.6 [2.5–6.5]	2.7* [1.6–6.1]
	16Hz	3.3 [2.1–7.5]	3.0* [1.7–7.8]	4.0 [1.8–6.9]	2.9* [1.6–6.2]	3.5 [1.8–7.5]	2.6* [1.6–7.0]	3.6 [1.9–6.2]	2.7* [1.6–5.9]
Xrs ($cmH_2O/L/s$)	8Hz	0.2 [−1.5–0.4]	0.4* [−1.3–0.6]	0.0 [−1.9–0.5]	0.2* [−0.6–0.7]	0.3 [−2.0–0.5]	0.4 [−0.7–0.6]	0.1 [−1.4–0.5]	0.1 [−1.0–0.5]
	12Hz	0.5 [−1.4–0.9]	0.8* [−1.1–1.2]	0.3 [−1.7–1.0]	0.6* [−0.5–1.3]	0.6 [−1.9–1.0]	0.9 [−0.4–1.1]	0.5 [−1.4–1.1]	0.5 [−0.9–1.2]
	16Hz	0.9 [−1.0–1.6]	1.3* [−0.6–1.8]	0.8 [−1.1–1.6]	1.0* [−0.1–1.9]	1.0 [−1.4–1.7]	1.3 [−0.2–1.8]	1.0 [−0.9–1.7]	1.0 [−0.3–1.8]

Rrs and Xrs values before (pre) and 180 minutes after (post) tiotropium inhalation at visit 1 and visit 2 (after 21 days of treatment). Data are expressed as median [range], * indicates $p < 0.05$ comparing pre and post values within the groups. Rrs and Xrs were not significantly changed by 21 days of treatment in both groups, nor was the change significantly different between the groups.

Effect of maintenance treatment with tiotropium

Spirometry. Treatment with 21 days tiotropium once daily increased baseline FEV_1 % predicted, but this increase was not significant (p = 0.13, **Figure 3A**). Also, the change in FEV_1 % predicted after 21 days of treatment was not significantly different between the groups. However, FEV_1/FVC ratio was significantly improved by 21 days of daily treatment with tiotropium (**Figure 3B**, p = 0.04), and the change in FEV_1/FVC ratio was significantly different between the groups (p = 0.002).

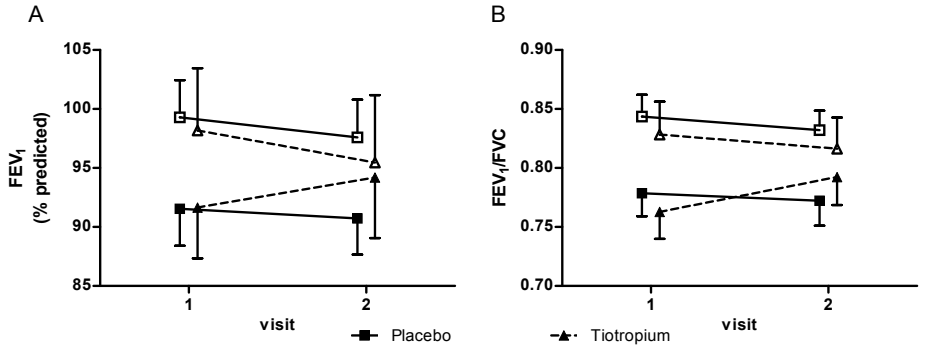

Figure 3. FEV_1 % predicted and FEV_1/FVC ratio values.
These figures show the mean ± SEM for FEV_1 % predicted and FEV_1/FVC ratio before (closed symbols) and 180 minutes after (open symbols) tiotropium inhalation at visit 1 (before treatment) and visit 2 (after treatment for 21 days) for the tiotropium treatment group (triangles) and the placebo group (squares). In the tiotropium treated group FEV_1 % predicted increased between visit 1 and 2, but this increase was not significant (p = 0.134). FEV_1/FVC ratio did significantly improve following 21 days of daily treatment with tiotropium (p = 0.013), but not in the placebo group (p = 0.754).

FOT. Rrs and Xrs were not significantly changed by 21 days of treatment with tiotropium once daily or placebo at all 3 frequencies (p >0.1), nor were the changes in Rrs or Xrs by 21 days of treatment significantly different between the groups (p > 0.3, **Table 2**). The change in Rrs at 8 Hz by a single dose of tiotropium at visit 1 was significantly related to the change in Rrs at 8 Hz by 21 days of tiotropium once daily (r = 0.67, p = 0.017.

Effect of tiotropium on airway hyperresponsiveness

Airway hyperresponsiveness as measured by the provocative concentration inducing a 20% fall in FEV_1 (PC_{20}) significantly increased by 21 days treatment with tiotropium or placebo (**Figure 4A**, mean change ± SD(mg/ml): tiotropium −2.6 ± 4.0; placebo −2.2 ± 2.5).; p < 0.01 for both groups). This effect was reflected in the maximal dose response curve (**Figure 4B**, mean change ± SD (mg/ml): tiotropium 10.5 ± 13.3; placebo 5.1 ± 8.0). In both groups PC_{20} significantly increased after pre-treatment with a single dose of tiotropium at visit 1, as compared to the unpretreated screening PC_{20} (mean change ± SD(mg/ml): tiotropium 2.2 ± 3.2; placebo 2.1 ± 3.3; p < 0.05 in both groups). In both groups PC_{20} returned to screening values at visit 2 (p = 0.53 in tiotropium group and p = 0.89 in placebo group).

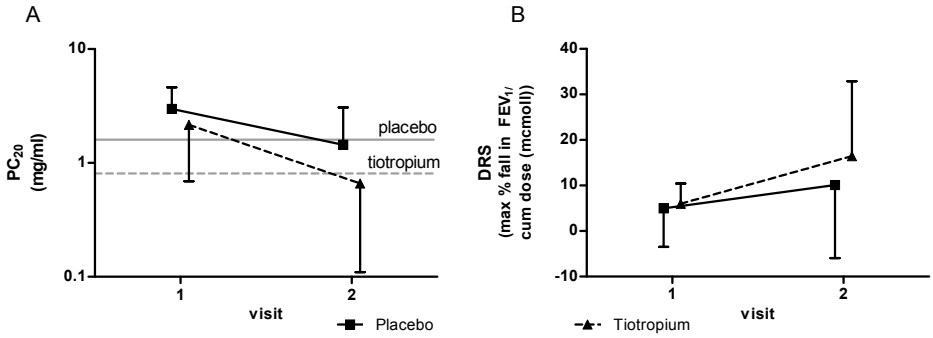

Figure 4. PC_{20} histamine and dose-response-slope.
Figure **4A** shows the geometric mean for PC_{20} histamine ± standard deviation in doubling doses. Figure **4B** shows the mean for dose-response-slope (DRS) ± standard deviation at each visit for the tiotropium group (triangles, dotted line) and placebo group (squares, solid line). The grey horizontal lines present the PC_{20} values of the screening visit without pre-treatment with tiotropium for each group. PC_{20} at visit 2 was significantly lower as compared to visit 1 ($p < 0.01$), but not as compared to screening in both groups ($p > 0.5$).

Effect of tiotropium on deep inspiration-induced bronchodilation

M/P ratio. Deep inspiration-induced bronchodilation as measured by M/P ratio measured at a given level of bronchoconstriction was not improved by 3 weeks treatment with tiotropium or placebo. This was shown at both a 20% fall in FEV_1 ($p = 0.66$), as well as a 40% fall in FEV_1 ($p = 0.89$).

Tabel 3. Change in Rrs and Xrs by a deep inspiration.

Median [range]		Visit 1 (before treatment)		Visit 2 (after treatment)	
		tiotropium	placebo	tiotropium	placebo
Rrs ($cmH_2O/L/s$)	8Hz	−0.6 [−3.5–1.3]	−0.3 [−2.4–3.5]	−0.4 [−2.8–2.0]	0.4 [−1.2–2.8]
	12Hz	−0.4 [−2.0–0.9]	−0.2 [−1.7–2.5]	0.0 [−1.6–1.4]	0.4 [−0.8–2.3]
	16Hz	−0.3 [−2.4–0.6]	−0.1 [−1.3–2.4]	0.0 [−1.0–1.1]	0.5 [−0.4–2.1]
Xrs ($cmH_2O/L/s$)	8Hz	2.5 [−0.3–5.4]	2.4 [−0.0–6.0]	1.0 [−1.5–4.8]	0.6 [−1.0–4.7]
	12Hz	1.6 [−0.2–4.2]	1.9 [0.1–3.9]	0.7 [−1.3–3.5]	0.3 [−1.2–2.8]
	16Hz	1.1 [−0.1–3.7]	1.2 [0.1–2.7]	0.7 [−1.1–3.0]	0.2 [−1.0–2.0]

Change in Rrs and Xrs by a deep inspiration following histamine-induced bronchoconstriction at visit 1 (baseline) and visit 2 (after 21 days of treatment with tiotropium or placebo). Data are expressed as median [range]. The change in Rrs and Xrs by deep inspiration was not significantly changed by 21 days of treatment in both groups, nor was the change significantly different between the groups.

FOT. Deep inspiration-induced bronchodilation as measured by a reduction in Rrs by deep inspiration following histamine challenge was not significantly improved by 21 days of treatment with tiotropium ($p > 0.2$) or placebo at the 3 frequencies. Median changes in Rrs and Xrs by deep inspiration at both visits are given in **table 3**.

Safety

27 patients were included in the study. 3 patients did not finish the study protocol due to minor adverse effects. 24 patients finished the study protocol.

Discussion

This study shows that in mild asthmatic patients a single dose of tiotropium significantly improves lung function as reflected by an increase in FEV_1 % predicted and a reduction in respiratory resistance. Maintenance treatment with tiotropium over 21 days improved FEV_1 % predicted and significantly increased FEV_1/FVC ratio as compared to placebo. Airway hyperresponsiveness was not affected by daily use of tiotropium. Although, interestingly, the first dose of tiotropium significantly increased PC_{20} histamine, but this effect diminished both in the placebo group as well as in the tiotropium group. Also, tiotropium once daily had no effect on deep inspiration-induced bronchodilation, both measured by respiratory resistance and M/P ratio. Thus, in mild asthmatics with a normal lung function tiotropium had a direct and sustained bronchodilatory effect.

This is the first study examining the effects of tiotropium on multiple outcomes of airway function (FEV1, FEV1/FVC, Rrs, Xrs, airway hyperresponsiveness to histamine and deep inspiration-induced bronchodilation) in patients with mild asthma. We found a significant increase in FEV_1 % predicted and reciprocal reduction in respiratory resistance 180 minutes following inhalation of 18mcg tiotropium at each visit. Moreover, daily use of 18mcg tiotropium reduced airway tone as reflected by an improvement in FEV_1/FVC ratio. Our data confirm[18] and extend previous observations. In severe persistent asthmatics tiotropium when added to conventional therapy (long-acting beta agonists and corticosteroid treatment) produced an extra improvement in FEV_1 (FEV_1 by >15% (or 200 ml) for at least 8 successive weeks in at least 30% of the subjects[19,20]. Also, exacerbation rate declined but this was not statistically evaluated since it was not a powered outcome parameter. Further, in severe persistent asthma FEV_1 improvement was inversely related to sputum eosinophils[21] after 4 weeks of treatment with tiotropium. Our study extrapolate these data from severe asthmatic patients showing that tiotropium once daily for 3 weeks reduces airway tone mild asthma.

Although we found a significant improvement in FEV_1/FVC ratio, the increase in FEV_1 % predicted by 3 weeks tiotropium did not reach significance. Nevertheless, the number of subjects per group was enough to show an improvement of 5% within the treatment group

with a power of 80%. It is likely that in this group of mild asthmatics there was not enough room for improvement. Whether a small increase in FEV_1/FVC ratio or FEV_1 % predicted is of clinical relevance can be debated. Unfortunately, we did not include an asthma control questionnaire to relate the changes to asthma symptoms or asthma control. However, the parallel and significant improvement in FEV_1/FVC ratio implicates that airway obstruction was improving.

Tiotropium once daily for 3 weeks did not affect airway hyperresponsiveness, nor did it improve deep inspiration-induced bronchodilation as we hypothesized. Most studies examining the effect of tiotropium on induced bronchoconstriction were focused on methacholine challenges[5,22], and thus on the direct antagonizing effect of tiotropium on muscarinic receptors. Predictably, O'Connor et al found a significant dose-dependent protection against methacholine challenge at 2 hours after inhalation, which persisted for 48 hours[23]. Surprisingly, we found that after 3 weeks of treatment airway hyperresponsiveness increased in both groups. This effect could be explained by a significant reduction in airway hyperresponsiveness after the first inhalation of tiotropium at visit 1, which returned back to screening level at visit 2. Acute protection against histamine-induced bronchoconstriction has been shown by pre-treatment with ipatropium in normal subjects[24]. Histamine leads to bronchoconstriction by direct stimulation of histamine receptors on airway smooth muscle, and in part indirectly via stimulation of vagal reflex causing release of actelycholine[25]. The latter is inhibited by anticholinergic drugs, thereby reducing histamine-induced bronchoconstriction. It is unclear why this effect was only seen after the first dose of tiotropium. Upregulation of histamine receptors by daily use of tiotropium seems unlikely, since in both groups airway responsiveness returned to screening levels. In addition, tachyphylaxis to histamine is not likely since the screening visit and visit 1 were at least 1 day (>18 hours) apart[26].

An inhibitory effect of tiotropium on airway smooth muscle cells has been found in ovalbumin challenged guinea pigs and not in non-challenged animals[27]. This might be due to an increased level of acetylcholine by an augmented acetylcholine release after allergen challenge, reduction of inhibitory M2 receptors, or nonneuronal release of acetylcholine in conditions of allergic inflammation[5]. In the absence of inflammatory mediators or growth factors increased levels of acetylcholine may not result in structural changes within the airways and thus no inhibitory effect of tiotropium can be observed. Therefore it is possible that in these mild asthmatic patients a lower level of inflammatory mediators was present within the airways in some patients and thereby reducing the inhibitory effect of daily tiotropium inhalation. Since we did not include induced sputum or bronchial biopsies as outcomes of this study we cannot relate this to the improvement in lung function we found in our study. Therefore, whether the observed changes in lung function and airway hyperresponsiveness is a result of sustained bronchodilation by muscarinic receptor blockage or inhibition of allergen-induced remodeling remains unanswered.

Previously, we have shown that the expression of specific smooth muscle markers was related to the level of deep inspiration-induced bronchodilation[28]. Tiotropium has been shown

to reduce smooth muscle-specific myosin expression[27]. Therefore, treatment with tiotropium could have been able to modulate airway responses to deep inspiration. However, deep inspiration-induced bronchodilation did not improve in our patients, which may be due to the lack of inhibition of tiotropium on smooth muscle proliferation and contractility. This, of course, can only be answered in additional study by taking bronchial biopsies before and after treatment.

What is the clinical relevance of this study? Many studies have shown that in asthma beta-2-agonists are usually more effective bronchodilators than anticholinergics[29]. Therefore, anticholinergics are not used in the guidelines for regular asthma treatment[1]. In animal studies anticholinergic drugs appear to be better in inhibiting allergen-induced airway smooth muscle remodeling and smooth muscle contractility than beta-agonists[27,30]. Whether this is the case in human asthma as well, needs further examination. Our results demonstrate that 3 weeks of daily use of tiotropium has a beneficial effect on lung function with no apparent effect on airway hyperresponsiveness in mild asthma. On the other hand, bronchodilation by anticholinergics varies widely in asthma. First, patients with intrinsic (non-allergic) asthma and those with a longer duration may respond better[2]. Second, genetic studies have suggested that patients with homozygosity for arginine (Arg/Arg) rather that glycine may benefit more from anticholinergic therapy than from beta-agonists[20]. And finally, viral infections may cause M2 receptor dysfunction and thereby increasing acetylcholine-mediated airway tone resulting in increased bronchodilator response to anticholinergics[5].

We conclude tiotropium has both acute and prolonged bronchodilatory effects in mild asthmatic patients. Whether patients with asthma may benefit from tiotropium as long-acting bronchodilator on top of inhaled corticosteroid needs further research. Also, it remains to be established whether the observed changes in lung function and airway hyperresponsiveness are a result of sustained bronchodilation by muscarinic receptor blockage or inhibition of allergen-induced remodeling.

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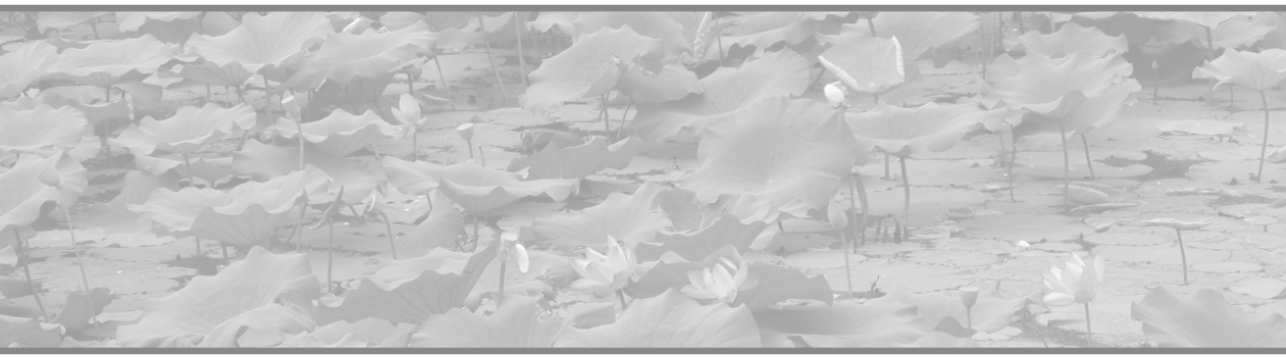

Chapter 7

Enhanced airway dilation by positive-pressure inflation of the lungs compared with active deep inspiration in patients with asthma

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Abstract

Background: Deep inspiration temporarily reduces induced airways obstruction in healthy subjects. This bronchodilatory effect of deep inspiration is impaired in asthma. Passive machine-assisted lung inflation may augment bronchodilation compared with an active deep inspiration in patients with asthma by either opening closed airways or by reducing fluid flux across the airway wall during deep inspiration, and thereby increasing the tethering forces on the airway wall.

Methods: We recruited 24 patients with asthma [18–46 yr old, forced expiratory volume in 1 second (FEV_1) > 70% predicted; provocative concentration of methacholine inducing a 20% fall in FEV_1 (PC_{20}) < 8 mg/ml], with either an impaired ($n = 12$) or an intact ($n = 12$) bronchodilatory response to deep inspiration. Two methacholine challenges were performed on separate days. At a 50% increase in respiratory resistance (forced oscillation technique at 8 Hz), the change in resistance by a positive-pressure inflation (computer-driven syringe) or an active deep inspiration was measured in randomized order.

Results: The reduction in resistance by positive-pressure inflation was significantly greater than by active deep inspiration in the impaired deep inspiration response group (mean change ± SE: −0.6 ± 0.1 vs. −0.03 ± 0.2 $cmH_2O \cdot l^{-1} \cdot s$, $P = 0.002$). No significant difference was found between positive-pressure inflation and active deep inspiration in the intact deep inspiration response group (−0.6 ± 0.2 vs. −1.0 ± 0.3 $cmH_2O \cdot l^{-1} \cdot s$, $P = 0.18$).

Conclusion: Positive-pressure inflation of the lungs can significantly enhance deep inspiration-induced bronchodilation in patients with asthma.

Introduction

The functional response of the airways to deep inspiration is different between healthy subjects and patients with asthma[27,41]. In healthy subjects deep inspirations reverse induced bronchoconstriction almost to baseline level[4,32,39]. In patients with mild asthma, however, dilation of constricted airways by deep inspiration is impaired[16,38,44]. The impairment of this bronchodilatory effect is related to asthma severity and disease status[28,40]. Restoring this physiological mechanism that reverses airways obstruction in patients with asthma might reduce the need of current asthma treatment.

During a deep inspiration the transpulmonary pressure is transmitted through the parenchymal tissue and distends all intrapulmonary structures, including the airways[42]. Airway wall thickening by chronic inflammation in asthma would decrease the strain transmission from the parenchyma to the airway wall, thereby reducing airway distension. Using a mathematical model it has been shown that peribronchial inflammation decreases both the load and the slope of the relationship between peribronchial and pleural pressure[30]. Furthermore, Burns and Gibson[12] showed that adding a resistance during deep inspiration, to enhance the subatmospheric intrathoracic pressure, decreased airway conductance (sGaw) in asthmatic patients. They concluded that the large subatmospheric pressures during deep inspiration may lead to extravasation of fluid in the inflamed asthmatic airway wall, thereby enhancing airway wall thickness. However, two studies using fast intravenous infusion of saline, resulting in airway wall thickening by edema and fluid flux[9,11], showed no effect on deep inspiration-induced bronchodilation[37].

On the other hand, the inflammation-induced remodeling processes in chronic asthma[13,25] may increase the airway wall stiffness[5] and make it more resistant to imposed stretch by deep inspiration[17,33,35,47]. Using high-resolution CT scans, Brown and Mitzner[8] demonstrated in mechanically ventilated dogs that increased airway wall stiffness by smooth muscle tone resulted in impaired dilation of the airways by positive pressure compared with relaxed airways. Even though it has been observed that the airways of asthmatic patients dilated to the same extent as those of healthy subjects by a deep inspiration, the airways of the patients with asthma constricted following the deep inspiration[10]. The latter may be a result of altered smooth muscle function in asthma leading to impaired bronchodilation by deep inspiration[2].

It can be postulated that airway wall distension can be improved by manipulation of the intrathoracic pressures by passive lung inflation in patients with asthma. Mechanical inflation of the lungs would induce stretch of the airways without large subatmospheric intrathoracic pressures and could therefore prevent extravasation of fluid in inflamed airways. In addition, the inflated volume may open closed airways, thereby redistributing tethering forces of the parenchyma on the airway wall.

Therefore, we hypothesized that positive-pressure machine-assisted lung inflation will reduce the level of airways obstruction in patients with asthma who do not feature bronchodilation

following active deep inspiration. To that end, we developed a computer-controlled syringe, which can inflate the lungs with a predetermined individual volume and inspiration time for each subject. This was connected to a forced oscillation device, which measured resistance (Rrs) and reactance (Xrs) of the respiratory system continuously during the breathing maneuvers.

The aim of this study was to compare changes in Rrs and Xrs in response to active or positive-pressure (syringe inflated) deep inspiration maneuvers in asthmatic patients who have an intact or an impaired bronchodilatory response to active deep inspiration while provoked with inhaled methacholine.

Methods

Subjects

For this study we recruited 24 patients with mild to moderate persistent asthma[32a]. All patients had a history of wheezing, breathlessness, or cough. They were all atopic, as demonstrated by a positive skin reaction to 1 of 10 common aeroallergen extracts (HAL, Haarlem, The Netherlands) and were hyperresponsive to methacholine [provocative concentration of methacholine inducing > 50% increase in Rrs (PC_{50} Rrs) < 8 mg/ml] (7). The subjects were clinically stable and used β_2-agonists on demand only or in combination with inhaled corticosteroids. Short- and long-acting β_2-agonists were stopped, respectively, 8 and 24 h before the challenges. None of the participants had a recent upper respiratory tract infection or other relevant diseases. The Medical Ethics Committee of the Leiden University Medical Center approved the study, and the subjects gave their written informed consent before entering the study.

Study design

Clinical status, atopy, and hyperresponsiveness to methacholine were assessed during a screening visit. The initial response of the airways to an active deep inspiration was measured at the end of the methacholine challenge of the screening visit. The response determined whether the subject was included in *group A* (intact response to deep inspiration, the "intact DI response group") or *group B* (impaired response to deep inspiration, the "impaired DI response group") (see also *Statistical analysis*). On two subsequent randomized visits two additional methacholine challenges were performed to measure the response of the airways to either an active deep inspiration or a positive-pressure inflation performed by the computer-controlled motor-driven syringe (**Figure 1**).

Methacholine challenges

The response of the airways to methacholine was measured using the forced oscillation technique (FOT) at 8 Hz[44]. At baseline, forced expiratory volume in 1 s (FEV_1) was measured three times following saline inhalation. One minute later, three measurements of Rrs during 30 s of

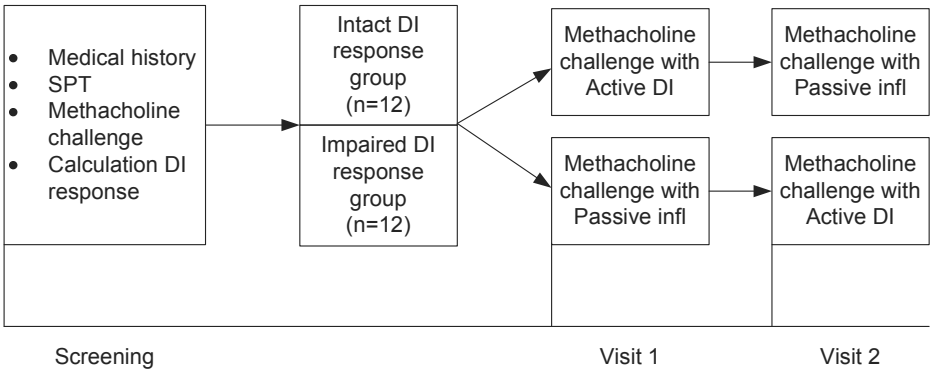

Figure 1. Study design.
During the screening visit the response to an active deep inspiration (DI) was calculated, and the patients were divided over the groups "intact" and "impaired" DI response based on these calculations. Next, all patients would return for 2 more challenges. Following these methacholine challenges, the airway responses to either an active DI or positive-pressure inflation were measured, in randomized order.

tidal breathing were performed. The mean of these three measurements was used as baseline Rrs. Serial doubling doses of methacholine bromide (0.03–9.6 mg/ml) in normal saline were aerosolized and inhaled for 2 min by tidal breathing at 5-min intervals. Following each dose Rrs was measured during 30 s of tidal breathing starting at 30 and 90 s after methacholine inhalation. The challenge was stopped when the highest Rrs of these two measurements was increased by more than 50% from baseline. Another measurement was performed to calculate the change in Rrs induced by deep inspiration. Finally spirometry was performed to measure the fall in FEV_1.

Machine-assisted positive-pressure inflation

We developed a computer-controlled motorized syringe system and software to perform a passive deep inspiration maneuver [artificial lung function generator (ALFG)]. The ALFG was connected to a forced oscillation device (**Figure 2**). We used valves to switch between FOT measurements and positive-pressure inflation. Therefore, the measurements taken before and after active deep inspiration were done under the exact same conditions as before and after positive-pressure inflation. The maneuver started with tidal breathing, followed by an expiratory reserve volume (ERV) maneuver. The ERV was calculated from the volume difference between last end-expiratory value and the plateau of the ERV maneuver. Using this ERV, the specific inspiratory capacity (IC) was calculated by subtracting the ERV value from the previously measured baseline vital capacity (VC). As a safety procedure to prevent overinflation, we used 90% of IC as the inspiratory volume to be inflated by the machine. In addition, if ERV following methacholine inhalation was less than baseline ERV, we used baseline ERV. The ERV maneuver was followed by tidal breathing to determine the starting point and duration of the positive-pressure inflation. The function of a cosine during a half period (period of π radians)

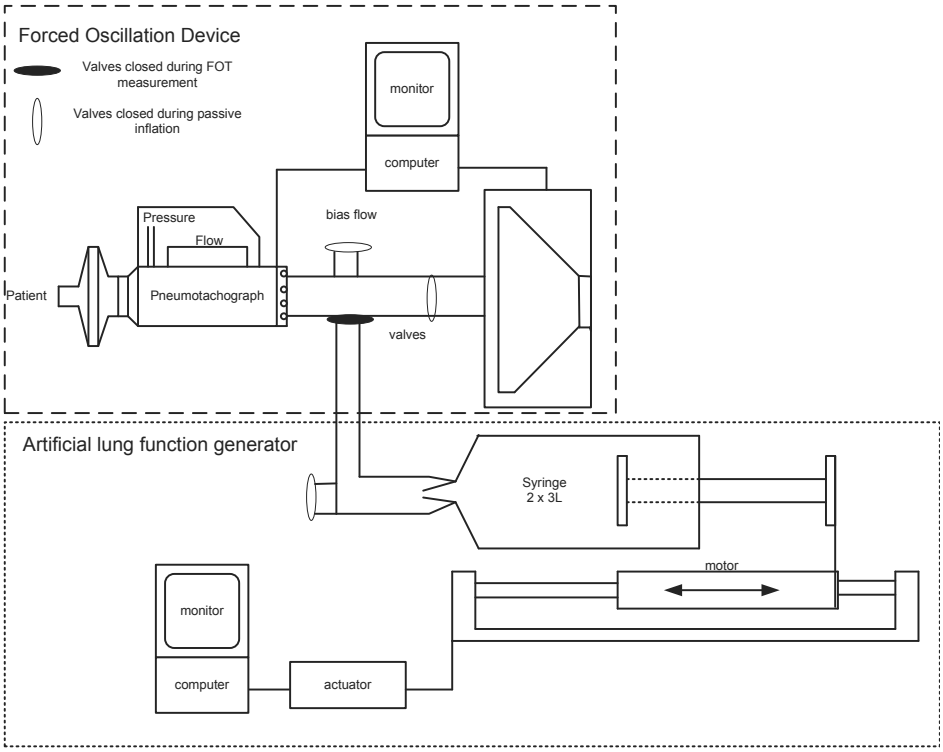

Figure 2. Schematic overview of artificial lung function generator (ALFG) and forced oscillation technique (FOT).

The top panel shows the forced oscillation device and the lower panel shows the connected ALFG. During all the measurements the black valve was closed and the white valves were open. In this situation the experimental setting resembles a regular FOT measurement. Just before the positive-pressure inflation the valves were switched (white valves closed and black valve opened), and at the end of the positive-pressure inflation (at total lung capacity) the valves were switched back and FOT measurements started again.

was used to simulate the flow-driven passive deep inspiration maneuver, with the inspiration time as signal period time, and the predetermined inspiration capacity as the amplitude of the cosine. Rrs and Xrs were measured continuously, except during the positive-pressure inflation itself. The active deep inspiration was preceded by the same maneuvers to keep the active and passive deep inspiration measurements comparable.

Forced oscillation technique

Rrs and Xrs were measured continuously during the breathing maneuvers using a forced oscillation device (Woolcock Institute)[38,44] with an applied oscillation frequency of 8 Hz and an amplitude of ±1 cmH_2O. Flow was measured using a 50-mm-diameter Fleisch pneumotachograph (Vitalograph, Maids Moreton, UK), and differential pressure was measured using a ±2.5 cmH_2O solid-state transducer (Sursense DCAL4; Honeywell Sensing and Control, Milpitas, CA). Mouth pressure was measured using a similar transducer with a higher range (±12.5 cmH_2O).

Analog pressure and flow signals were digitized at 400 Hz. The time- and frequency-dependent respiratory impedance Zrs was estimated based on the hypothesis that random errors occur in both pressure and flow. This yields a total least squares (TLS) estimate of respiratory impedance as a function of time and frequency and allows an estimation of confidence intervals in the course of time. This method has been fully described in a previous study[44].

Statistical analysis

Mean Rrs and Xrs were calculated from all data points during three tidal inspirations (Rrs_{insp} and Xrs_{insp}) and three tidal expirations (Rrs_{exp} and Xrs_{exp}) separately. The response of the airways was calculated as the difference between Rrs and Xrs following and preceding the deep inspiration. An impaired response to deep inspiration was defined as a decrease in Rrs_{exp} of less than 2 SDs of Rrs_{exp} preceding the deep inspiration, whereas an intact response to deep inspiration was defined as a decrease in Rrs_{exp} of more than 2 SDs (**Figure 3**).

The sample size of 12 patients per group was based on our data with regard to Rrs measurements[44], allowing the detection of a 1 $cmH_2O \cdot l^{-1} \cdot s$ difference within and between the groups,

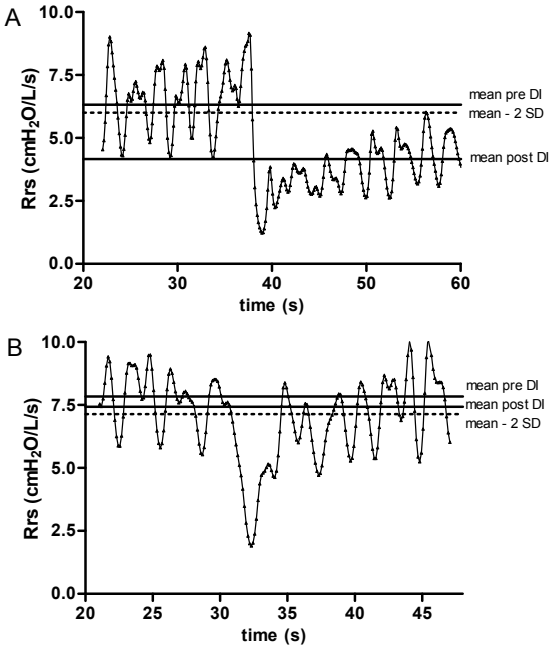

Figure 3. Example of calculation of DI response.
2 examples of Rrs measurements at the end of the methacholine challenge of the screening visit. **A**: the change in resistance of the respiratory system (Rrs) in a patient with an intact DI response. The top solid line represents the mean of the 3 tidal expirations before DI, the dashed line represents the mean minus 2 SDs, and the bottom solid line represents the mean of 3 tidal expirations following DI. This example clearly shows that the DI decreased Rrs by more than 2 SDs. **B**: the change in Rrs in a patient with an impaired DI response. In this case the active DI did not decrease Rrs by more than 2 SDs.

if $\alpha = 0.05$, and $1 - \beta = 0.80$. Between-group differences were analyzed using Mann-Whitney *U*-tests. Within-group differences were explored using Wilcoxon signed-rank tests. Correlations were examined using Spearman's rank correlation coefficients. We used SPSS version 12.01 for all analyses (SPSS, Chicago, IL). *P* values < 0.05 were considered statistically significant.

Results

All patients performed the measurements without any personal or medical problems. The patient characteristics are given in **Table 1**. The groups were not significantly different with regard to sex, age, steroid usage, lung volumes (VC, ERV, IC), or lung function (FEV_1% predicted). A methacholine-induced increase of more than 50% in Rrs could not be reached in all patients. This occurred in 6 of 24 patients: 2 in the intact DI response group, and 4 in the impaired DI response group. The challenge was then stopped if apparent breathlessness or wheezing occurred in combination with a further decrease in Xrs. FEV_1 dropped by more than 20% in all cases (**Table 1**). We calculated mean resistance and reactance over tidal inspirations (Rrs_{insp}, Xrs_{insp}) and tidal expirations (Rrs_{exp}, Xrs_{exp}) separately. However, the parameters showed the same results, indicating that deep inspiration and positive-pressure inflation altered both parameters in the same way. We have therefore only presented the results of Rrs_{exp} and Xrs_{exp}.

Table 1. Patient characteristics

	Intact DI Response Group	Impaired DI Response Group
Sex, M/F	3/9	3/9
Age, yr	28.1±9.1	26.7±9.7
ICS usage, yes/no	5/7	7/5
FEV_1, %predicted	96.6±15.2	90.6±11.7
VC, liters	4.6±1.1	4.2±0.8
ERV, liters	1.6±0.5	1.5±0.4
IC, liters	2.9±0.7	2.7±0.7
PC_{50} Rrs methacholine, mg/ml	0.73±1.6	0.45±1.5
Fall in FEV_1, %	30.9±10.9	37.4±11.5
IV post-methacholine, %		
Active deep inspiration	81.6±12.2	70.5±13.1*
Positive-pressure inflation	82.0±8.7	85.2±5.6

Data are expressed as number [male/female (M/F); inhaled corticosteroid (ICS) usage] or means ± SD. DI, deep inspiration; FEV_1, forced expiratory volume in 1 s; VC, vital capacity; ERV, expiratory reserve volume; IC, inspiratory capacity; Rrs, resistance of the respiratory system; PC_{50}, provocative concentration of methacholine inducing a 50% increase in Rrs. Only the inspiratory volume as a percentage of baseline inspiratory capacity (IV %) for the active deep inspiration was significantly different between the groups (*P = 0.027).

Baseline measurements

Baseline Rrs_{exp} and Xrs_{exp} were not significantly different between the groups at both visits ($P > 0.1$). Active deep inspiration significantly decreased Rrs_{exp} at baseline in the intact DI response group (mean change ± SE: −0.12 ± 0.06 $cmH_2O{\cdot}l^{-1}{\cdot}s$, $P = 0.04$) but not in the impaired DI response group (+0.05 ± 0.10 $cmH_2O{\cdot}l^{-1}{\cdot}s$). Positive-pressure inflation had no effect on baseline Rrs_{exp} in both groups ($P = 0.9$). Xrs_{exp} was not significantly changed by either deep inspiration or positive-pressure inflation ($P > 0.2$).

Methacholine-induced changes

Methacholine significantly increased Rrs_{exp}, and decreased Xrs_{exp}, in both groups ($P < 0.01$; **Figure 4A and 4B**) at both visits. Both Rrs_{exp} and Xrs_{exp} were not significantly different between the groups following methacholine inhalation before deep inspiration or positive-pressure inflation ($P > 0.11$). The changes in Rrs_{exp} and Xrs_{exp} by methacholine and deep inspiration or positive-pressure inflation are summarized in **Table 2**.

Active deep inspiration

Active deep inspiration significantly decreased Rrs_{exp} in the intact DI response group ($P = 0.003$, **Figure 4A**) but not in the impaired DI response group ($P = 0.9$), which confirmed the findings from the screening visit. Also the change in Rrs_{exp} by active deep inspiration was significantly larger in the intact DI response group compared with the impaired DI response group ($P < 0.01$), which resulted in a significantly lower Rrs_{exp} during the three tidal expirations following the active deep inspiration in the intact DI response group ($P < 0.01$). Interestingly, Xrs_{exp} was significantly increased by active deep inspiration in both groups ($P < 0.02$; Fig. 4B), and the change in Xrs_{exp} was not significantly different between the groups ($P = 0.5$). However, it resulted in a significantly higher Xrs_{exp} following active deep inspiration in the intact DI response group ($P = 0.02$).

Positive-pressure inflation

Rrs_{exp} was significantly decreased by positive-pressure inflation in both groups ($P < 0.02$), and the change induced by the positive-pressure inflation was not significantly different between the groups ($P = 0.8$). Also, Xrs_{exp} was significantly increased by positive-pressure inflation in both groups ($P < 0.01$; **Figure 4B**) with no significant differences between the groups. Notably, the change induced in Rrs_{exp} by the positive-pressure inflation was significantly larger than the change induced by active deep inspiration ($P = 0.002$) in the impaired DI response group, but not in the intact DI response group ($P = 0.18$) (**Table 3**).

Inspiratory volumes

VC, ERV, and IC were measured at baseline to calculate the volume to be inflated by the machine. Also, the actual inspired volume during both the active and positive-pressure inflation

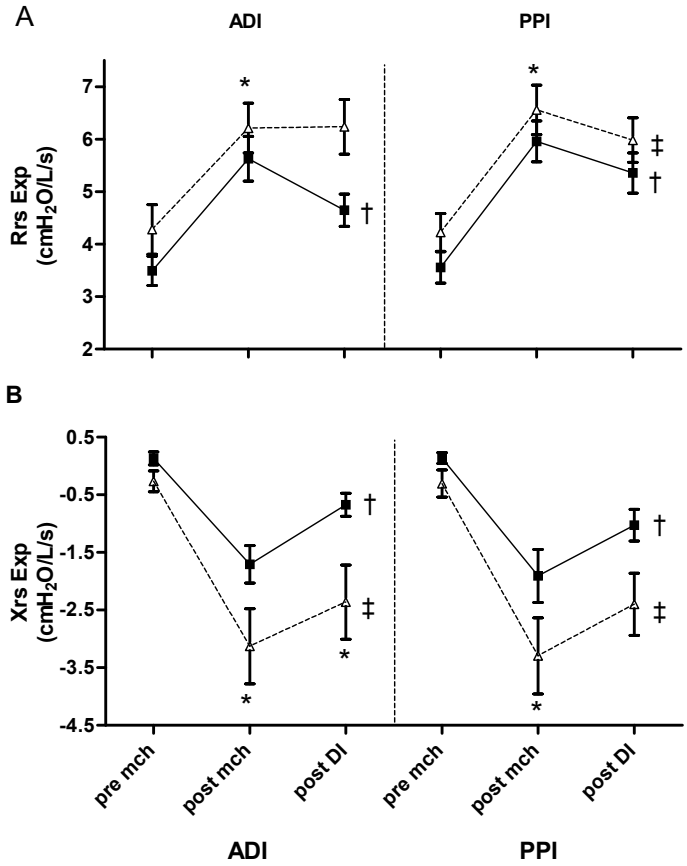

Figure 4. Mean resistance of the respiratory system during tidal expiration (Rrs_{exp}) during the challenges. Mean ± SE for Rrs_{exp} (A) and reactance of the respiratory system during tidal expiration (Xrs_{exp}; B) before methacholine inhalation (pre-mch), after methacholine inhalation (post-mch), and following deep inspiration (post-DI) for the intact DI response group (■) and the impaired DI response group (△) are shown. The data points in the left panels represent the measurements of the active deep inspiration (ADI), and those in the right panels represent the measurements of the positive-pressure inflation (PPI). **A:** Rrs_{exp} was significantly increased by methacholine at both visits (*P < 0.01) in both groups. In the intact DI response group, Rrs_{exp} was significantly decreased by both the active deep inspiration and positive-pressure inflation (†P < 0.01). In the impaired DI response group Rrs_{exp} was only significantly reduced by positive-pressure inflation (‡P < 0.02). **B:** Xrs_{exp} was significantly decreased by methacholine at both visits (*P < 0.01) in both groups. In contrast with the Rrs results, Xrs_{exp} was significantly increased by both the active deep inspiration and positive-pressure inflation in both groups (†‡P < 0.02). However, Xrs_{exp} following active deep inspiration was significantly higher in the intact DI response group (*P = 0.02).

was measured. Using these values we calculated the percentage inspired volume of baseline inspiratory capacity during the maneuvers following methacholine inhalation. The percentage inspiratory volume of the active deep inspiration following methacholine inhalation was significantly lower in the impaired DI response group (mean ± SD: 71 ± 13%) compared with the intact DI response group (82 ± 12%, $P = 0.027$) and was significantly increased by positive-pressure

Table 2. Rrs and Xrs values during tidal breathing and deep inspiration or positive-pressure inflation

Rrs, $cmH_2O{\cdot}l^{-1}{\cdot}s$	Intact DI		Impaired DI	
	ADI	PPI	ADI	PPI
Baseline				
3 Tidal exp (pre-DI)	3.5±0.3	3.6±0.3	4.3±0.5	4.2±0.4
At TLC	1.7±0.3		1.4±0.1	
At FRC (post-DI)	2.7±0.2	2.9±0.3	3.1±0.3	3.5±0.3
3 Tidal exp (post-DI)	3.4±0.3	3.6±0.3	4.3±0.4	4.2±0.4
Post-methacholine				
3 Tidal exp (pre-DI)	5.6±0.4	6.0±0.4	6.2±0.5	6.6±0.5
At TLC	1.9±0.1		2.1±0.2	
At FRC (post-DI)	3.5±0.4	3.9±0.3	3.8±0.1	4.3±0.4
3 Tidal exp (post-DI)	4.6±0.3	5.4±0.4	6.2±0.5	6.0±0.4
Xrs, $cmH_2O{\cdot}l^{-1}{\cdot}s$	Intact DI		Intact DI	
	ADI	PPI	ADI	PPI
Baseline				
3 Tidal exp (pre-DI)	0.1±0.1	0.1±0.1	0.3±0.2	0.3±0.2
At TLC	0.4±0.1		0.7±0.1	
At FRC (post-DI)	0.3±0.1	0.3±0.1	0.1±0.1	0.2±0.2
3 Tidal exp (post-DI)	0.1±0.1	0.1±0.1	0.4±0.2	0.5±0.3
Post-methacholine				
3 Tidal exp (pre-DI)	−1.7±0.3	−1.9±0.5	−3.1±0.7	−3.3±0.7
At TLC	−0.8±0.1		−1.0±0.2	
At FRC (post-DI)	−0.3±0.2	−0.4±0.2	−0.3±0.2	−0.9±0.5
3 Tidal exp (post-DI)	−0.7±0.2	−1.0±0.3	−2.4±0.6	−2.4±0.5

Data are expressed as means ± SE. All the Rrs and reactance of the respiratory system (Xrs) data measured at baseline and following methacholine inhalation (Post-methacholine), during active deep inspiration (ADI) and positive-pressure inflation (PPI), are shown for each group separately. Rrs at total lung capacity (TLC) could not be measured during PPI due to valve switching at that time point. Exp, expiration; FRC, functional residual capacity; pre-DI, before deep inspiration; post-DI, after deep inspiration ($^{*}P = 0.006$). The change in Rrs_{exp} by PPI was significantly larger than by ADI in the impaired DI response group $^{\dagger}(P = 0.002)$.

inflation (mean change ± SD: 15 ± 14%; $P = 0.011$). Notably, although the reduction in Rrs_{exp} was not related to the percentage inspiratory volume ($P > 0.1$; **Figure 5**), the increase in percentage inspiratory volume by positive-pressure inflation correlated with the increase in reduction of Rrs_{exp} by positive-pressure inflation ($P < 0.01$; **Figure 6**).

Table 3. Changes in Rrs and Xrs induced by methacholine and deep inspiration

	ADI		PPI	
	Rrs_{exp}	Xrs_{exp}	Rrs_{exp}	Xrs_{exp}
Intact DI response group				
Change by methacholine	2.1±0.3	−1.8±0.3	2.4±0.2	−2.0±0.4
Change by DI	−1.0±0.3*	1.0±0.2	−0.6±0.2	0.9±0.3
Impaired DI response group				
Change by methacholine	1.9±0.3	−2.9±0.5	2.3±0.3	−3.0±0.5
Change by DI	0.03±0.2	0.8±0.3	−0.6±0.1†	0.9±0.2

Data are expressed as means ± SE in $cmH_2O \cdot l^{-1} \cdot s$. Rrs_{exp}, Rrs measurements during tidal expiration; Xrs_{exp}, Xrs measurements during tidal expiration. The change in Rrs_{exp} by ADI was significantly larger in the intact DI response group compared with the impaired DI response group The changes in Xrs_{exp} induced by both active deep inspiration and positive-pressure inflation were not significantly different between the groups (P = 0.38). Also in the impaired DI response group no significant difference was seen in the change induced in Xrs_{exp} by either active deep inspiration or positive-pressure inflation (P = 0.48).

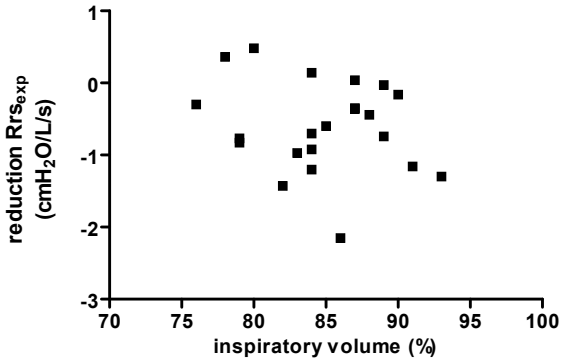

Figure 5. Reduction in Rrs_{exp} in relation to inspiratory volume by positive-pressure inflation. Percentage inspiratory volume of baseline inspiratory capacity in relation to the reduction of Rrs_{exp} by positive-pressure inflation. No significant association was found between the inspired volume and the reduction in airways obstruction (Spearman rho = −0.11, P = 0.62).

Discussion

This study shows that airways obstruction can be reduced by positive-pressure inflation of the lungs in asthma. Interestingly, this could also be achieved in patients with asthma who were not capable of reducing respiratory resistance by an active deep inspiration. These results suggest that influencing transpulmonary pressures by mechanical inflation of the lung can restore the beneficial bronchodilatory effects of lung inflation in patients with asthma.

To our knowledge this is the first study that used a computer-controlled syringe to inflate the lungs of conscious subjects in the upright position, and at the same time continuously measure the change in airways obstruction by lung inflation by the positive-pressure inflation. The mean

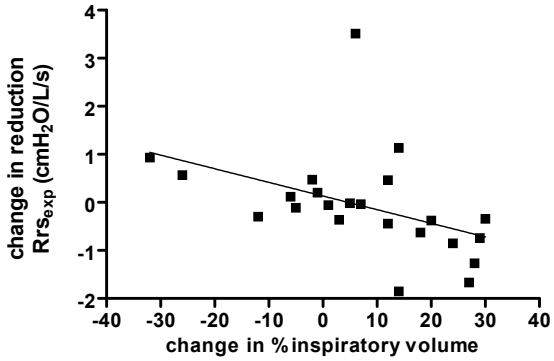

Figure 6. Correlation between the changes in reduction in Rrs_{exp} and percentage inspiratory volume by positive-pressure inflation.
We calculated the changes in % inspiratory volume (of baseline inspiratory capacity) and bronchodilation (reduction in Rrs_{exp}) as induced by positive-pressure inflation compared with active deep inspiration. This figure shows the correlation between the changes in the percentage inspiratory volume and the changes in bronchodilation (reduction of Rrs_{exp}) (Spearman rho = 0.6, P = 0.04; y = –0.029x + 0.151). Thus an increase in the percentage inspiratory volume by positive-pressure inflation was associated with more bronchodilation.

reduction in Rrs_{exp} in all patients by the active deep inspiration was 0.7 $cmH_2O{\cdot}l^{-1}{\cdot}s$, which is in line with our previous data measured by FOT showing a reduction of 0.6 $cmH_2O{\cdot}l^{-1}{\cdot}s$[44]. Our data further extend the findings by Burns and Gibson[12], who showed that deep inspiration through an added resistance, compared with a regular deep inspiration, resulted in lower airway conductance in patients with asthma, but not in healthy subjects. They proposed that inflation by positive pressure could prevent the occurrence of edema. And indeed, positive-pressure inflation of the lungs resulted in an improvement of bronchodilation in these patients with asthma.

In our study we included 24 patients with mild to moderate persistent asthma. However, only in 12 patients was Rrs_{exp} significantly reduced by active deep inspiration. We questioned whether this difference was due to either limited dilation of the airways or to a difference in the response of the airways to stretch. Therefore, we calculated the Rrs at the TLC and FRC level. We found that Rrs at TLC was not significantly different between the groups (mean ± SE: impaired DI response group 2.1 ± 0.2 $cmH_2O{\cdot}l^{-1}{\cdot}s$; intact DI response group 1.9 ± 0.1 $cmH_2O{\cdot}l^{-1}{\cdot}s$; P = 0.6), but tended to be higher at FRC in the impaired DI response group (3.8 ± 0.1 vs. 3.5 ± 0.4 $cmH_2O{\cdot}l^{-1}{\cdot}s$; P = 0.09). Following active deep inspiration Rrs was significantly higher in the impaired DI response group (**Table 2**). This suggests that positive pressure and active deep inspiration dilated the airways to the same extent, but following active deep inspiration airways reconstricted more in the patients form the impaired DI response group.

Our data are in line with the results by Brown et al[10] using high-resolution CT scans. They demonstrated that distension of constricted airways (>3 mm) by deep inspiration is not significantly different between healthy subjects and patients with mild asthma. However, following deep inspiration, the airways of healthy subjects remained dilated, whereas bronchoconstriction

occurred in the asthmatic patients. These data, together with ours, suggest that the airway-parenchyma interdependence is not the reason for impaired bronchodilation following deep inspiration in patients with mild asthma. Other studies that used the FOT also demonstrated rapid renarrowing of airways following deep inspiration in mild asthma[38]. A reduction in dilation of airways was only demonstrated in severe asthma[26]. Therefore, airway distension during deep inspiration may become impaired with increasing asthma severity, whereas the airway response following deep inspiration is already impaired in mild asthma. This is also observed with the loss of bronchoprotection[41,43]; therefore these two phenomena could clearly be linked.

We succeeded in safely inflating the airways of conscious sitting patients with a volume and inspiration time specific for each patient, and measured Rrs and Xrs continuously during these measurements. We used 8 Hz to measure impedance of the respiratory system, because this is close to the resonance frequency[34] and thus would represent airway caliber predominantly. Respiratory resistance is frequency dependent. At lower frequencies (0.1–2 Hz) inhomogeneity and tissue resistance may increasingly affect impedance (constant-phase model)[24]. However, these frequencies are too close to the breathing frequency. The changes in Rrs_{exp} and Xrs_{exp} by deep inspiration and positive-pressure inflation were different. Possibly, the changes in Xrs_{exp} represent opening of closed airways[4,14] or a change in compliance of the airway wall[36]. On the other hand, Xrs_{exp} may be more sensitive, compared with Rrs_{exp}, to measure changes in airway caliber or airway compliance. We did not measure FRC before the deep inspiration or positive-pressure inflation, and therefore we cannot exclude a difference in hyperinflation between the patients. However, we randomized the order of the inspiratory maneuvers, while both maneuvers were performed in the exact same way. Thus a difference in hyperinflation or calculated IC is unlikely to explain the differences between the groups. Unfortunately we did not include the measurement of transpulmonary pressures as well. Although it is most likely that the pressures within the alveoli and airways were supra-atmospheric during the inflation, we do not have access to these data. In addition, we asked the patients to completely relax and not to assist during the positive-pressure inflation. We have not observed any obvious active inspiration during the positive-pressure inflation, but we cannot exclude that this may have occurred. Nevertheless, the AFLG was able to reduce airways obstruction, as expected, and therefore can be used in future study designs to further investigate the pathophysiological mechanism of airway dilation by lung inflation.

The patients were selected based on their medical history regarding asthma symptoms, atopy, and airway hyperresponsiveness. Interestingly, the number of patients on inhaled steroids was equal among the two groups, confirming that under steroid treatment patients with asthma can exhibit both impaired and intact deep inspiration-induced bronchodilation[45]. The patients were assigned to the impaired or intact DI response group based on the reduction in Rrs following methacholine inhalation on the screening visit. However, the results shown for the reduction in Rrs_{exp} by active deep inspiration were measured on a randomized subsequent visit. The improvement in bronchodilation following positive-pressure inflation compared with

active deep inspiration was therefore not a result of regression to the mean. The response of the airways to either active deep inspiration or positive-pressure inflation was measured following methacholine inhalation only, since baseline intrinsic airway tone can be highly variable. The dose of methacholine that increased Rrs by 50% (PC_{50} Rrs) was not significantly different between the groups at both visits and was not significantly different between the visits in each group. Therefore, we believe that the level of bronchoconstriction was similar between the groups, and between the two visits. Nevertheless, smooth muscle activity can still be variable even at the same fall in respiratory resistance, which cannot be excluded in this human in vivo study.

We aimed to inflate the lungs by 90% of baseline IC (VC minus ERV) for safety reasons to prevent overinflation. The mean percentage inspiratory volume of baseline IC by positive-pressure inflation was 83.5%. The discordance between the actual inflated percent volume and the calculated volume can be due to leakage of air between the lips and mouth piece, early start of the apparatus, or an increased ERV. Surprisingly, the positive-pressure inflation actually reached a higher percentage of baseline IC than an active deep inspiration in the impaired DI response group. Therefore, we do believe that the inflated volume was enough to dilate the airways adequately.

How could positive-pressure inflation of the lungs induce bronchodilation in patients who cannot achieve this by an active deep inspiration? First, positive-pressure inflation may have opened closed airways that could not be opened by active deep inspiration[3,29]. Indeed, the improvement in reduction of airways obstruction by positive-pressure inflation over active deep inspiration was related to an increase in the percent inspired volume (**Figure 6**). However, the reduction in Rrs_{exp} by active deep inspiration was not related to the percentage inspired volume. In addition, both active deep inspiration and positive-pressure inflation led to a significant increase in Xrs_{exp} in the impaired DI response group. Both observations may therefore not represent a causal relationship, but parallel consequences of the positive-pressure inflation. However, in both cases increased inspired volume by positive-pressure inflation may have led to an increase in tethering of the alveolar attachments, thereby improving the distension of the intraparenchymal airways.

On the other hand, positive-pressure inflation may have led to an increase in stretch of smooth muscle within the airway wall. Using high-resolution CT scans Brown and Mitzner8 showed in dogs that airways, with increased smooth muscle tone, cannot be dilated to their maximal diameter by transpulmonary pressures of up to 25 cmH_2O. This suggests that under physiological conditions it may be impossible to stretch constricted airways to the maximal diameter. This may be augmented by increased stiffness of the airway wall in patients with asthma as a result of chronic inflammation and remodeling[5]. Many in vitro and in vivo animal studies have shown that length oscillations of smooth muscle cells are necessary to reduce stiffness and contractility of the cells[18,22,23]. Positive-pressure inflation may have induced greater stretching forces on the airways, and thereby increased stretch of smooth muscle cells and thus reduced airway wall stiffness.

What may be the clinical implication of our study? Although we developed the ALFG in an experimental setting, noninvasive mechanical ventilation is a realistic treatment option in asthma at the emergency department[15,31,46]. Especially during exacerbations of asthma it has been shown that deep inspirations can induce bronchoconstriction[28]. Whether this is due to altered smooth muscle function that further constricts on stretch, or to acute inflammatory edema enhanced by large subatmospheric pressures during deep inspiration, remains unclear. However, in both conditions occasional inflation of the lungs (mimicking a deep inspiration) may perturb the ongoing pathophysiological process and act synergistically with pharmaceutical bronchodilators to reduce the airway narrowing[21]. In the intensive care setting is has been shown that high-volume ventilation recruits closed airways, and prevents closure in combination with positive end-expiratory pressures at the mouth[1,19,20]. Further studies are required before the use of positive-pressure deep inspirations could be implemented as an actual additional treatment option in the clinical setting.

In conclusion, positive-pressure inflation of the lungs can significantly enhance the reduction in airways obstruction compared with active deep inspiration in patients with asthma. In addition, the inspired volume during the active deep inspiration was significantly lower in patients who were not capable of reducing airways obstruction by deep inspiration compared with patients with an intact bronchodilatory effect of deep inspiration. This suggests that the tethering forces of the parenchyma during active deep inspiration, possibly in relation to the magnitude of the inspired volume, are not strong enough to adequately stretch the airway wall, which may be overcome by positive-pressure inflation.

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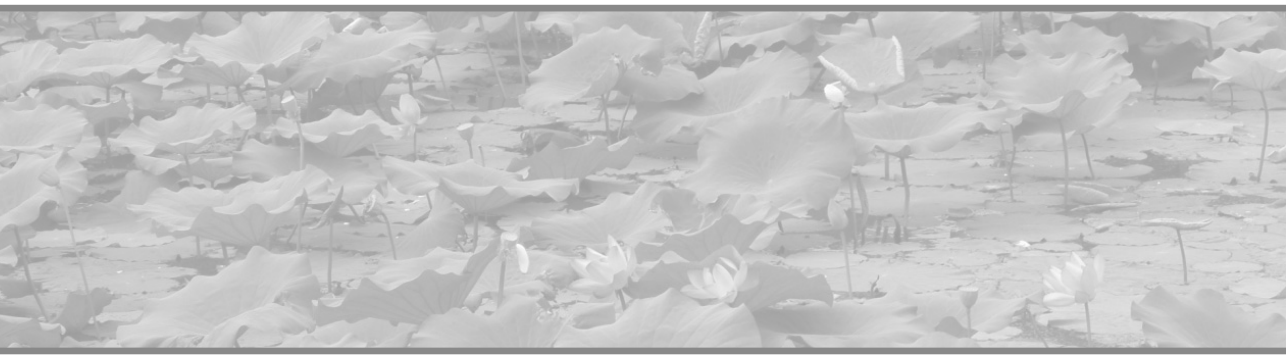

Chapter 8

Fluctuations and determinism of respiratory impedance in asthma and chronic obstructive pulmonary disease

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Abstract

Asthma and COPD are chronic respiratory diseases that fluctuate widely with regard to clinical symptoms and airways obstruction, complicating treatment and prediction of exacerbations. Time series of respiratory impedance obtained by the forced oscillation technique are a convenient tool to study the respiratory system with high temporal resolution. In previous studies it was suggested that power-law like fluctuations exist also in the healthy lung and that Zrs variability differs in asthma. In this study we elucidate such differences in a population of well-characterized subjects with asthma (n=13, GINA 1+2), COPD (n=12, GOLD I+II) and controls (n=10) from time series at single frequency (12 min, f=8 Hz). Maximum likelihood estimation did not rule out power-law behavior, accepting the null hypothesis in 17/35 cases ($p>0.05$), and significant differences in exponents for COPD ($p<0.03$). Detrended fluctuation analysis exhibited scaling exponents close to 0.5, indicating few correlations, with no differences between groups ($p>0.14$). In a second approach, we considered asthma and COPD as dynamical diseases, corresponding to changes of unknown parameters in a deterministic system. The similarity in shape between the combined probability distributions of normalized resistance and reactance was quantified by Wasserstein distances and reliably distinguished the two diseases (cross-validated predictive accuracy 0.80; sensitivity 0.83, specificity 0.77 for COPD). Wasserstein distances between 3+3 dimensional phase space reconstructions resulted in marginally better classification (accuracy 0.84, sensitivity 0.83, specificity 0.85). These latter findings suggest that the dynamics of respiratory impedance contains valuable information for the diagnosis and monitoring of patients with asthma and COPD, whereas the value of the stochastic approach is not clear presently.

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are the two most common chronic respiratory diseases, affecting millions of people worldwide[19,20]. A characteristic of these diseases is their variability in clinical symptoms and in the degree of airways obstruction. Airway caliber is fluctuating due to a complex interplay of environmental and endogenous stimuli and the dynamics of airway smooth muscle (ASM). In addition, these dynamics are affected by inflammatory processes that are difficult to assess noninvasively[40]. Recent models also indicate that bronchoconstriction might lead to self-organized cascades of ventilatory breakdown[57] and the sporadic nature of this phenomenon might explain the difficulties in predicting life-threatening exacerbations in both diseases.

Daily measurements of peak expiratory flow (PEF), on the other hand, exhibit long-range correlations over the course of months, indicating the existence of memory in the respiratory system[14,15]. Interestingly, the variability in these PEF time series seems to correlate with predictability of airway obstruction, where increased airway instability occurs when correlations become weaker. Although the physiological origin of these long-range baseline correlations is mostly unknown, these findings highlight the importance of fluctuations in the respiratory system and suggest the existence of similar phenomena also on shorter time-scales. Indeed, a well known example is provided by inter-breath intervals which exhibit characteristic scaling exponents[13,42,53].

Here we focus on signals obtained by the forced oscillation technique (FOT). Due to its relative ease, non-invasiveness and good tolerance, FOT has become a valuable tool to investigate airway properties[38]. A small pressure perturbation is superimposed on the inflowing air and the integrated response of the airways recorded. Assuming a linear response allows to estimate (input) respiratory system impedance (Zrsin), a complex quantity whose real and imaginary parts are respiratory resistance (Rrs) and reactance (Xrs), such that $Zrsin = Rrs + j\,Xrs$[32]. Commonly, only the magnitude $(Rrs^2 + Xrs^2)^{1/2}$ is referred to as impedance Zrs, complemented by the phase angle arctan(Rrs/Xrs), and we also adopt this convention in the rest of the paper. Clinically, these quantities have proven useful to assess and study lung diseases[21], and this has been investigated in a plethora of studies. However, apart from a few exceptions, only the mean values of Zrs, averaged over a few breathing cycles, are used[28]. It is the aim of the present study to investigate whether more involved analysis techniques allow to distinguish between asthma, COPD or controls better with increased accuracy, compared to by just using mean Zrs.

For completeness, we mention that factors influencing Zrs include exercise, posture and sympathetic tone[4]. Within-breath measurements of Zrs show a marked bi-phasic pattern that is the result of volume and flow dependence[9,55] with slightly distinct behavior for inspiratory and expiratory phases[39]. This modulation is partially attributed for by interference with the larynx and glottis[51], but also hints at hysteresis, i.e., dependence on the volume history, in the respiratory system[59]. On top of these, Zrs is influenced by ventilatory inhomogeneities[17], airway

calibre[43], interactions between airways[61], and various artifacts, the most problematic being the upper airways shunt[5,38].

Separation of Zrs into contributions from various airways and tissue components is possible by the use of mathematical compartment models[12]. However, this necessitates the use of complex excitation signals and estimation procedures, and imposes limits on the temporal resolution, which is on the order of the inverse of the excitation frequency. Therefore, single frequency excitation signals are preferred to track Zrs in real-time, and the role of deep inspirations and methacholine challenge has been studied thereby[29,49,55].

From the above we postulate that the temporal course of Zrs should contain valuable information that is differentially affected by respiratory diseases. In contrast to most studies that consider significance probabilities of differences on the group level, we estimate predictive accuracy when classifying individual subjects with regard to group membership. Employing cross-validated linear discriminant analysis (LDA) allows to quantify the amount of functionally differentiated information contained in the Zrs signals.

We present results obtained by two distinct approaches. In the first, Zrs time series are considered to arise from a stochastic process and we analyze their "noise" component[9] by distributional analysis of fluctuations, similar to the analysis of Que et al.[45]. Based on recent recommendations, we employ state-of-the-art maximum likelihood estimation[8,60]. Furthermore, we employ detrended fluctuation analysis (DFA)[41] to quantify the extent to which Zrs signals are correlated in time and how self-similar these fluctuations appear; to our knowledge this method has not been applied to impedance measurements before.

In a second approach, the Zrs signal is considered to arise from a dynamical system. Thereby we will assume the respiratory system to contain a deterministic component. Moreover, we will make the assumption that the dynamical evolution of this deterministic component changes in distinct ways in airways influenced by respiratory diseases. This idea of a "dynamical disease" postulates that diseases correspond to changes in control parameters that subsequently modify the dynamical behavior of the system[18]. Utilizing the methods of nonlinear time series analysis[25.52], we quantify differences between phase space reconstructions of normalized impedance time series.

Methods

Subjects

We analyzed data collected during the baseline phase of a previous study[50]. The population consisted of 13 patients with intermittent and mild persistent asthma, characterized by GINA guidelines as step 1 and 2[19], 12 patients with mild to moderate COPD, characterized as GOLD type I and II[20], and 12 healthy control subjects.

The patients with asthma were all non-smokers or ex-smokers with less than five pack years exposure, and had a history of episodic wheezing or chest tightness. Baseline forced expiratory volume in 1s (FEV_1) was more than 70% of predicted, and the provocative concentration of methacholine for a 20% fall in FEV_1 (PC_{20}) was less than 8 mg/mL. All asthma patients were atopic, which was determined by one or more positive skin prick tests against 10 common aeroallergens.The COPD patients were all smoker or ex-smokers with more than ten pack years exposure and had a history of chronic cough or dyspnea. Their FEV_1/FVC ratio was less than 70% predicted post bronchodilator, and the reversibility of FEV_1 by salbutamol was less than 12% of predicted.All patients were clinically stable, used β2-agonists on demand only, and had no history of respiratory tract infection or other relevant diseases up to two weeks prior to the study. None of the asthma or COPD patients had used inhaled or oral corticosteroids up to three months prior to the study.The healthy controls had no history of respiratory symptoms and were non-smokers or ex-smokers with less than five pack years exposure. Baseline FEV_1 was more than 80% of predicted and PC_{20} methacholine was more than 16 mg/mL. They showed no positive reaction to the skin prick test.The baseline characteristics of the three groups have been listed previously[50]. The institutional review board for human studies approved the protocol, and the subjects gave their written informed consent before entering the study.

Forced oscillation method

A forced oscillation device (Woolcock Institute, Australia) with a fixed oscillation frequency of 8 Hz and an amplitude of ±1 cm H_2O was used after calibration with tubes of known resistance. Subjects breathed through an antibacterial filter with a resistance of 0.2 cm H_2O/L/s and respiratory flow was measured by a Fleisch pneumotachograph (diameter 50 mm, Vitalograph Ltd, Maids Moreton, UK). Differential pressure was measured by a ±2.5 cm H_2O solid-state transducer (Sursense DCAL4; Honeywell Sensing and Control, Milpitas, USA). A similar transducer with a higher range (±12.5 cm H_2O) was used to measure mouth pressure[50]. The pressure generator was connected by a 50 cm long inertive tube.

The pressure and flow signals were digitized at 400 Hz, and the resulting time series were transformed to the time-frequency domain by a maximal overlap discrete Fourier transform that acts as a band-pass filter for the frequency 8 Hz (filter width 100 samples, i.e., a time window of 0.25 s). Time- and frequency-dependent complex respiratory impedance Zrsin was then estimated, based on the hypothesis that random errors occur in both pressure and flow signals, by a total least squares (TLS) fit, which is equivalent to maximum likelihood estimation. Further details can be found in the online supplement of reference 50.

Time series and artifact removal

Respiratory impedance was measured three times during 60 s of tidal breathing on four distinct days, during the course of a few weeks, yielding 12 time series per subject. Before further analysis the signals were downsampled to 16 Hz, i.e., the Nyquist frequency for the applied pressure oscillation, to decrease the computational effort (this is admissible here where we were mainly interested in properties for larger time scales). Artifacts were removed automatically by a custom-written algorithm. Each respiratory cycle was considered individually and rejected if: (i) negative resistance values occurred or the TLS estimation did not converge (indicative of artifacts), or (ii) flow limitation occured. The latter was detected if the difference in mean Xrs for inspiratory and expiratory phases was greater than 3 $cmH_20/L/s$ or if the difference in peak Xrs for inspiratory and expiratory phases exceeded 8 $cmH_20/L/s$ (reference 10; with slightly adjusted threshold values). These events occurred infrequently and only a few percent of breathing cycles were thereby rejected.

Analysis

Statistical properties

In addition to mean and standard deviation (SD) over time, the time series were characterized by two functions of higher moments. Excess kurtosis, defined as the fourth central moment divided by the square of the variance minus 3, was used as a measure of peakedness of the distributions, and skewness, defined as the third central moment divided by the third power of SD, was used as a measure of asymmetry of the distributions. Distributions of parameters are shown in box- and whisker plots for each group separately (with labels A: asthma, C: COPD, N: controls), that also show significance probabilities of differences in mean (unpaired two-sample nonparametric Wilcoxon tests) for all pairwise contrasts and additionally between controls and a "diseased" group D that comprises both asthmatics and COPD patients. Statistical significance was tested at the p=0.05 level.

Power-law analysis

Fluctuations of Zrs were defined by

$$Zvar(i) = (Zrs(i)-mean(Zrs))^2, \quad (1)$$

as in reference 45. The probability distribution f(x) of Zvar follows a power-law if it is proportional to x^{α}, with an exponent $\alpha \leq -1$. Thereby, it is assumed that power-law behavior is only present for values $x \geq x_{min}$, for a finite threshold $x_{min} > 0$. The probability density of the two-parameter power-law distribution is then

$$f(x) = -(\alpha+1)x_{min}^{(\alpha+1)}x^{\alpha}, \quad (2)$$

which is also known as the Pareto distribution. The value of α is determined by its maximum likelihood estimate[60]. The threshold x_{min} is estimated by optimizing the goodness-of-fit of the estimated power-law with the empirical distribution of the data, quantified by the Kolmogorov-Smirnov statistic D[8]. To avoid spurious minima for very short tails, only values of x_{min} were considered such that at least 200 values of Zvar remained in the tail. **Figure 1** shows an example of the estimation procedure. To compare with results obtained in reference 45, we also extrapolate the probability distribution for unit fluctuations, i.e., consider $\log_{10} f(1) = \log_{10}(-\alpha-1) + (\alpha+1)\log_{10}(x_{min})$.

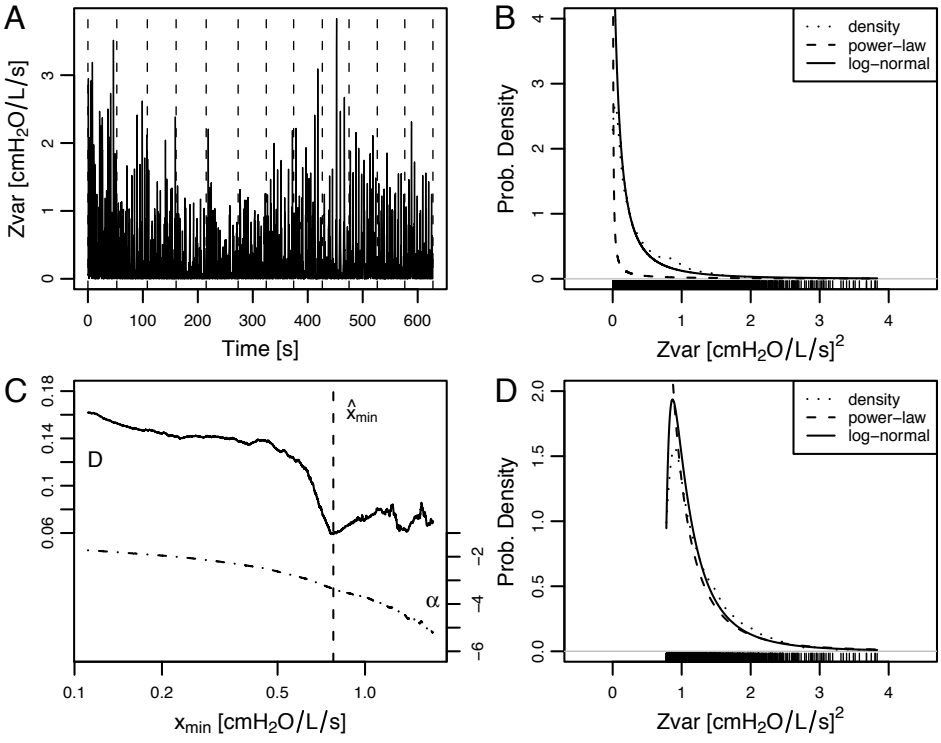

Figure 1. Power-law behavior in the distribution of Zrs fluctuations (**Zvar, eq. (1)**).
(A) Time series of Zvar; the broken lines indicate the 12 distinct measurements. **(B)** Estimate of the probability density of Zvar (dotted line). The three-parameter log-normal distribution (solid line) fits the data best, the power-law distribution (broken line) fits the tail somewhat better but is not a good model for small values of Zvar. **(C)** Estimated power-law exponent α (right coordinate axis) and Kolmogorov-Smirnov statistic D (left coordinate axis). The optimal value of the threshold x_{min} is located at the minimum of D (broken vertical line). **(D)** Estimate of the probability density of Zvar for the tail with $x \geq x_{min}$ (dotted line). The (truncated) power-law distribution (dotted line) fits the tail best, but the (three-parameter) log-normal distribution (solid line) is comparable.

To test whether the power-law hypothesis is a viable description of the data, we employed the permutation test of Clauset et al.[8]. A number of synthetic dataset were generated with similar distributional properties than the original data. The power-law estimation was repeated independently for each of these and the fraction of values of the Kolmogorov-Smirnov statistic D that were larger than its value for the observed data forms the significance probability (p-value) of this test. For a conservative test, if $p < 0.05$ the null hypothesis of power-law behavior was rejected, otherwise power-law behavior was accepted to be a valid model of the experimental data. We employed this test with 100 Monte Carlo datasets for each Zvar time series, but did not correct for multiple comparisons (n=35). Note that accepting the null hypothesis does not prove the existence of power law behavior, but the compatibility of this model, i.e., that there is no significant evidence against the power-law hypothesis.

As an alternative to the power-law behavior we fitted two- and three-parameter log-normal distributions[47] both to the tail and to the complete set of Zvar values, and considered the three-parameter truncated power-law distribution[1] that introduces an additional upper bound x_{max} and allows for general exponents $\alpha < 0$. The significance of an improvement in fitting the data was assessed by the asymptotic likelihood ratio test.

Detrended fluctuation analysis

Individual time series of Zrs were submitted to DFA[41] to assess self-similar behavior. Thereby, the deviations of the X(i)=Zvar(i) time series from the mean were first integrated,

$$Y(i) = \sum_{j=1}^{i} (X(j) - \bar{X}) \tag{3}$$

The profile Y(i) was then divided into N_s=int(N/s) nonoverlapping segments of length s. Since the length N of the time series is usually not a multiple of the scale s, a short part at the end of the profile may remain. In order not to disregard this part of the series, the procedure was repeated starting from the opposite end, leading to a total of 2N segments[24]. For each such segment either a linear (DFA1) or a quadratic (DFA2) trend was estimated by least-squares regression and subtracted from the data. The squares of the residuals were integrated and divided by the length to yield the mean-square error $F^2(j,s)$ of the j-th segment at scale s. The second order fluctuation function is given by the total root-mean square (RMS) error,

$$F(s) = \left(\frac{1}{2N_s} \sum_{j=1}^{2N_s} F^2(j,s) \right)^{1/2} \tag{4}$$

Scaling behavior of F(s) was assessed in a double logarithmic plot for a variety of scales. The smallest scale considered was s=8 samples. The scale was successively increased by a factor of $\sqrt{2}$ until s was at most half of the length of the time series.

Scaling behavior results in a line in the double logarithmic plot of F(s) against s, which was estimated by weighted linear regression. Weights proportional to the inverse of scale were used to account for the fact that the larger scales are estimated from less segments, with increased variance. The occurence of two separate scaling regimes (crossover phenomenon) was tested by assuming each scale individually as a change point and separately fitting lines to F(s) for both scales smaller and for scales larger. If the introduction of a change point led to an decrease in total RMS error of more than a factor 5, the existence of a crossover phenomenon was assumed. **Figure 5A** shows an example.

Wasserstein distances

The time series were alternatively assumed to be projections of a dynamical system, considering Rrs and Xrs as two dynamical variables. In the simplest case the two-dimensional scatterplot of Rrs versus Xrs captures the dynamical range of the impedance over the course of time. To allow for comparisons of distinct systems and to put the two parameters on equal standing, we normalized their marginal distributions independently to zero mean and unit variance and considered the resulting two-dimensional joint probability distributions (**Figure 2**).

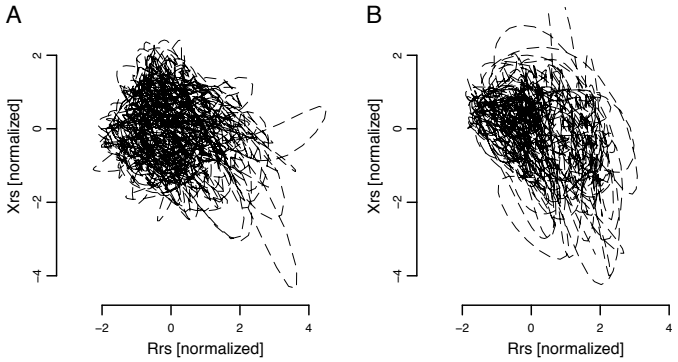

Figure 2. Wasserstein distances of mixed resistance (Rrs) and reactance (Xrs) time series. **(A)** Trajectory of Rrs/Xrs in a 1+1 dimensional embedding for a subject with asthma, normalized to zero mean and unit variance for each component separately. To improve visualization, stippled lines instead of individual sample points are shown. **(B)** Analogous trajectory for a subject with COPD. The Wasserstein distance quantifies the work needed to transform one of these embeddings into the other, and thereby robustly quantifies differences in shape. For this example, the mean Wasserstein distance was 0.412 ± 0.029 SE (bootstrapped 25 times from 512 sample points each).

To quantify differences between two such empirical probability distributions we employed quadratic Wasserstein distances[35,36]. Assume X(i) and Y(i) (i=1, ..., N) to be two vector-valued time series (here with normalized Rrs and Xrs as its two components) of the same length N. In this case the quadratic Wasserstein distance between X and Y reduces to the optimal transportation problem

$$W_2(X,Y) = \arg\min_{\sigma} \left(\sum_{i=1}^{N} \| X(i) - Y(\sigma(i)) \|^2 \right)^{1/2} \quad (5)$$

over all permutations σ of the set {1,2,...,N}. The value $W_2(X,Y)$ can be interpreted as the average work (in terms of distance) needed to transform one distribution into the other after optimally matching sample points. This convex optimization problem was solved numerically by a network simplex algorithm[30].

Since the computation of $W_2(X,Y)$ is time-consuming, and the two series X and Y do not have the same length in general, we bootstrapped all distances by randomly sampling 512 points from both probability distributions a total of K=25 times each, using the mean of the K distance values so obtained as an unbiased estimator of $W_2(X,Y)$.

Nonlinear analysis

Generalizing the approach of the previous section, we recovered the state space of the impedance dynamics by delay vector embedding, as common in nonlinear time series analysis[25,52]. Let X(i) be a (normalized) time series, then

$$X_m(i) := (X(i) , X(i+\tau), \ldots, X(i+(m-1)\tau)) \quad (6)$$

is a m-dimensional delay vector with time lag τ. The lag guarantees that successive components of $X_m(i)$ contain essentially new information on the state of the system, and a convenient choice for the optimal lag τ is the decorrelation time, i.e., the lag at which the autocorrelation function (ACF) of X falls to 1/e.

The embedding dimension m should be large enough to unfold the dynamics properly, and was here estimated by the method of false nearest neighbors (FNN)[26]. If the distance between a point $X_m(i)$ and its nearest neighbor $X_m(k(i))$ increases by a factor of more than 10 when increasing the embedding dimension m, then $X_m(k(i))$ is classified as a (relative) false neighbor. The optimal embedding dimension was assessed as the value of m where the fraction of false nearest neighbors dropped below the 1 percent threshold.

Combing two m-dimensional delay vector series for Rrs and Xrs into a 2m dimensional vector-valued time series X^{2m}, we did again employ the Wasserstein distances (see above) in 2m dimensions to robustly quantify differences in the shape of their impedance dynamics for each pair of two subjects. This approach has been pioneered by Murray and Moeckel[34].

Multidimensional scaling

Measuring distances between phase space distributions for each pair of subjects (n=35) leads to a n-by-n matrix of distances Dij = W_2(Xi,Xj). To analyze these statistically it is advantageous to represent these distances by points in an Euclidean space R^k, which is achieved by metric multidimensional scaling (MDS)[3]. The coordinates of points in the space R^k are ordered according

to their contribution to the variance of the distances, and can often be assigned functional meaning or lead to the formulation of hypotheses about it[35]. This dimension reduction method introduces misrepresentation errors, which were quantified by stress-per-point; for the i-th point with k coordinates x_i the stress-per-point is the average of $(||x_i-x_j||-D_{ij})^2$ over all points $j \neq i$.

Discriminant analysis

In order to assess the predictive value of observed differences between groups of subjects, we employed linear discriminant analysis (LDA) as implemented in the statistical software R[56]. A linear discriminant function is obtained that projects each of the k data items (from c distinct groups) onto r numerical scores, where r=min(c-1,k), corresponding to the optimal linear separation between the classes. The latter is given by a linear combination that maximizes a generalized signal-to-noise ratio, leading to r canonical variates that identify a vector subspace containing the variability across the c classes. From the r scores from data items are classified according to the nearest group centroid, measured in terms of Mahalanobis distance. These centroids are estimated from the sample covariance matrix and LDA makes the assumption that there are no differences between the group covariances. Here we will use LDA on the k MDS coordinates (derived from the Wasserstein distances), an idea that goes back to reference 2.

Results are given in terms of total accuracy, i.e., the fraction of correctly identified group memberships, and for binary classifications additionally in terms of sensitivity and specificity relative to the diagnosis of a positive condition (mostly COPD in the following) against a negative (mostly asthma). Let TP denote the number of true positives, FP the number of false positives, and TN, FN analogously for the negatives. Then

$$\text{sensitivity} = \text{TPR} = \text{TP}/(\text{TP}+\text{FN}), \quad \text{specificity} = 1\text{-FPR} = \text{TN}/(\text{FP}+\text{TN}), \tag{7}$$

where TPR is the true positive rate and FPR the false positive rate. For such binary classifications receiver-operator characteristics can be given that elucidate the relationship between FPR and TPR, depending on the decision boundary.

Cross-validation and worst-case classifier

All classification methods suffer from the fact that resubstitution accuracy, i.e., predictive accuracy of the data used to derive a classifier, invariably improves as the prediction model becomes more complex. Eventually the prediction model can distinguish data items by irrelevant chance differences, i.e., by using the noise in measurements to classify. To control for such overfitting we employed leave-one-out cross-validation. When using LDA on coordinates derived by MDS of Wasserstein distances, it is then necessary to estimate the MDS coordinates of a time series in the MDS space defined by the remaining n-1 time series. This is achieved by estimating the fallible scalar products by a nonlinear optimization procedure[54] and is the reason why we consider metric MDS here instead of nonmetric generalizations[3].

All classification accuracies should be judged in the light of (i) the null accuracy, achieved by randomly "guessing" class membership, of 1/3=0.33, and (ii) the worst-case classification accuracies, classifying all subjects as belonging to the largest group, which were 13/35=0.37 (all three groups) and 13/25=0.52 (asthma versus COPD)

Multiple response permutation procedures

Apart from being used for classification, the Wasserstein distances were assessed in terms of how significant the separation between classes was. This was achieved by a multiple response permutation procedure (MRPP)[33]. The test statistic δ is the overall weighted mean of within-group means of all pairwise distances. It is first calculated under the known true group assignment, and then by randomly permuting the group labels. The number of values of δ obtained under the permutation distribution that are larger than the original δ defines the significance probability (p-value) of this test. All MRPP tests were run with 10^5 randomly generated permutations.

Additionally, the chance-corrected within-group agreement

$$A = 1 - \delta/E[\delta] \tag{8}$$

was determined, where E[δ] is the average of δ over the permutations. This quantifies the proportion of the distances explained by group structure and is analogous to a coefficient of determination.

Software used

All data analysis was performed in the statistical computing environment R[46], utilizing the *MASS* package[56], the *fractal* package (W Constantine W and D Percival, unpublished), the *vegan* package (J Oksanen, R Kindt, P Legendre, B O'Hara and MHH Steven, unpublished) and the *ROCR* package[48]. Note that the implementation of DFA in *fractal* is faulty and was replaced by a custom-written algorithm which can be obtained from the authors on request. The two matrices of Wasserstein distances (one shown in **Figure 6A**) are available as Data Supplements in the online journal.

Results

Statistical results and predictive accuracies

The mean values of respiratory impedance (Zrs), resistance (Rrs) and reactance (Xrs) are shown in **Figure 3.** There were significant differences between COPD patients and asthmatics (p=0.035) or healthy controls (p=0.014) in mean Zrs, but not between asthmatics and controls (p=0.61). This increase in mean Zrs was reflected in significant decreases in Xrs for the COPD

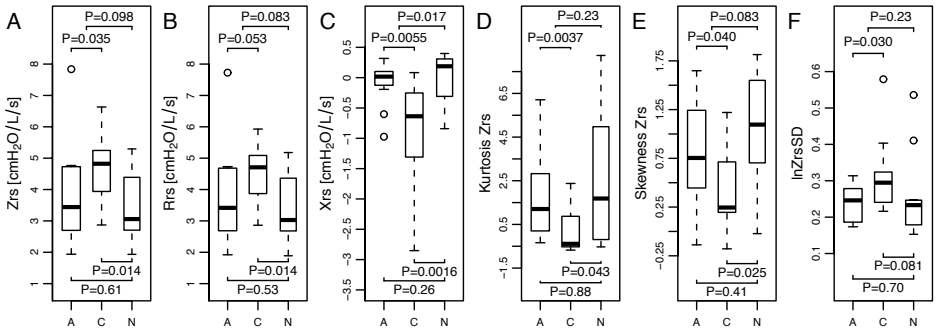

Figure 3. Statistical summary of impedance data.
Significance probabilities are indicated in the boxplots. Groups are labeled (A: asthma, C: COPD, N: healthy controls).**(A)** Mean values of respiratory impedance Zrs, **(B)** resistance Rrs and **(C)** reactance Xrs. Higher moments of Zrs: **(D)** kurtosis and **(E)** skewness. As a measure of variability, **(F)** lnZrsSD.

group (p=0.0055/p=0.0016) and to a lesser degree in increases in Rrs (p=0.053/0.014). Although some of these differences were highly significant (e.g., mean Xrs for COPD versus controls), classification of COPD versus controls by LDA of mean Xrs only achieved an accuracy of 0.77, and accuracies of 0.73 for both mean Rrs and Zrs. Discrimination between asthma and COPD was possible with accuracies of 0.72 (mean Xrs) and 0.64 (mean Rrs or Zrs).

Mean lnZrs and lnZrsSD were considered for completeness (to compare with the results of references 11 and 44) and differed significantly between asthma and COPD (p=0.046/p=0.030) and led to similar classification (lnZrs) or slighly worse (lnZrsSD) classification results. No significant differences between asthmatics and controls were detected (p=0.49/p=0.70), consistent with the findings of reference 11, but COPD showed marginal increases in lnZrs (p=0.017), but not in lnZrs variability (p=0.081). Regarding higher moments, there were significant differences in kurtosis (peakedness) and skewness (asymmetry) of Zrs between COPD and the other groups (A: p=0.0037/p=0.040, N: p=0.043/p=0.025). These allowed for an accuracy of 0.68 (kurtosis) and 0.64 (skewness) when distinguishing asthma from COPD.

Power-law analysis

Estimated power-law exponents and thresholds are shown in **Figure 4** for the Zvar fluctuations. There were significant differences in exponents between COPD and asthma or controls (p=0.018/p=0.023), with slightly smaller exponents α in COPD, indicating that relatively larger fluctuations in COPD occur less often than in asthmatics or healthy controls. The threshold x_{min} was significantly higher in COPD than for the other groups (p<0.008). The latter could be explained by a slightly larger variability in COPD (cf. **Figure 3F**), and resulted in a classification accuracies of 0.72 (asthma/COPD) and 0.73 (COPD/controls). The logarithm of the extrapolated probability density at Zvar=1.0 $(cmH_20/L/s)^2$ showed a significant increase for COPD with respect to the other groups (p<0.002; **Figure 4D**), and this indeed resulted in classification accuracies of 0.80 (against asthma) and 0.82 (against controls), whereas classification of asthma

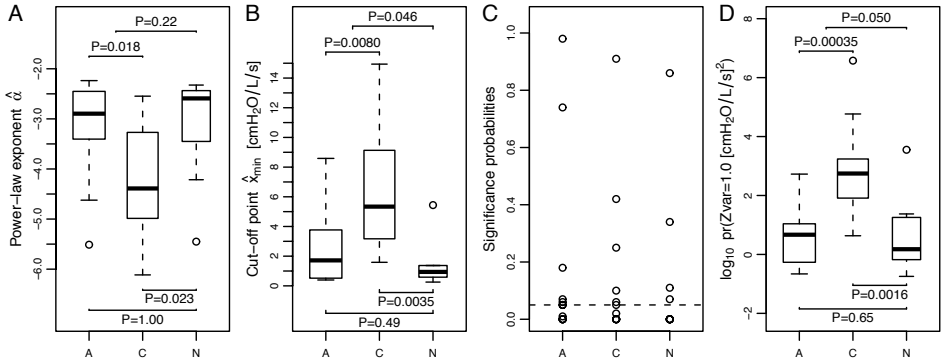

Figure 4. Estimated power-law behavior.
Exponent **(A)** and onset threshold **(B)** in a group-wise comparison (A: asthma, C: COPD, N: healthy controls). Significance probabilities are indicated. **(C)** Evidence for power-law behavior by permutation test (100 bootstraps; see text for details) and estimated intercept **(D)** to compare with the findings of Que et al[45]. The null hypothesis of power-law behavior is not rejected (0.05 level, broken line) for 17 out of 35 cases, indicating compatibility with the power-law hypothesis.

versus controls was more or less random with an accuracy of 0.57, in contrast to the earlier findings of reference 45.

The null hypothesis of power-law behavior was accepted for 17/35 subjects, distributed almost evenly among the three groups (**Figure 4C**). Fitting a three or two parameter log-normal distribution to the same data in the tail of the Zvar distribution resulted in a comparable fit, with an insignificant likelihood ratio in all cases. The truncated power-law distribution generally resulted in the best fit of the tail data, but also with an insignificant increase in likelihood. It can be concluded that both log-normal and power-law distributions are plausible models for the tails (compare **Figure 1D**).

Regarding the complete set of Zvar values, the log-normal distribution achieved the largest likelihood. However, this is still not a significant improvement over the power-law distribution, which seems to model the more extreme values better (cf. **Figure 1B**)

Detrended fluctuation analysis

Although DFA is to a certain extent robust against the removal of segments[7,31], it is advisable to analyze only contiguous data. In contrast to the previous section we therefore employed DFA on individual 1 min measurements only, with about 800-900 data points each. The quasi-periodic nature of the breathing cycle introduces spurious residuals due to the detrending for scales that are smaller than the average breathing period[37]. In particular, the scaling exponents obtained from DFA1 and DFA2, respectively, differ largely. Above this period, the scaling behavior changes (**Figure 5A**) and results for DFA1 and DFA2 mostly agree. We chose the values of the more robust DFA1 for the larger scales and averaged this for all 12 measurements of each subject.

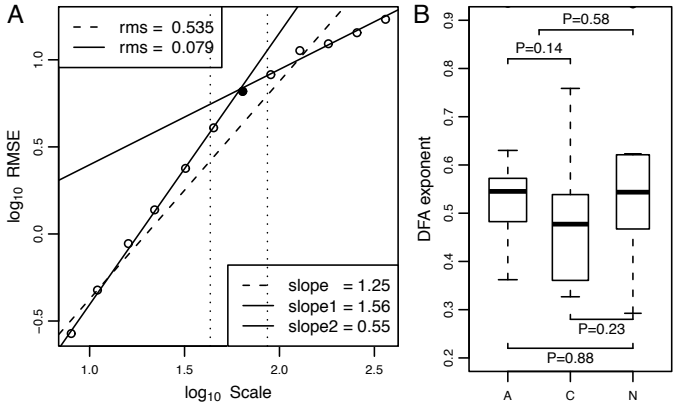

Figure 5. Detrended fluctuation analysis of Zrs time series.
(A) Crossover phenomenon exhibited by most Zrs time series. Vertical lines (dotted) correspond to the timescale of the average breathing period (5.4 s, 86.4 values) and half its value. Variation of RMSE with scale should lead to a linear scaling relationship (stippled line), which is not the case here globally. Introducing a change point, the scales below and above it are fitted separately, and the lowest sum of RMS errors for the weighted linear regression lines is achieved in the situation shown (location of changepoint filled). The slope of the two lines (slope1/slope2) is the respective scaling exponent. **(B)** Group-wise comparison of DFA exponents (slope2) for the larger time scales.

There were no significant differences in scaling exponents between groups (p>0.14), compare **Figure 5B**, although it seems that scaling behavior might be closer to the value 0.5 of Brownian motion for COPD than for asthma and controls.

Distance-based analysis

The Wasserstein distances for the 1+1 dimensional joint probability distributions of Rrs and Xrs (both normalized independently) allowed to distinguish asthma and COPD with above chance accuracies. The eigenvalue distribution indicated that the distances can be represented reasonably well in k=2 dimensions (explaining a fraction 0.65 of their variance) with an intermediate misrepresentation error (a fraction of 0.10 of the average distance) more or less uniformly distributed among the points. The group structure in this functional space was significantly clustered (p=0.002), but a within-group agreement A=0.06 suggests that only about 6% of the variance among distances is explained by group structure. Including more reconstruction dimensions, the cross-validated classification accuracies decreased. LDA in two MDS dimensions classified with accuracy 0.51 in the full contrast, and with accuracy 0.76 between asthma/COPD. The best asthma/COPD classification was achieved in just one-dimension, leading to an accuracy of 0.80, sensitivity 0.83 and specificity 0.77.

Nonlinear analysis

Assuming the Rrs and Xrs time series to result from an underlying dynamical system, the proper time lag for delay vector reconstruction was assessed by the decorrelation time of the

autocorrelation functions, with mean values of 14±13 SD and 12±9 SD, respectively. Due to the high variability, and since stochastic contributions to the signal might bias these estimates to larger values, the median values of 10 (for Rrs and Xrs alike) were chosen, corresponding to 0.625 s as characteristic time scale of the impedance dynamics, i.e., about one-fourth of a breathing cycle. Assessment of false nearest neighbours (FNN) suggested an embedding dimension of three to four (FNN Rrs: relative 3.8±0.6 SD, Xrs: relative 3.9±0.7 SD) and m=3 was chosen, as balancing the influence of noise seemed more important than improved resolution of the dynamics.

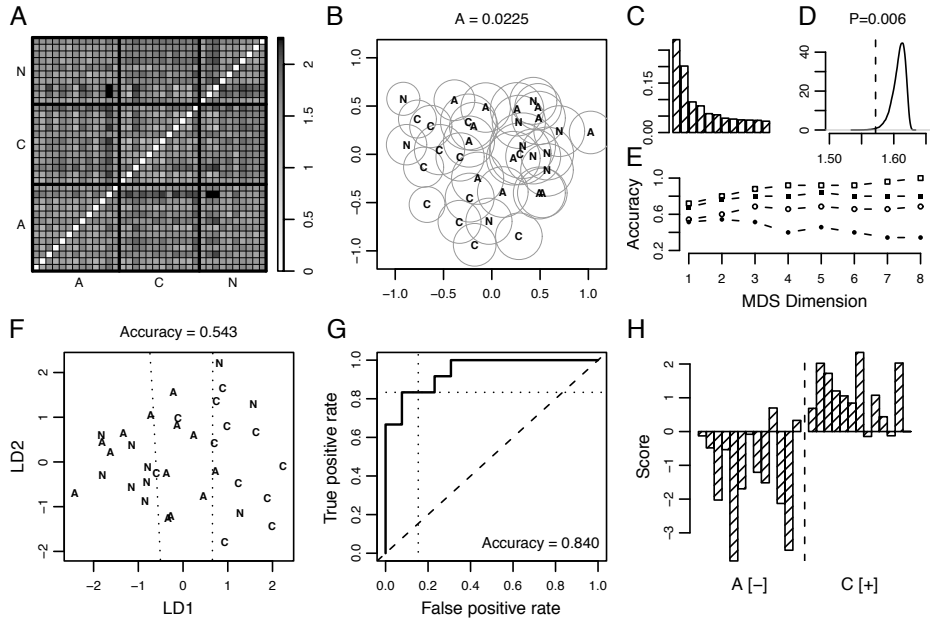

Figure 6. Distance-based analysis of normalized probability distributions of combined Rrs and Xrs in a 3+3 dimensional embedding.
(A) Distance matrix for all subject-wise comparisons of signals by Wasserstein distances. **(B)** Two-dimensional MDS representation depicting the two major axes of variation. Each subject is represented by a point in this functional space (A: asthma, C: COPD, N: healthy controls), approximating the measured distances as close as possible. Misrepresentation error is indicated (by circles whose area is equal to stress-per-point), and the value of A (eq. (8)) given. **(C)** Relative size of eigenvalues of the scalar product matrix obtained from the distances, as a measure of explained variance. **(D)** Distribution of the MRPP statistic δ. The value of δ for the original groups is indicated (broken line). The fraction of permutations to the left of this is the significance probability (P-value) that the distances are not structured with respect to group membership. **(E)** Classification accuracies with respect to the number of MDS dimensions. Circles: full contrast, cross-validated (●) and resubstitution accuracy (○). Squares: asthma/COPD contrast, cross-validated (■) and resubstitution accuracy (□). **(F)** Discriminant functions for full classification in a two-dimensional reconstruction. The decision boundaries are indicated (dotted lines). **(G)** Receiver-operator-characteristic for the discrimination of asthma (negatives) against COPD (positives) in the optimal five-dimensional reconstruction. Sensitivity (true positive rate, 0.83) and specificity (1-false positive rate, 0.85) for the optimal threshold are indicated (broken lines), resulting in an accuracy of 0.84. **(H)** Corresponding discriminant scores for all subjects.

As in the 1+1 dimensional case, we quantified differences between the 3+3 dimensional delay vector distributions of Rrs (three delay coordinates) and Xrs (the other three coordinates), normalizing the two to zero mean and unit variance independently. Results are shown in **Figure 6**. The eigenvector distribution (**Figure 6C**) suggests that although two dimensions captured most of the variance of the distances (a fraction of 0.48), quite a few more are needed to represent the distances faithfully. Indeed, for a two-dimensional MDS reconstruction the misrepresentation error was relatively large (**Figure 6B**, about a fraction of 0.16 of the average distances). The group structure was still significant (p=0.006; **Figure 6D**), even under a lower within-group agreement A=0.023. The classification accuracies for the full contrast attained their maximum of 0.54 for two dimensions and for the asthma/COPD contrast in five reconstruction dimensions (**Figure 6E**), which resulted in an accuracy of 0.84, sensitivity 0.83 and specificity 0.85 in five reconstruction dimensions (**Figure 6G-H**).

Discussion

We have attempted to distinguish between asthma, COPD and healthy controls either by assessing fluctuations and scaling behavior, or by robustly comparing probability distributions of the dynamical behavior of Rrs and Xrs, implicitly assuming an underlying dynamical system.

Main findings

Evidence for the controversial power-law hypothesis[45] was found. That is, the power-law null hypothesis could not be rejected for 17/35 subjects at the 5 percent significance level, and their Zvar fluctuations were consistent with power-law behavior when this was fitted to the tail of the distributions. However, although there was no evidence against the power-law distribution, the two- or three-parameter log-normal distribution described the tail almost equally well. Without larger time series it is difficult to conclude this issue.

Consistent with earlier findings we did not detect significant changes between power-law exponents for asthmatics versus controls ($p > 0.99$), but COPD showed significantly different exponents. In contrast to Que et al.[45], we did not detect significant differences in power-law intercepts between asthmatics and controls, although the extrapolated intercepts were significantly larger for COPD. The earlier analysis was done with methods that are now known to be potentially unreliable[60], and these earlier findings should therefore be carefully reconsidered. The final analysis of this data in[44] in terms of log-normal distributions is consistent with our results.

Detrended fluctuation analysis did not obtain any significant differences in scaling exponents. Moreover, the scaling exponents were close to 0.5, the value obtained for Brownian motion, although it seems that exponents in COPD might be somewhat closer to 0.5 than for asthma and controls, indicating increased randomness. Due to the quasi-periodic nature

of breathing, DFA exhibited two different scaling regimes. For scales lower than the average breathing period the DFA exponent depends strongly on the method of detrending and has to be considered unreliable. For scales above the breathing period, we have considered the exponents to be reliable. At even larger timescales it seems likely that respiratory impedance exhibits yet another crossover into scaling exponents significantly larger than 0.5, since similar phenomena were found in time series of tidal breathing parameters[6]. However, to conclude this issue necessitates much longer recordings than presently available.

The distance-based analysis between probability distributions further evidenced that there exist subtle differences in respiratory properties. Since the Rrs and Xrs time series were normalized for this analysis, only differences in the shape of the dynamical behavior were thereby quantified. Interestingly, these were sufficiently large to allow robust (cross-validated) classification of 80 percent of subjects in the asthma/COPD contrast, which was better than classification based on mean Zrs, lnZrsSD, skewness and kurtosis of Zrs, etc., individually. Only the estimated intercept of the power-law behavior of the tail resulted in similar classification between asthma and COPD. This finding confirms our hypothesis that the two diseases differentially affect the within-breath dynamics of respiratory impedance.

Regarding the 3+3 dimensional delay embedding and its Wasserstein distances, these did only improve classification marginally (to 84 percent in the asthma/COPD contrast) with respect to the 1+1 dimensional distributions. In the light of the largely increased noise level (due to the sparseness of delay vectors) this indicates that such delay reconstruction might possibly incorporate additional information that is not present when only using the 1+1 dimensional distributions. However, it seems necessary to reduce the influence of noise considerably before this could be convincingly demonstrated and is left to future studies.

In contrast to the largely successful classification of asthma versus COPD, predictive classification of asthmatics versus healthy controls was problematic, due to large overlap between those two groups, both in the statistical properties of Zrs as well as in their dynamical behavior.

Clinical implications

The distance-based time series analysis of respiratory impedance led to a correct distinction between patients with asthma and COPD in at least 80 percent of cases, i.e., the forced oscillation technique can capture discriminative aspects of airway disease from recordings during simple, tidal breathing. The differential diagnosis of asthma and COPD can be a challenge in clinical practice[23] as it appears that both diseases can exhibit overlapping pathological and physiological features[16]. Part of this overlap may be due to real co-existence of both diseases in some patients, whereas in others the current diagnostic techniques apparently fail to pick up the difference[16]. Alternatively, it cannot be excluded that the classical diagnoses of asthma and COPD are not capturing existing and clinically relevant phenotypic differences among patients with obstructive airway diseases. Our data suggest that characterization of patients based

on evidence from objective measurements, such as respiratory impedance time series, may become more informative in clinical assessment than traditional diagnostic labels.

Our patients were used as a so-called 'training-set'[27], thereby being representative of gold-standard patients of either disease. The presently observed discriminative capacity of the dynamic time series analysis is, therefore, promising with regard to differential diagnosis and monitoring of asthma and COPD. The fully non-invasive nature of the measurements, without the requirement of artificial breathing maneuvers, offers great prospect for clinical application in chronic, often elderly patients. However, this still requires validation experiments, in independently recruited patients with an intention-to-diagnose[27], in order to establish the diagnostic accuracy of the dynamic time series analysis of respiratory impedance in clinical practice.

Conclusion

Instead of evaluating Zrs signals with respect to the mechanical properties of airways, we have attempted a stochastic and nonlinear analysis. The distance analysis showed that there exist subtle differences in these signals that can only be partially attributed to statistical properties, such that the nature of the differential behavior of respiratory impedance is mostly unclear. Self-similar fluctuations were not detected in the signals at this timescale, and the evidence for power-law behaviour was not conclusive. The distance-based analysis however has proved useful and detected clustering in functional space, indicating functional changes in respiratory impedance that are characteristic with respect to disease. Reverse-engineering of these patterns is a possibility, since the interpolation properties of Wasserstein distances[58,ch.5,1], in combination with nonlinear modeling techniques[22], principally allow to compute characteristic dynamical models for each group of subjects. This would potentially lead to further insights into how the respiratory system is affected in disease and possibly also allow to assess and track changes in airway caliber over the course of time.

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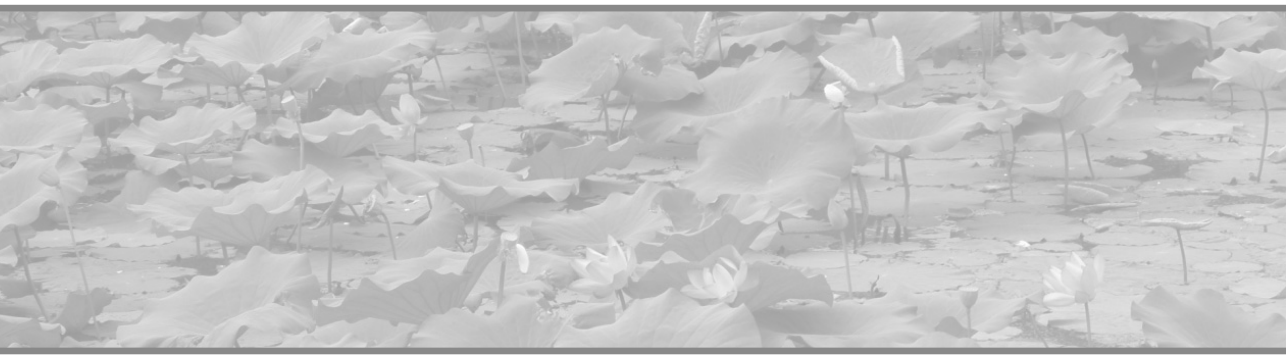

Chapter 9

Summary and general discussion

9.1. Introduction

Asthma is defined as a chronic inflammatory disorder, associated with airway hyperresponsiveness. As described in the general introduction deep inspirations modulate airway responses to bronchoconstrictor agents and can therefore be considered as a very strong endogenous protective mechanism against airway narrowing. The airways of asthmatic patients respond differently to lung inflation by deep inspiration resulting in less bronchodilation of constricted airways. The loss of this protective mechanism may be involved in the pathophysiology of asthma. The studies described in this thesis were all directed at either further elucidating the (patho)physiological mechanism underlying deep inspiration-mediated bronchoprotection, or to restoring this protective mechanism in asthma. A summary of the conclusions of the studies will be followed by a general discussion and directions for future research.

9.2. Summary

In chapter 2 and 3 the results are shown from an observational study to examine airway responses to deep inspiration in patients with asthma, COPD and healthy control subjects. We found that the bronchodilatory effect of deep inspiration is impaired in patients with asthma, and even more markedly impaired in patients with COPD, as compared with healthy subjects. It appears that the loss of deep inspiration-induced bronchodilation is not asthma specific. Whether it occurs from the same pathophysiological mechanism is unclear. However, in asthma, this impairment was related to the inflammatory cell numbers within the submucosa and airway smooth muscle layer. Namely, reduced deep inspiration-induced bronchodilation was associated with increased numbers of mast cells within the airway smooth muscle bundles and increased $CD4^+$ lymphocyte counts in the bronchial lamina propria (chapter 2). This association was not found in patients with COPD. In addition, in asthma impaired bronchodilation by deep inspirations was related to a lower level of expression of calponin, desmin, and MLCK expression in bronchial biopsies, whereas increased airway hyperresponsiveness was associated with a higher level of expression of α-SM-actin, desmin, and elastin in bronchial biopsies. Thus, airway hyperresponsiveness, lung function, and airway responses to deep inspiration are associated with the level of expression of some, but not all, of the smooth muscle contractile and structural proteins, as well as the composition of the extracellular matrix within the airway wall (chapter 3).

In chapter 4, we examined the effect of airway wall edema on airway responses to deep inspiration. This was done in patients with mitral valve regurgitation needing mitral valve repair surgery, since they are expected to have pulmonary congestion in the absence of allergic airway inflammation. We expected to observe an increase in respiratory resistance following a deep inspiration, as is seen in patients with asthma with spontaneous airways obstruction[1],

as a result of fluid flux across the airway wall as a result of large transpulmonary pressures during deep inspiration. However, in patients with mitral valve disease a deep inspiration did not lead to bronchoconstriction, although lung function was diminished as compared to healthy subjects. Thus, this suggests that airway wall edema *per se* may not lead to bronchoconstriction following deep inspiration (chapter 4).

Chapters 5, 6 and 7 show the results of studies addressing ways to restore the beneficial protective mechanism of deep inspirations against airway narrowing. First, we investigated whether maximal reduction of airway inflammation by a course of high-dose oral prednisone on top of inhaled corticosteroids in well controlled asthmatic patients would further improve deep inspiration-induced bronchodilation. Indeed, the degree of deep inspiration-induced bronchodilation at a given level of airways obstruction was improved by this treatment regimen. The improvement was not related to concurrent reductions in airway hyperresponsiveness or to changes in the level of exhaled NO (chapter 5). Second, anticholinergic drugs have been shown to protect against airway wall remodelling in animal models of allergic inflammation. It inhibited both airway smooth muscle proliferation, as well as smooth muscle contractility. We hypothesized that 21 days of treatment with tiotropium would improve lung function, airway hyperresponsiveness and deep inspiration-induced bronchodilation by inhibiting allergy-driven airway inflammation in asthma. Treatment with tiotropium did not significantly improve deep inspiration-induced bronchodilation or airway hyperresponsiveness, but a significant effect on baseline bronchial tone was observed. This was shown by improvements in both FEV_1/FVC ratio and FEV_1 % predicted (chapter 6). And finally, in chapter 7 we aimed to dilate constricted airways by using passive inflation with positive-pressure inflation in mild asthma. In addition, we examined whether this would restore bronchodilation by lung inflation in patients with asthma who showed no significant bronchodilation by an active deep inspiration. We showed that airways obstruction can indeed be reduced by positive-pressure inflation of the lungs in asthma, comparable to active deep inspiration. And this could also be achieved in patients with asthma who were not capable of significantly reducing airways obstruction by an active deep inspiration (chapter 7).

Finally, in chapter 8 we have used time series of the respiratory system impedance data from the studies shown in chapter 2 and 3 to study the respiratory system with high temporal resolution. Fluctuations in time series of respiratory system impedance measurements by forced oscillation technique exist in the healthy lung, and the variability of these fluctuations differs from an asthmatic lung[2]. We hypothesized that the temporal course of respiratory system impedance is differentially affected by respiratory disease. In addition, we considered the impedance signal to arise from a dynamic system, and assumed that this system contains a deterministic component, that changes in distinct ways in different respiratory disease. In other words, a specific respiratory disease corresponds to changes in the control parameters that modify the dynamic behavior of the system. Using cross-validated linear discriminant analysis on mean Zrs, Rrs and Xrs enabled us to classify COPD vs. healthy controls, and asthma vs. COPD.

The distance-based analysis shows further evidence that there are differences in respiratory properties between asthma and COPD. Differences in the shape of the dynamic behavior were sufficient to correctly classify 80% of subjects to be either asthmatic or COPD patient (cross-validated). These findings are in keeping with the hypothesis that the two diseases affect the within-breath dynamics of respiratory impedance in a different way (chapter 8).

Taken together, we may conclude that specific inflammatory cells within the airway submucosa and airway smooth muscle layer, as well as the level of expression of specific smooth muscle and extracellular proteins may alter the airway responses to deep inspirations, but

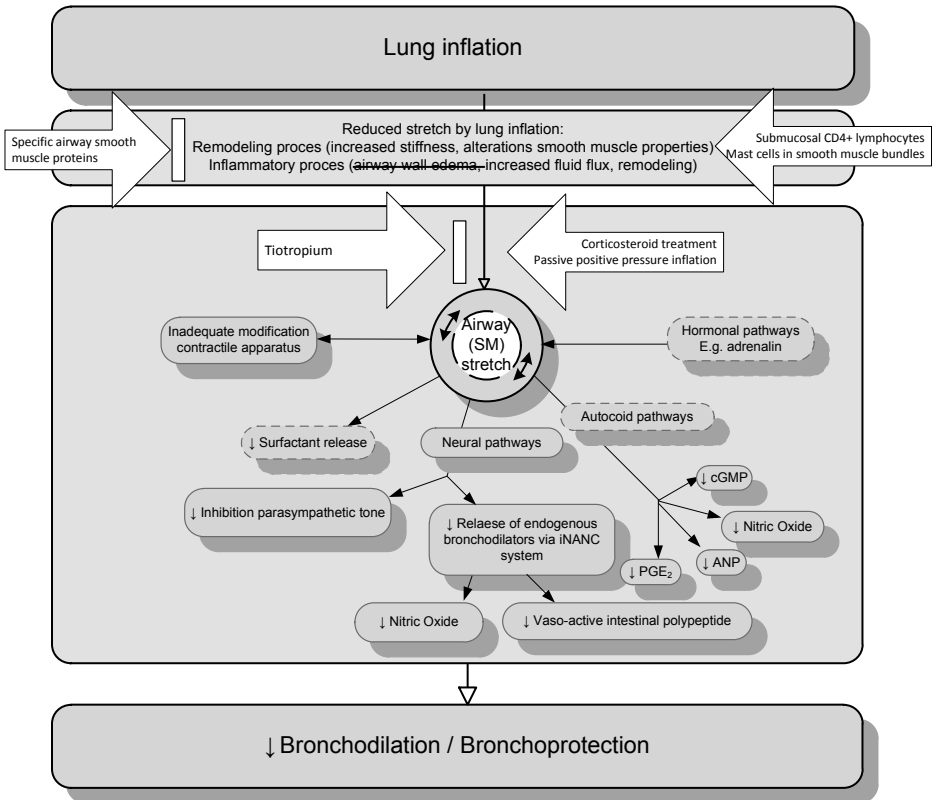

Figure 1. Possible mechanisms explaining impaired beneficial effects of deep inspiration.
This figure is derived from **figure 5 in chapter 1**. It shows the possible mechanisms explaining impaired beneficial effects of deep inspiration. In addition, the arrows show the main results of our studies and where they interact with these mechanisms. First, the level of expression of specific airway smooth muscle proteins, nor treatment with tiotropium-bromide, seems to interfere with the amount of stretch of the airways that is induced by lung inflation, and thus the degree of bronchodilation upon deep inspiration is not affected. On the other hand, the number of CD4+ lymphocytes in submucosa and mast cells in smooth muscle bundles may affect the remodeling and/or inflammatory process in the airway wall and thereby affect airway distensibility upon deep inspiration. Maximal steroid therapy and passive positive pressure inflation increased bronchodilation, most likely through increasing airway stretch upon deep inspiration.

edema *per se* does not. In addition, steroids and passive positive pressure inflation improve bronchodilation of constricted airways by deep inspiration, whereas tiotropium does not exert this effect (**Figure 1**). And finally, studying impedance data with high temporal resolution shows that the dynamic behavior of the respiratory system differs between asthma and COPD. These findings will be further discussed in relation with other published data in the following sections.

9.3. Deep inspiration-induced bronchodilation, airway inflammation and steroid responses

In chapter 2 we found that in asthma increased numbers of CD4 + lymphocytes within the submucosa, and mast cells within the airway smooth muscle bundles, are related to less bronchodilation following a deep breath. Furthermore, in chapter 5 we showed that high dose prednisolone on top of inhaled corticosteroids significantly improved deep inspiration-induced bronchodilation of constricted airways. Are these phenomena linked? In other words is the improvement by steroid therapy likely a result of inhibition of submucosal CD4+ lymphocytes or mast cells in the smooth muscle layer?

9.3.1. CD4+ lymphocytes

First, steroids have been shown to reduce CD4+ lymphocytes. For example, in allergic asthmatic patients pre-treatment with prednisone (3 days prednisone 30mg twice daily) inhibited influx of inflammatory cells, specifically eosinophils, basophils and CD4+ lymphocytes, in broncho-alveolar lavage fluid after segmental allergen challenge[3]. Another placebo-controlled study showed that a longer treatment protocol with oral corticosteroids (prednisolone 20mg o.d. for 2 weeks followed by 10mg for 4 weeks) significantly reduced asthma symptoms, albuterol usage, and increased FEV1 in steroid naïve asthmatic patients. This was accompanied by a reduction in submucosal eosinophils (81%), mast cells (62%) and CD4+ lymphocytes (68%), whereas placebo treatment resulted in no significant changes in cell numbers in the bronchial biopsies. The reduction in CD4+ lymphocytes was related to a decrease in airway hyperresponsiveness[4]. Also, inhaled corticosteroids suppress airway inflammation by ongoing allergen challenge with low-dose house dust mite in mild asthmatic patients, especially eosinophils, neutrophils, and lymphocytes[5].

On the other hand, in patients with asthma withdrawal of inhaled corticosteroid therapy (1760 mcg/day), until peak flow dropped by 25%, FEV1 dropped by 15% or 6 weeks elapsed, showed that half of the patients exacerbated and half of them did not. In both groups eosinophils increased in bronchial biopsies by steroid withdrawal, but CD4+ lymphocytes increased only in the groups that exacerbated[6]. This suggests that the steroid withdrawal-induced increase of CD4+ lymphocytes exert a greater influence on the airway function than eosinophils, and may

explain why we did not find a relationship between eosinophils and airway responses to deep inspiration. Taken together, it is plausible that steroid-induced improvement in bronchodilation by deep inspiration is mediated through inhibition of submucosal CD4 lymphocyte infiltration.

9.3.2. Mast cells in airway smooth muscle bundles

What about mast cells in airway smooth muscle bundles? In asthma, as compared to non-asthmatic subjects, the airway smooth muscle bundles are infiltrated by increased numbers of mast cells and lymphocytes[7]. Mast cells are recruited to the airway smooth muscle bundles by numerous chemo attractants (stem cell factor, chemokines, cytokines, CCR3 and CxCR1)[8]. In contrast, non-asthmatic airway smooth muscle releases mediators that inhibit mast cell migration towards asthmatic airway smooth muscle[8]. The presence of mast cells within the airway smooth muscle layer has been associated with airway hyperresponsiveness in asthma, but not in subjects with eosinophilic bronchitis[9,10]. Furthermore, dexamethasone-treated smooth muscle cells were less effective in enhancing C3a-induced mast cell degranulation and thus may lead to less bronchoconstriction[11]. But whether mast cell migration to airway smooth muscle is reduced by steroid treatment has not been investigated yet. Therefore, no direct conclusions can be made whether steroid-treatment in asthma improves deep inspiration-induced bronchodilation by reducing mast cell infiltration or degranulation in airway smooth muscle bundles.

9.3.3. Steroids and airway smooth muscle cell relengthening

Although the beneficial effects of corticosteroids have been attributed to suppression of airway inflammation it is possible that steroid-treatment exerts direct action on airway smooth muscle cells as well and thereby improves airway hyperresponsiveness and deep inspiration-induced bronchodilation. It has been shown that force fluctuations imposed on contracted airway smooth muscle cells in vitro results in relengthening of the cells[12], and is regulated through the p38MAPkinase signaling pathway[13]. Corticosteroids inhibit p38MAPkinase signaling and indeed augment force fluctuation-induced relengthening of airway smooth muscle cells *in vitro*[14]. Steroid-treatment could therefore have improved deep inspiration-induced bronchodilation by augmenting relengthening of airway smooth muscle cells upon stretch by inhibiting p38MAPkinase. However, this is still speculative since we did not measure p38MAPkinase in our studies.

9.4. Deep inspiration-induced bronchodilation and airway smooth muscle cells

As shown in the introduction, there are many hypotheses on the role of airway smooth muscle in the (patho)physiological of deep inspiration-induced bronchodilation (chapter 1; 3.1 and

4.2). By examining the relationship between airway responses to deep inspiration and the expression of structural and contractile markers of airway smooth muscle cells in bronchial biopsies of patients with asthma as shown in chapter 3 we aimed to further elucidate this role. We found that more bronchodilation by deep inspirations was related to higher levels of expression of calponin, desmin, and MLCK in bronchial biopsies, whereas increased airway hyperresponsiveness was associated with a higher level of expression of α-SM-actin, desmin, and elastin. Are our results in support of the previously presented hypotheses?

9.4.1. Plasticity

Plasticity refers to the ability of airway smooth muscle cell to adapt its contractile apparatus to the length at which it is activated. Smooth muscle cells with longer actin filaments show a more elastic behavior by increasing the range of myosin-actin overlap, which can generate the same amount of force after being stretched[15] and may therefore increase airway hyperresponsiveness. A higher level of α-SM-actin expression in bronchial biopsies may indicate more actin monomers that can form longer actin filaments by polymerization in the asthmatic inflammatory environment, and thus supports the relationship we found with airway hyperresponsiveness. In contrast, we would have expected to find increased levels of α-SM-actin expression in patients with less bronchodilation after a deep breath, but found the opposite. Increased α-SM-actin expression, in combination with increased levels of desmin and elastin expression, may therefore determine force generation upon stimulation but not stretch-induced bronchodilation. It is possible that more factors, such as extra-cellular matrix composition, airway wall thickness, or number of alveolar attachments determine the net bronchodilatory effect of lung inflation.

9.4.2. Increased smooth muscle tone

Interestingly, we found a positive relationship between FEV_1% predicted and the positive staining intensity for the smooth muscle proteins calponin, desmin, and MLCK, as well as a negative relationship between these markers and deep breath–induced reduction in respiratory resistance. It has been shown that cultured smooth muscle cells with increased tone produce enhanced levels of contractile proteins, such as myosin, MLCK and desmin, when cultured under cyclic stretch conditions[16]. The positive correlations between FEV_1% predicted and deep breath–induced bronchodilation could therefore reflect the effect of stretch on contractile protein production in these patients with asthma, rather than the influence of increased expression of these contractile markers on airway function. Most *in vitro* studies on the effect of length changes have been performed in isolated tracheal smooth muscle strips and provide length oscillations[17,18]. A recent study in an isolated intact bronchial airway with luminal volume oscillations showed no bronchodilation by increasing oscillatory strains. It demonstrates the difficulty of testing airway wall contraction and dilation at airway smooth muscle cellular level, since the lung has such a unique geometry and structure[19]. Therefore, human *in vivo*

experiments, such as the recent studies, are most likely to reflect the "true" pathophysiology of the airways in asthma.

9.5. Intervention studies

Although research on (patho)physiological mechanisms is both interesting and necessary, in clinical research the inevitable question is "what is the clinical relevance?'. Since deep inspirations have shown to provide a physiological protective mechanism against airway narrowing (chapter 1) and that this beneficial effect is lost in asthma this clinical relevance is very close. Namely, if this mechanism can be restored in asthma it could provide the best combat to bronchospasms, and thus symptoms, and possibly reduce the need of current medical treatment. Unfortunately, restoring deep inspiration-induced bronchodilation and/or bronchoprotection in asthma of which the (patho)physiological mechanism is not completely understood may not be possible yet. On the other hand, trying to restore this mechanism could also lead to new insights on underlying pathophysiology.

Several studies in this thesis have intervened with parts of the pathophysiological mechanism in order to at least improve deep inspiration-induced bronchodilation. We studied the effect of maximal steroid treatment (chapter 5), anticholinergic treatment (chapter 6), and passive inflation (chapter 7) on airway responses to deep inspiration. Each of these studies interacted with possible causes of impaired stretch of the airways by lung inflation **(Figure 1)**. Maximal steroid treatment could reduce airway wall remodeling as well as inflammatory induced airway wall thickening. Anticholinergic treatment has shown to reduce allergy driven airway smooth muscle proliferation and contractility. And, passive inflation may stretch the airways more effectively plus to a larger extent by 'pushing from the inside' with positive pressure inflation than what can be achieved by 'pulling from the outside' with negative intra-thoracic pressure. In addition, anticholinergic treatment and passive inflation could also have changed the effect of stretch on the contractile apparatus of the smooth muscle cell, although we have not directly measured that. Below, these interventions are discussed, as well as bronchial thermoplasty, a novel option in asthma treatment.

9.5.1. Maximal steroid treatment

The improvement in deep inspiration-induced bronchodilation, as measured by improvement in M/P ratio, in asthma patients by the use of systemic steroid treatment on top of maintenance therapy with inhaled steroids was a novel finding. Previously, several studies already showed that inhaled corticosteroid in steroid-naïve asthma patients improved bronchodilation following deep inspiration[20,21], whereas another study found no effect of inhaled corticosteroids on the bronchodilatory effect of a deep inspiration in a study with asthmatic patients with mild-to-severe airway hyperresponsiveness. The methods of assessing the airway responses to deep

inspiration differed among these three studies, as well as the dose and type of inhaled steroids used. This emphasizes the need for more standardized measurements of deep inspiration-induced bronchodilation, preferably measurements not including a deep breath such as the forced oscillation technique. Furthermore, the results implicate that patients with asthma, who are regularly treated with inhaled corticosteroids, can have residual airway inflammation[22,23] that impairs the mechanical properties of the airways even during clinically stable episodes. Patients with impaired airway responses to deep inspiration, although being clinically stable, may therefore be more at risk for the development of exacerbations. As airway responses to deep inspiration tend to be related to asthma severity[24] and the severity of breathlessness[25], long-term studies are required in order to address the prognostic implications of impaired responses of the airways to deep inspiration in asthma patients.

9.5.2. Anticholinergic treatment

Recent meta-analysis indicating that usage of long-acting beta-agonists without concomitant inhaled corticosteroids increases the risk of asthma-mortality[26,27] has given way for developing studies on alternative bronchodilatory treatment in asthma, such as tiotropium. Interestingly, anticholinergics have also been shown to inhibit airway smooth muscle proliferation and contractility in models of allergic inflammation[28,29]. Therefore, we hypothesized that in allergic asthma daily use of tiotropium reduces smooth muscle contractility, thereby improving lung function, airway hyperresponsiveness and deep inspiration-induced bronchodilation. Treatment with tiotropium did not significantly improve deep inspiration-induced bronchodilation or airway hyperresponsiveness, but a significant effect on baseline bronchial tone was observed. Improvements in lung function have been shown by tiotropium added to conventional therapy in severe asthma. Long-term effects of anticholinergic treatment on airway hyperresponsiveness to a non-cholinergic agent has not been investigated before, although acute protection against histamine-induced bronchoconstriction has been shown by pre-treatment with ipatropium-bromide in normal subjects[30], possibly via inhibition actelycholine after vagal reflex release by histamine[31]. An inhibitory effect of tiotropium on airway smooth muscle cells has been found in ovalbumin challenged guinea pigs and not in non-challenged animals[28]. This might be due to an increased level of acetylcholine by an augmented acetylcholine release after allergen challenge, reduction of inhibitory M2 receptors, or nonneuronal release of acetylcholine in conditions of allergic inflammation[32]. In the absence of inflammatory mediators or growth factors increased levels of acetylcholine may not result in structural changes within the airways and thus no inhibitory effect of tiotropium can be observed. Therefore it is possible that in these mild asthmatic patients a lower level of inflammatory mediators was present within the airways and thereby reducing the inhibitory effect of daily tiotropium inhalation. Since we did not include induced sputum or bronchial biopsies as outcomes of this study we cannot relate this to the improvement in lung function we found in our study. Taken together, tiotropium has bronchodilatory effects both acute and prolonged in mild asthmatic patients

and may therefore be considered as alternative long-acting bronchodilator. Whether the observed changes in lung function and airway hyperresponsiveness are a result of sustained bronchodilation by muscarinic receptor blockage or inhibition of allergen-induced remodeling remains to be established.

9.5.3. Improving stretch by positive pressure lung inflation

We postulated that airway wall distension can be improved by manipulation of the intrathoracic pressures by passive lung inflation in patients with asthma. Mechanical inflation of the lungs would induce stretch of the airways without large subatmospheric intrathoracic pressures and could therefore prevent extravasation of fluid in inflamed airways. In addition, the inflated volume may open closed airways, thereby redistributing tethering forces of the parenchyma on the airway wall. Indeed, we showed that positive pressure inflation resulted in more bronchodilation, as compared to an active deep inspiration in patients with asthma. The opposite has been done as well by adding a resistance to a deep inspiration, which resulted in lower airway conductance in patients with asthma as compared to a regular deep inspiration, but not in healthy subjects[33]. Since we did not measure transpulmonary pressures it is difficult to discriminate whether the bronchodilatory effect of positive pressure inflation was a result of actual dilation of the airway tree or reopening of closed airways that could not be opened by active deep inspiration. The relationship between the increase in inspiratory volume and improvement in bronchodilation following positive pressure inflation, suggests that a lower inspiratory volume by any cause could result in reduced bronchodilation following lung inflation. Instead of improving lung inflation-induced stretch of the airways, the reverse has been investigated as well. For example by strapping the chest wall, which reduced lung function[34], increased airway constriction to methacholine[35], and indeed reduced deep inspiration-induced bronchodilation[36]. Also, several studies on airway responses to deep inspiration have been performed in obese patients, who are known to have reduced inspiratory capacity. Obese asthmatics, but not lean asthmatics or non-asthmatic obese subjects, show an increase in airway resistance following a deep breath[37]. Also, obese non-asthmatic subjects show no bronchoprotective effects of deep inspiration as compared to non-obese non-asthmatic subjects, indicating that obesity alone alters airway mechanics during deep inspirations[38], possibly as a result of lower inspiratory volume capacities. Our data suggest that the tethering forces of the parenchyma during active deep inspiration, possibly in relation to the magnitude of the inspired volume, are not strong enough to adequately stretch the airway wall, which may be overcome by positive-pressure inflation, and could be applicated to non-asthmatic disorders resulting in lower inspiratory volumes as well, such as obesity.

9.5.4. Bronchial thermoplasty

Airway smooth muscle has been mentioned to be the appendix of the lung, in other words it may be useless and possibly harmfull[39]. A new intervention called bronchial thermoplasty

delivers controlled thermal energy to the airway wall resulting in prolonged reduction/loss of airway smooth muscle mass[40]. There is some evidence that it improves overall asthma control, as shown by improvement in quality of life, asthma symptoms, severe exacerbations, and health care utilization[41-44]. In addition, initially it appeared that airway hyperresponsiveness was reduced by bronchial thermoplasty as well[45], but placebo controlled studies could not demonstrate this. In dogs, bronchial thermoplasty led to increased airway size in both relaxed and contracted states over a normal range of inflation pressures[46], and reduced airway closure to methacholine[47] as shown with high-resolution CT scans. Whether airway responses to deep inspiration are affected by bronchial thermoplasty remains to be investigated. However, it can be postulated that if indeed airway smooth muscle content is decreased in the treated airways, that in pharmacologically constricted airways the contraction can more easily be overcome by lung inflation, and might even be comparable to the response of airways to deep inspiration in healthy controls.

9.6. Dynamic behavior of the respiratory system

Another approach to lung disease is by system biology[48,49]. The lung is a complex organ, and system biology integrates information from all levels of structure and function of the system[50-52]. A characteristic of biological systems is their temporal behavior. Studying the temporal behavior of the lung by analysis of fluctuations in airway function over time is a way to approach lung disease as a biological system[53]. Variability analysis of the respiratory system has been successfully applied on impedance data[2] and peak flow measurements before[54]. We used time series of impedance measurements by forced oscillation technique to examine this temporal behavior in asthma and whether this method allowed us to discriminate between asthma and COPD. The non-linear distance-based time series analysis of respiratory impedance led to a correct classification of patients with asthma or COPD in at least 80% of cases. This suggests that the within-breath dynamics of respiratory impedance as captured by forced oscillation technique provides alternative information on characterizing patients with obstructive lung disease. In addition, this method could be used to monitor airway disease and evaluate treatment.

9.7. Future research

The studies described in this thesis have helped to provide more insight into the (patho)physiological mechanism of deep inspiration-induced bronchodilation and have shown that standard therapy (glucocorticosteroids), and novel therapy (passive positive pressure inflation) improves the airway responses to deep inspiration. However, many questions remain before fully understanding the bronchoprotective effects of deep inspirations. First of all, the contractile

apparatus of the airway smooth muscle and its function over different lengths needs to be clarified in order to understand how deep inspiration-induced stretch can influence it. Second, the interaction of inflammatory cells with smooth muscle cells and the functional consequences should be addressed. And finally, direct interventional studies intended to improve airway function and airway responses to deep inspiration should be extended. These considerations and the results of the studies in this thesis led to these research questions:

- Are mast cells within the smooth muscle layer influenced by standard asthma therapy, and is this related to improvements in airway function and deep inspiration dynamics?
- Is it useful to create new therapies targeting specific airway smooth muscle proteins, such as actin or desmin to reduce airway hyperresponsiveness?
- Is extra-cellular matrix deposition in airway smooth muscle bundles responsible for impaired airway responses to deep inspiration?
- Do steroids also have an acute effect on deep inspiration-induced bronchodilation *in vivo* by stretch-induced relengthening of airway smooth muscle cells as has been shown *in vitro*?
- Could passive positive pressure inflation be useful in clinical practice, such as in the emergency unit for acute asthma exacerbations?
- Would passive positive pressure inflation improve airway responses to deep inspiration in other pulmonary diseases or disorders associated with lower inspiratory volume capacity as well?
- Does tiotropium indeed inhibit allergy driven airway smooth muscle proliferation *in vivo* in asthma?
- Do changes in the temporal behavior of airway function provide information on the course of the disease and treatment effects?

9.7. Final remarks

Deep inspirations provide a strong physiological endogenous protection against airway narrowing, which is lost in asthma, and other pulmonary diseases such as COPD. This thesis has provided further insight in the (patho)physiological mechanism underlying the beneficial effects of deep inspirations, as well as treatment options to restore this mechanism.

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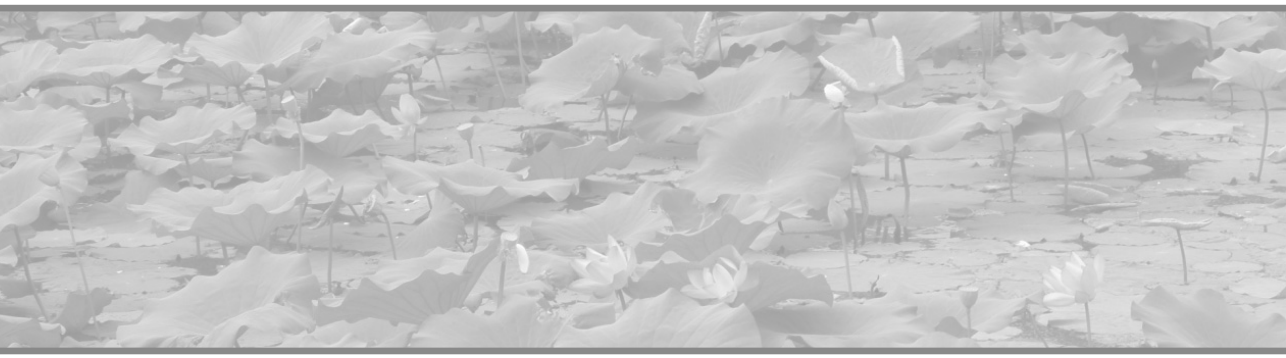


Chapter 10

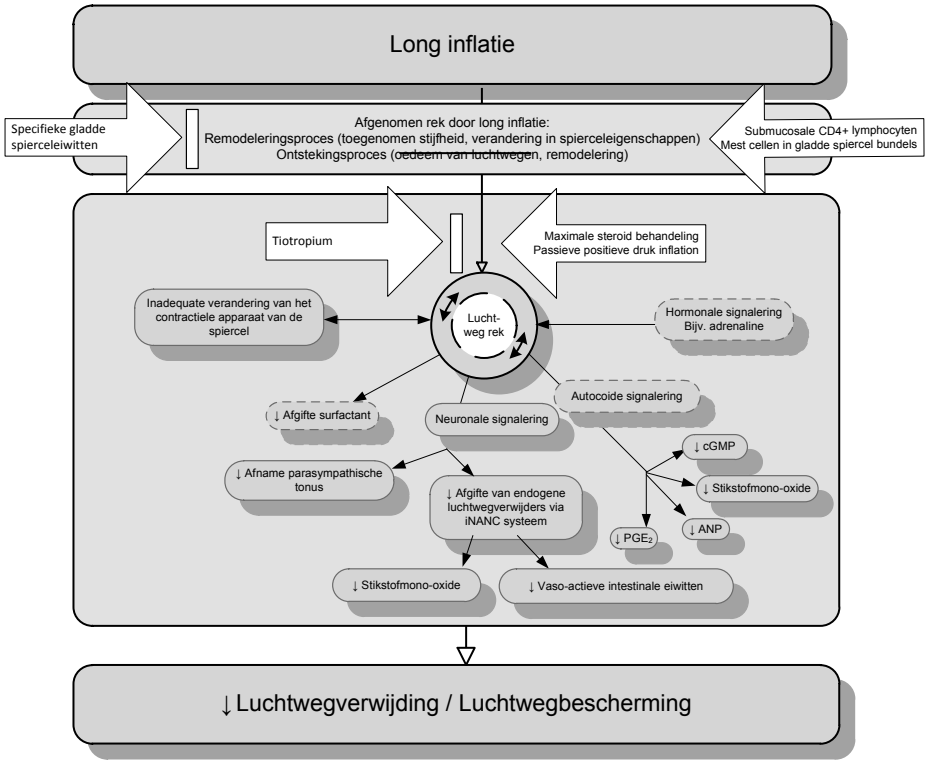
Nederlandse samenvatting en discussie

10.1. Inleiding

Astma wordt gedefinieerd als chronische ontstekingsziekte, geassocieerd met bronchiale hyperreactiviteit, wat leidt tot terugkerende episodes van piepen, benauwdheid en hoesten. Bronchiale hyperreactiviteit is een term, die wordt gebruikt wanneer de luchtwegen te veel en te makkelijk vernauwen als reactie op een uitlokkende stimulus, zoals allergenen of sigarettenrook. Een diepe inademing kan de reactie van de luchtwegen op uitlokkende prikkels beïnvloeden, leidend tot minder luchtwegvernauwing en kan daarom worden beschouwd als een sterk beschermingsmechanisme tegen luchtwegvernauwing. Patiënten met astma hebben een chronische ontsteking van de luchtwegen. Deze ontsteking uit zich in de aanwezigheid van bepaalde ontstekingscellen (zoals mest cellen, eosinofielen en lymfocyten) in de luchtwegwand, maar ook door structurele veranderingen als reactie op de chronische ontsteking (remodelering). Zowel de aanwezigheid van ontstekingscellen, als de remodeleringsprocessen hebben invloed op de mechanische eigenschappen van de luchtwegen.

Iedereen zucht ongeveer 3 tot 6 keer per uur. Over het algemeen wordt dit geïnterpreteerd als teken van moeheid en/of verveling, maar dit heeft ook een fysiologische betekenis. Tijdens een diepe inademing beweegt het middenrif omlaag en de borstkas omhoog en naar buiten. De longen vullen zich dan met lucht en daardoor worden de luchtwegen die tussen de longblaasjes in liggen opgerekt (**Figuur 3 uit hoofdstuk 1**). Nu blijkt, dat het regelmatig oprekken van de luchtwegen door een diepe inademing van invloed is op de reactie van de luchtwegen op een prikkelende stof. De luchtwegen van patiënten met astma reageren anders op een diepe inademing, in vergelijking tot controle personen, waardoor er minder luchtwegverwijding optreedt na de diepe inademing. Uit eerder onderzoek is gebleken, dat wanneer mensen zonder astma gedurende een periode van 20 minuten niet diep zuchten, de reactie van de luchtwegen op een normaal niet prikkelende stof ineens verergert. Deze reactie is dan ongeveer vergelijkbaar met die van een astma patiënt. Mag dezelfde persoon vlak voor het inademen van diezelfde prikkelende stof toch een aantal maal diep zuchten dan treedt er nauwelijks een reactie op. Diepe inademingen kunnen dus beschermen tegen luchtwegvernauwing. Bij patiënten met astma maakt het voor de reactie op de luchtwegvernauwende stof niet uit of zij wel of geen diepe zuchten vooraf hebben genomen. Uit meerdere onderzoeken blijkt dat bescherming tegen luchtwegvernauwing door een diepe inademing al bij mild astma verloren is gegaan. Anderzijds, wanneer men bij gezonde personen toch luchtwegvernauwing opwekt kan een enkele diepe inademing deze luchtwegvernauwing tijdelijk doen afnemen, terwijl bij patiënten met astma veelal geen effect wordt gezien en soms zelfs leidt tot verdere luchtwegvernauwing. Diepe inademingen kunnen dus ook luchtwegverwijding geven en ook dit effect is minder aanwezig bij patiënten met astma.

Het verlies van het beschermende mechanisme van een diepe inademing tegen luchtwegvernauwing zou dus een rol kunnen spelen in het ziekteproces bij astma. Hoe diepe inademingen beschermen tegen luchtwegvernauwing en waarom dit niet goed werkt bij patiënten met astma is tot op heden niet opgehelderd. Eigenlijk kan elke factor die voorkomt dat de luchtwegen worden opgerekt tijdens een diepe inademing (zoals vochtophoping in de luchtwegwand, chronische ontsteking of remodelering van de luchtwegwand) leiden tot een verminderd luchtwegverwijdend effect van een diepe inademing. Daarnaast kan elke factor die het effect van rek van de luchtwegen verminderd (zoals een veranderd contractiel apparaat, minder vrijkomen van luchtwegverwijdende stoffen (surfactant, stikstofmonoxide) en minder activatie van bepaalde neurogene of hormonale processen) ook leiden tot een afgenomen beschermend effect van diepe inademing (**Figuur 1**).



Figuur17. Deze figuur is een afgeleide van **figuur 5 uit hoofdstuk 1**. Het toont de mogelijke mechanismen die het verminderde effect van een diepe inademing kunnen verklaren. Daaraan toegevoegd zijn de blokpijlen waarin de belangrijkste resultaten uit de studies van dit proefschrift staan en geven daarbij aan op waarop zij aangrijpen. Zo is te zien dat tiotropium en specifieke spiercelmarkers niet aangrijpen op het veronderstelde onderdeel van het pathofysiologisch mechanisme. Aan de andere kant zie je dat de hoeveelheid CD4+ lymfocyten in de submucosa en mestcellen in de spierbundels van bronchus biopten waarschijnlijk te maken hebben met het remodeleringsproces en daarmee de rek van de luchtwegen door long inflatie beïnvloeden. Ook zie je dat maximale steroïd behandeling en passieve positieve druk inflatie juist de rek bevorderen.

De studies die in dit proefschrift beschreven worden, waren voornamelijk gericht op het verder ophelderen van dit mechanisme, hoe dit beschermende effect verloren gaat bij patiënten met astma en of het hersteld kan worden. Een samenvatting van de conclusies van deze studies wordt hieronder weergegeven, gevolgd door een algemene discussie en aandachtspunten voor verder onderzoek.

10.2. Samenvatting verschillende hoofdstukken

In hoofdstuk 2 en 3 worden de resultaten getoond van een observationele studie, waarin de reactie van de luchtwegen op een diepe inademing werd bekeken bij patiënten met astma, chronische obstructieve longziekte (COPD) en gezonde controlepersonen. We vonden dat het luchtwegverwijdende effect van een diepe inademing verminderd was bij patiënten met astma, maar zelfs nog sterker verminderd was bij patiënten met COPD, in vergelijking met de gezonde controlepersonen. Het verlies van het luchtwegverwijdende effect van een diepe inademing is dus niet astmaspecifiek, maar kan ook bij andere longziekten zoals COPD optreden. Of dit dan te maken heeft met hetzelfde onderliggende ziekteproces is nog niet duidelijk.

Bij de patiënten met astma en COPD hebben we ook kleine stukjes weefsel uit de luchtwegen gebiopteerd. In deze bronchus biopten hebben we gekeken naar ontstekingscellen in de weefsellaag (submucosa) direct onder het bekledende cellaag (epitheel) en naar mestcellen in de spierbundels. Daarnaast hebben we gekeken naar specifieke gladde spierceleiwitten en structurele eiwitten uit de luchtwegwand (extracellulaire matrix). Deze spierceleiwitten kunnen betrokken zijn bij de samentrekking van de spiercellen op een prikkel (actine, myosine, calponine, MLCK), maar sommige eiwitten zijn meer betrokken bij de stevigheid van het contractiele apparaat (desmine) of zijn gerelateerd aan proliferatie van de spiercellen (vimentine). Daarna werd bekeken of er een relatie was tussen het aantal ontstekingscellen, of de spierceleiwitten in deze biopten, en de reactie van de luchtwegen op een diepe inademing, bronchiale hyperreactiviteit en longfunctie.

Bij de patiënten met astma zagen we dat het verlies van het luchtwegverwijdende effect van een diepe inademing gerelateerd was aan het aantal ontstekingscellen in de bronchus biopten. Namelijk, een toename van de CD4+ lymfocyten in de submucosa en het aantal mestcellen in de spierbundels was gerelateerd aan een afname van luchtwegverwijding na een diepe inademing. Deze associatie werd niet gevonden bij patiënten met COPD (hoofdstuk 2). Daarnaast vonden we bij patiënten met astma, dat minder luchtwegverwijding na een diepe inademing gerelateerd was aan minder expressie van specifieke spierceleiwitten (calponine, desmine en MLCK) in de bronchus biopten, terwijl toegenomen bronchiale hyperreactiviteit en een betere longfunctie gerelateerd was aan meer expressie van andere spiercelmarkers (alfa-SM-actin en desmine) en extracellulaire matrix (elastine) in deze biopten. Dus bronchiale hyperreactiviteit, longfunctie en de reactie van de luchtwegen op een diepe inademing zijn geassocieerd met de

mate van expressie van sommige contractiele- en structurele spierceleiwitten, evenals met de compositie van de extracellulaire matrix in de luchtwegwand (hoofdstuk 3).

In hoofdstuk 4 hebben we het effect van vochtophoping (oedeem) in de luchtwegwand op de reactie van de luchtwegen op een diepe inademing bekeken. Uit eerder onderzoek is gebleken dat bij patiënten met spontane luchtwegvernauwing een diepe inademing kan leiden tot verdere luchtwegvernauwing. Een van de hypotheses hiervoor is dat de vochtophoping ten gevolge van de luchtwegwandontsteking verplaatst of verergert als reactie op de hoge drukken over de luchtwegen tijdens een diepe inademing. Dit onderzoek werd daarom gedaan bij patiënten met mitralisklepinsufficiëntie, die daarvoor een mitralisklep-hersteloperatie kregen, omdat bij deze patiënten oedeem van de luchtwegwand werd verwacht zonder de aanwezigheid van allergische luchtwegwandontsteking. We hadden verwacht dat de weerstand in het luchtwegsysteem zou toenemen na een diepe inademing, zoals ook wordt gezien bij astma patiënten met spontane luchtwegobstructie. Echter, bij patiënten met mitralisklepijden leidde een diepe inademing niet tot toename van de luchtwegsysteeemweerstand, ook al was de longfunctie wel afgenomen ten opzichte van gezonde controles. Dit suggereert dat oedeem in de luchtwegwand *op zich* niet hoeft te leiden tot luchtwegvernauwing na een diepe inademing (hoofdstuk 4) en dus dat de ontsteking in de luchtwegen zoals bij patiënten met astma ook een rol speelt.

In de hoofdstukken 5, 6 en 7 zijn de resultaten weergegeven van de studies waarin werd getracht het beschermende mechanisme van een diepe inademing tegen luchtwegvernauwing te herstellen. Allereerst werd onderzocht of maximale behandeling van luchtwegwand ontsteking met ontstekingsremmers, in dit geval een stootkuur prednison, bovenop de onderhoudsbehandeling met ontstekingsremmers via inhalatie (inhalatiesteroïden) bij patiënten met goed gecontroleerd astma zou leiden tot verdere verbetering van het luchtwegverwijdende effect van een diepe inademing. Inderdaad, de mate van luchtwegverwijding na een diepe inademing bij een gegeven mate van luchtwegvernauwing verbeterde onder dit behandelregime. Daarnaast zagen we een afname van de bronchiale hyperreactiviteit en uitgeademd stikstofmonoxide. Stikstofmonoxide wordt gezien als een maat van ontsteking in de luchtwegen die in de uitgeademde lucht gemeten kan worden. De verbetering in het luchtwegverwijdende effect van een diepe inademing door een stootkuur prednison was niet gerelateerd aan gelijktijdige verbetering van bronchiale hyperreactiviteit en de verandering in uitgeademd stikstofmonoxide (hoofdstuk5).

Vervolgens hebben we het effect van tiotropium behandeling bij patiënten met mild astma op longfunctie, hyperreactiviteit en luchtwegverwijdende effect van diepe inademing onderzocht. Tiotropium is een inhalatiemiddel dat specifieke (cholinerge) receptoren op de spiercel blokkeert en daarmee het samentrekken van de spiercellen in de luchtwegen, en dus luchtwegvernauwing, tegengaat. Onderzoek in een muismodel van allergisch astma heeft aangetoond dat ook de structurele veranderingen in de luchtwegwand (remodelering) ten gevolge van allergische ontsteking worden tegengegaan door tiotropium. Zowel de toename, als de

contractiliteit, van gladde spiercellen in de luchtwegen werd hierdoor geremd. Wij hadden als hypothese dat 21 dagen behandeling met tiotropium zou leiden tot een verbetering van longfunctie, bronchiale hyperreactiviteit en diepe inademing-geïnduceerde luchtwegverwijding door de allergiegedreven luchtwegwandontsteking te remmen bij patiënten met astma. De behandeling met tiotropium had geen significant effect op het luchtwegverwijdende effect van een diepe inademing of bronchiale hyperreactiviteit, maar wel werd een significante verbetering gezien in de longfunctie. De hoeveelheid lucht die in 1 seconde kan worden uitgeademd (FEV_1) als percentage van voorspeld is een maat voor luchtwegvernauwing en deze verbeterde zowel direct na inname van het medicijn, als na 3 weken behandeling. Ook een andere maat van luchtwegvernauwing, de FEV_1 ten opzichte van het totale volume dat een persoon maximaal kan in- en uitademen (geforceerde vitale capaciteit; FEV_1/FVC ratio), verbeterde (hoofdstuk 6).

In de studie uit hoofdstuk 7 hadden we als doel om bij patiënten met astma vernauwde luchtwegen te verwijden door deze passief 'op te blazen' met positieve druk inflatie. Speciaal daarvoor hadden we een apparaat ontwikkeld dat een specifiek per patiënt berekend volume de longen in blies. Tegelijkertijd konden we de weerstand in het luchtwegsysteem meten. We wilden aantonen dat bij patiënten, bij wie geen significante luchtwegverwijding na diepe inademing optreedt, dit wel gegenereerd kan worden met positieve druk inflatie. We lieten zien dat passieve druk inflatie inderdaad leidt tot luchtwegverwijding bij patiënten met astma waarvan de luchtwegen vernauwd zijn, vergelijkbaar met een actieve zelfuitgevoerde diepe inademing. Vooral bij de patiënten, waarbij een actief zelfuitgevoerde diepe inademing niet leidt tot een significante luchtwegverwijding, doet een passieve druk inflatie dit wel.

Tot slot hebben we in samenwerking met de faculteit wiskunde een studie verricht naar het gebruik van de niet-invasieve meting van luchtwegsysteemweerstand in de tijd met de forced oscillation technique (FOT) als instrument om het lange-termijn gedrag van een systeem (in dit geval een longziekte) te benaderen. Hierbij wordt de ziekte als dynamisch systeem gezien, dat met fluctuatie-analyse kan worden ontleed. Middels Wasserstein afstanden kan het verschil tussen deze systemen berekend worden en hieruit bleek dat astma en COPD als dynamisch systeem van elkaar verschillen. Deze methode zou gebruikt kunnen worden als diagnostisch hulpmiddel, maar nog belangrijker als monitor van de ziekte om te zien of therapie van invloed is op het systeem en of bijvoorbeeld het lange-termijn gedrag van het systeem een exacerbatie zou kunnen voorspellen (hoofdstuk 8).

Samengevat mogen we concluderen dat specifieke ontstekingscellen in de submucosa en spiercelbundels van de luchtwegen en de mate van expressie van specifieke spiercel- en extracellulaire matrixeiwitten de respons van de luchtwegen op een diepe inademing kunnen veranderen, maar dat oedeem in de luchtwegwand op zich dit niet doet. Daarnaast kunnen steroïdbehandeling en positieve druk inflatie luchtwegverwijding na een diepe inademing verbeteren, terwijl behandeling met tiotropium dit niet doet (figuur 7). Deze bevindingen zullen verder bediscussieerd worden in relatie tot eerder gepubliceerde data in de volgende secties.

10.3. Luchtwegverwijding door diepe inademing, ontsteking van de luchtwegen en reactie op ontstekingsremmers

In hoofdstuk 2 lieten we zien dat in bronchus biopten van patiënten met astma een toename van het aantal CD4+ cellen in de submucosa en een toename van het aantal mestcellen in de spierbundels gerelateerd was aan minder luchtwegverwijding na een diepe inademing. Daarnaast laten de resultaten uit hoofdstuk 5 zien dat een stootkuur prednison bovenop inhalatiecorticosteroïden significant het luchtwegverwijdende effect van een diepe inademing verbeterd. Zijn deze twee bevindingen aan elkaar verbonden? Met andere woorden, is de verbetering na een stootkuur prednison te wijden aan afname van CD4+ lymfocyten in de submucosa of aan afname van mestcellen in de spierbundels?

10.3.1. CD4+ lymfocyten

Van steroïden is aangetoond dat zij naast het verminderen van astma symptomen ook ontstekingscellen, waaronder het aantal CD4+ lymfocyten, in de luchtwegen kunnen verminderen. Een studie laat zien dat de daling in CD4+ lymfocyten was gerelateerd aan de verbetering in bronchiale hyperreactiviteit. Aan de andere kant, het tijdelijk stoppen van inhalatiesteroïden bij patiënten met mild astma, resulteerde in een exacerbatie van astma bij de helft van de patienten. Bij alle patiënten steeg het aantal eosinofielen in de bronchus biopten, maar het aantal CD4+ lymfocyten steeg alleen bij de patiënten die een exacerbatie kregen. Dit suggereert dat de stijging van CD4+ lymfocyten in de luchtwegwand na stoppen van steroïden een grotere invloed heeft op longfunctie dan eosinofielen. Dit kan dan ook verklaren waarom wij geen relatie vonden tussen eosinofielen en het luchtwegverwijdende effect van een diepe inademing, maar wel met het aantal CD4+ lymfocyten in de luchtwegwand. Samengevat is het heel goed mogelijk dat de verbetering in het luchtwegverwijdende effect van een diepe inademing door een stootkuur prednison inderdaad komt door verlaging van het aantal CD4+ lymfocyten in de submucosa.

10.3.2. Mestcellen in de spierbundels

De spierbundels in de luchtwegen van patiënten met astma, in vergelijking met die van gezonde controlepersonen, zijn meer geïnfiltreerd door mestcellen en lymfocyten. De mestcellen worden gerekruteerd naar de spierbundels door meerdere chemo-attractoren (stamcelfactor, chemokines, cytokines, CCR3, CxCR1). Daarentegen, geven gladde spiercellen uit de luchtwegen van niet-astmatische luchtwegen juist mediatoren af die de migratie van mestcellen naar de spierbundels tegen gaat. De aanwezigheid van de mestcellen in de spierbundels in de luchtwegen is geassocieerd met bronchiale hyperreactiviteit bij patiënten met astma. Echter, of mestcel migratie naar de spierbundels in luchtwegen wordt tegengegaan door steroïd behandeling is niet onderzocht. Daarom kunnen geen directe conclusies worden getrokken of de verbetering in luchtwegverwijdende effect van diepe inademing door een

stootkuur prednison kan komen door verminderde mestcel infiltratie van de spierbundels of minder degranulatie van de mestcellen in de spierbundels.

10.3.3. Steroïden en gladde spiercel verlenging.

Hoewel de gunstige effecten van steroïden bij astma worden toegeschreven aan de onderdrukking van luchtwegwand ontsteking, is het mogelijk dat steroïden ook direct effect hebben op de functie van gladde spiercellen in de luchtwegen en daardoor verbetering kunnen geven van bronchiale hyperreactiviteit en luchtwegverwijding na diepe inademing. *In vitro* onderzoek op gecontraheerde gladde spiercellen uit luchtwegen heeft laten zien dat krachtfluctuaties leiden tot verlenging van deze cellen. Verlenging van gladde spiercellen leidt tot minder stijfheid van de luchtwegwand en tot minder luchtwegvernauwing. Dit proces wordt gereguleerd via het enzym p38MAPkinase. Steroïden verhinderen de p38MAPkinase signalering en verbeteren daarmee de door krachtfluctuaties-geïnduceerde verlenging van gladde spiercellen. Het is daarom mogelijk dat een stootkuur prednison heeft geleid tot verbetering van luchtwegverwijding na een diepe inademing door het verbeteren van verlenging van gladde spiercellen na rek door een diepe inademing via het verhinderen van p38MAPkinase signalering. Dit is echter wel speculatief gezien het feit dat wij geen p38MAPkinase hebben gemeten in een van onze studies.

10.4. Luchtwegverwijding na diepe inademing en gladde spiercellen

Tijdens de ademhaling worden de luchtwegen steeds een klein beetje opgerekt en dus ook de gladde spiercellen die in de luchtwegen aanwezig zijn. Deze rek zorgt er voor dat de spiercel in een ontspannen en minder contractiele staat blijft. De functie van spiercellen is waarschijnlijk in evenwicht met deze dynamische omgeving. De rol van gladde spiercellen in de luchtwegen in relatie tot het luchtwegverwijdende effect van een diepe inademing is tot op heden niet helemaal duidelijk. Hiervoor zijn meerder hypotheses, waaronder de dynamiek van rek op het aantal verbindingen tussen actine en myosine, het bestaan van plasticiteit (de cel kan de contractiele elementen herrangschikken waardoor de cel zich aanpast aan nieuwe dimensies) en de mate van proliferatie van spiercellen afhankelijk van de manier waarop deze gerekt worden (bi-axiaal versus uni-axiaal). We hadden als doel een van deze hypotheses verder te onderbouwen door de expressie van verschillende contractiele en structurele spierceleiwitten in bronchus biopten te analyseren in relatie tot het luchtwegverwijdende effect van een diepe inademing. We vonden dat er meer luchtwegverwijding optreedt na een diepe inademing bij patiënten met meer expressie van calponine, desmine en MLCK in bronchus biopten, terwijl bronchiale hyperreactiviteit was geassocieerd met een hogere expressie van actine, desmine en elastine. Zijn deze resultaten in lijn met de eerdere hypotheses?

10.4.1. Plasticiteit

Plasticiteit refereert aan de mogelijkheid van gladde spiercellen in de luchtweg om het contractiele apparaat aan te passen aan de lengte waarop het wordt geactiveerd. Gladde spiercellen met langere actine filamenten tonen meer elastisch gedrag door toename in actine-myosine overlap. Hierdoor kan dezelfde hoeveelheid kracht worden opgebracht wanneer het bij een grotere lengte wordt geactiveerd. Dit zou kunnen leiden tot toename van bronchiale hyperreactiviteit. Een hogere mate van expressie van alfa-SM-actine in bronchus biopten zou kunnen wijzen op meer actine monomeren waardoor langere actine filamenten kunnen worden gevormd. Dit ondersteunt de relatie die we vonden tussen alfa-SM-actine expressie en bronchiale hyperreactiviteit. Echter, we hadden ook verwacht, een hogere mate van expressie van actine te vinden bij patiënten met minder luchtwegverwijding na een diepe inademing, maar vonden dit niet. Toegenomen actine expressie, in combinatie met hogere mate van expressie van desmine en elastine, zouden daarom de kracht generatie na stimulatie kunnen bepalen, maar niet de mate van rek en dus luchtwegverwijding na diepe inademing. Het is mogelijk dat meer factoren, zoals extracellulaire matrix compositie, luchtwegwanddikte, en/of het aantal alveolaire verbindingen het netto effect van rek van de luchtwegen en de daaropvolgende luchtwegverwijding bepalen.

10.4.2. Toegenomen gladde spiercel tonus

We vonden een positieve relatie tussen het geforceerde expiratoire volume in 1 seconde (FEV_1) als percentage van voorspeld en de mate van expressie van de gladde spierceleiwitten calponine, desmine en MLCK. Echter, we vonden ook een negatieve relatie tussen deze eiwitten en het luchtwegverwijdende effect van een diepe inademing. Bij gekweekte gladde spiercellen is aangetoond dat wanneer deze cellen een toegenomen spanning (door een extra prikkel) bevatten en cyclisch opgerekt worden, zij meer contractiele eiwitten, zoals myosine, MLCK en desmine, produceren. De positieve correlatie tussen FEV_1% van voorspeld en het luchtwegverwijdende effect van een diepe inademing kan daarom het effect van rek op de productie van contractiele eiwitten weergeven in deze patiënten met astma, in plaats van dat deze eiwitten een effect hebben op de functionaliteit van de luchtwegen. De meeste *in vitro* studies naar het effect van lengte verandering van gladde spiercellen zijn gedaan op spiercelstrips uit de grote luchtpijn (trachea) en met lengte oscillaties. Een recente studie toont echter aan dat in een geïsoleerde intacte luchtweg met luminale volume oscillaties geen verwijding optrad bij toename van de oscillaties. Dit toont aan hoe moeilijk het is om luchtwegwand vernauwing en verwijding op spiercel niveau te testen, gezien de unieke geometrie en structuur van de long en daarom lijkt het waarschijnlijk dat humane *in vivo* studies meer de 'echte' pathofysiologie van de luchtwegen in astma weergeven.

10.5. Interventie studies

Hoewel het onderzoek naar pathofysiologische mechanismen zowel interessant als noodzakelijk is, blijft de onvermijdelijke vraag bij klinisch wetenschappelijk onderzoek "wat is de klinische relevantie?". Aangezien diepe inademingen hebben laten zien dat deze bescherming bieden tegen luchtwegvernauwing (hoofdstuk 1) en dat dit gunstige effect verdwenen is bij patiënten met astma, is de klinische relevantie duidelijk zichtbaar. Namelijk, wanneer dit mechanisme hersteld kan worden bij patiënten met astma kan dit een directe en nieuwe behandeling betekenen tegen luchtwegvernauwing en dus benauwdheid. Tevens zou het kunnen leiden tot vermindering van de huidige medicatie die nodig is om astma goed te controleren. Herstel van diepe inademing-geïnduceerde luchtwegverwijding bij astma, waarvan het pathofysiologische mechanisme nog niet helemaal is opgehelderd, is misschien nog een brug te ver. Aan de andere kant, proberen om dit mechanisme te herstellen kan ook weer leiden tot nieuwe inzichten in het pathofysiologische mechanisme.

Meerdere studies uit dit proefschrift hebben aangegrepen op verschillende delen van het pathofysiologische mechanisme van diepe inademing met als doel dit te herstellen. We onderzochten het effect van maximale ontstekingsremmende (steroïd) behandeling (hoofdstuk 5), anticholinergische behandeling met tiotropium(hoofdstuk 6) en passieve druk inflatie (hoofdstuk 7) op de reactie van de luchtwegen na een diepe inademing. Elk van deze studies had een interactie met een mogelijke oorzaak van afgenomen rek van de luchtwegen door diepe inademing (**figuur 1**). Maximale steroïd behandeling zou de luchtwegwand remodelering en verdikking tegen kunnen gaan. Anticholinerge therapie kan allergeen gedreven gladde spiercelproliferatie en contractiliteit verminderen. En positieve druk inflatie kan de luchtwegen effectiever en meer oprekken door 'vanbinnenuit te drukken' in plaats van 'trekken van buitenaf' door negatieve druk in de borstkas zoals bij een actieve diepe inademing. Daarnaast zouden anticholinergica en positieve druk inflatie ook het effect van de rek op het contractiele apparaat van de gladde spiercel kunnen veranderen, hoewel we dit niet direct gemeten hebben (**figuur 1**). Hieronder worden deze interventies bediscussieerd, evenals een andere nieuwe behandeloptie bij astma, namelijk bronchiale thermoplastie.

10.5.1. Maximale steroïd behandeling

De verbetering in diepe inademinggeïnduceerde luchtwegverwijding bij patiënten met astma door systemische steroïd behandeling bovenop onderhoudsbehandeling met inhalatiesteroïden was een nieuwe bevinding. Eerder hebben verscheidene studies aangetoond dat inhalatiesteroïden bij astmapatiënten, die nog geen steroïden gebruiken, luchtwegverwijding na een diepe inademing kunnen verbeteren. Onze bevindingen (hoofdstuk 5) impliceren dat patiënten met astma die goed gecontroleerd zijn met inhalatiesteroïden nog steeds ontsteking van de luchtwegen kunnen hebben, die de mechanische eigenschappen van de luchtwegen beïnvloeden. Patiënten met een afgenomen luchtwegverwijding na diepe inademing, ook al

zijn zij klinisch stabiel, kunnen daarom een groter risico lopen op een verergering (exacerbatie) van astma. Aangezien de reactie van luchtwegen op een diepe inademing gerelateerd lijkt aan de ernst van astma en de ernst van benauwdheid zijn lange termijn studies nodig om te kijken of verminderde luchtwegverwijding na een diepe inademing prognostische betekenis heeft bij de behandeling van astma.

10.5.2. Behandeling met anticholinergica

Recente meta-analyses laten zien dat het gebruik van langwerkende luchtwegverwijders die de bètareceptoren stimuleren (bèta-agonisten), zonder gelijktijdig gebruik van inhalatie-steroïden, het risico op overlijden aan astma verhoogt. Deze bevindingen geven ruimte voor nieuwe onderzoeken naar alternatieve luchtwegverwijdende therapieën, zoals tiotropium (een langwerkend anticholinergicum). Interessant genoeg hebben anticholinergica laten zien dat zij in een model van allergische ontsteking de proliferatie en contractiliteit van gladde spiercellen kunnen remmen. Daarom hadden wij als hypothese, dat in allergisch astma dagelijks gebruik van het anticholinergicum tiotropium de contractiliteit van de spiercellen in luchtwegen zou worden geremd en daarmee longfunctie, bronchiale hyperreactiviteit en het luchtwegverwijdende effect van een diepe inademing zou verbeteren (hoofdstuk 6). Behandeling met tiotropium verbeterde de luchtwegverwijding na een diepe inademing, noch bronchiale hyperreactiviteit. Het had wel een significant effect op de longfunctie, zoals gezien in de FEV_1/FVC ratio. Verbetering in longfunctie is eerder laten zien, wanneer tiotropium wordt toegevoegd aan de conventionele behandeling bij patiënten met ernstig astma. Lange termijn effecten van anticholinerge behandeling op bronchiale hyperreactiviteit is niet eerder onderzocht. Een remmend effect van tiotropium op gladde spiercellen uit de luchtwegen is gevonden in een cavia model van allergisch astma. Dit kan komen door toegenomen aanwezigheid van acetylcholine onder allergische ontsteking. In de afwezigheid van ontstekingsmediatoren kan toename van acetylcholine wellicht niet leiden tot structurele veranderen in de luchtwegen en dus zie je ook geen remmend effect van tiotropium hierop. Het is dus mogelijk dat bij de milde astmapatiënten uit onze studie een lager niveau van ontstekingsmediatoren aanwezig was in de luchtwegen en daardoor het remmende effect van tiotropium op spiercelproliferatie en contractiliteit minder was. Aangezien we geen parameters van ontsteking hebben meegenomen in deze studie kunnen we de verbetering in longfunctie niet hieraan relateren. Samengevat kunnen we stellen dat tiotropium luchtwegverwijdende effecten heeft zowel acuut (na 3 uur) als op lange termijn (3 weken) bij patiënten met mild astma. Of de geobserveerde veranderingen in longfunctie en bronchiale hyperreactiviteit een gevolg zijn van aanhoudende luchtwegverwijding door muscarine receptor blokkade of door inhibitie van allergeen-geïnduceerde remodelering moet nog verder worden bevestigd.

10.5.3. Verbetering van rek van de luchtwegen door positieve druk long inflatie

We postuleerden dat luchtwegwandrek door long inflatie kan worden verbeterd door manipulatie van drukken in de borstkas met passieve long inflatie bij patiënten met astma. Mechanische inflatie van de longen zou rek van de luchtwegen kunnen geven zonder grote subatmosferische drukken in de borstkas en kan daarmee voorkomen dat vochtophoping of -verplaatsing in ontstoken luchtwegen optreedt. Daarnaast zou het ingeblazen volume afgesloten luchtwegen weer kunnen openen en daarmee de trekkrachten van het omgevende longweefsel op de luchtwegen kunnen redistribueren. Inderdaad vonden we in onze studie (hoofdstuk 7) dat positieve druk inflatie resulteerde in meer luchtwegverwijding in vergelijking met een actieve diepe inademing bij patiënten met astma.

Aangezien we geen drukken over de luchtwegen of in de borstkas hebben gemeten tijdens onze studie is het moeilijk te differentiëren of het luchtwegverwijdende effect van een positieve druk inflatie het gevolg is van daadwerkelijke verwijding van de luchtwegen of van het heropenen van afgesloten luchtwegen. De relatie tussen de toename in het ingeademde volume en verbetering in luchtwegverwijding na positieve druk inflatie suggereert dat een lager ingeademd volume, door welke oorzaak dan ook, kan leiden tot minder luchtwegverwijding na long inflatie. Bijvoorbeeld, door het tijdelijk beperken van de borstkasuitzetting tijdens rustademhaling daalt de longfunctie, neemt bronchiale hyperreactiviteit toe, en is er inderdaad minder luchtwegverwijding na een diepe inademing. Ook hebben verscheidene studies gekeken naar de reactie van luchtwegen op een diepe inademing bij patiënten met ernstig overgewicht (obesitas), waarvan we weten dat zij een afgenomen inademingsvolume hebben. Obese astmapatiënten tonen een toename van weerstand na een diepe inademing en hebben geen bescherming tegen luchtwegvernauwing middels diepe inademingen. Dit geeft aan dat overgewicht op zich een verandering geeft van longmechanica tijdens diepe inademing, mogelijk als gevolg van een lager inademingsvolume. Onze data suggereren dat de trekkrachten van het longweefsel tijdens een actieve diepe inademing, mogelijk in relatie tot de grootte van het geïnspireerde volume, niet sterk genoeg is om adequaat de luchtwegen te rekken, wat wel kan door positieve druk inflatie. Dit zou ook gebruikt kunnen worden bij niet astmatische aandoeningen waarbij lagere inademingsvolumes worden gezien, zoals bij obesitas.

10.5.5. Bronchiale thermoplastie

Gladde spiercellen in de luchtwegen zijn eerder wel eens beschreven als 'de appendix van de long', met andere woorden de aanwezigheid ervan zou wel eens functieloos en mogelijk schadelijk kunnen zijn. Een nieuwe interventie, bronchiale thermoplastie, levert gecontroleerde warmte energie aan de luchtwegen, waardoor afname of zelfs verdwijnen van gladde spiercellen in de luchtwegen bij proefdieren wordt gezien. Er zijn enkele aanwijzingen, dat het astma controle verbetert, zich uitend in een verbetering van kwaliteit van leven, astma symptomen, minder ernstige exacerbaties en minder gebruik van gezondheidszorg. Daarnaast,

leek aanvankelijk dat ook bronchiale hyperreactiviteit werd geremd door bronchiale thermoplastie, maar latere placebo gecontroleerde studies konden dit niet aantonen. Bij honden leidt bronchiale thermoplastie tot een toename in luchtweggrootte, zowel wanneer de luchtwegen ontspannen zijn, als wanneer deze vernauwd zijn. Ook was er minder vernauwing na de luchtwegvernauwende stof methacholine zichtbaar. Of de reactie van de luchtwegen op een diepe inademing veranderd door bronchiale thermoplastie is nog niet onderzocht, maar het kan gepostuleerd worden, dat als er minder spiercellen in de luchtwegwand aanwezig zijn, deze met een diepe inademing makkelijker gerekt kunnen worden en dus het luchtwegverwijdende effect van een diepe inademing toeneemt.

10.6. Dynamisch gedrag van het luchtwegsysteem

Een andere benadering van longziekte is door het te beschouwen als een biologisch system. De long is een complex orgaan, en systeem biologie integreert informatie van alle niveaus van structuur en functie van het systeem. Een van de karakteristieken van een biologisch system is het gedrag in de tijd. Het bestuderen van het gedrag van de long in de tijd, door het meten van fluctuaties in longfunctie in de tijd, is een manier om longziekte als een biologisch system te analyseren. Analyses van variabiliteit van het luchtwegsysteem zijn met succes toegepast op impedantie data en peak flow metingen. Voor ons onderzoek hebben we series van impedantie metingen in de tijd met de geforceerde oscillatie technique gebruikt om het temporale gedrag van dit systeem bij astma te bestuderen en of deze methode gebruikt zou kunnen worden om astma van COPD te onderscheiden. Het lukte met deze methode inderdaad om astma en COPD patiënten correct van elkaar te onderscheiden in 80% van de gevallen. Dit suggereert dat de impedantie metingen van het luchtwegsysteem alternatieve informatie bevat die astma en COPD van elkaar kan onderscheiden. Daarnaast zou deze methode gebruikt kunnen worden om longziektes te monitoren en het effect van behandeling te beoordelen.

10.7. Toekomstig onderzoek

De studies die in dit proefschrift beschreven worden hebben bijgedragen meer inzicht te krijgen in het (patho)fysiologische proces van luchtwegverwijding door diepe inademing en hebben laten zien dat standaard therapie (steroïden) en nieuwe behandelvormen (passieve druk inflatie) verbetering kunnen geven van de reactie van de luchtwegen op een diepe inademing. Maar er zijn nog veel vragen voordat we volledig begrijpen hoe het beschermende effect van diepe inademing werkt op de luchtwegen. Allereerst, het contractiele apparaat van de gladde spiercel in de luchtwegen en de functie ervan over verschillende lengtes moet eerst opgehelderd worden, voordat we goed kunnen begrijpen hoe rek de functie beïnvloedt. Ten

tweede, de interactie van ontstekingscellen in en met de gladde spiercellen en de functionele consequenties hiervan moet worden bekeken. Tenslotte, directe interventionele studies die als doel hebben het luchtwegverwijdende effect van een diepe inademing te herstellen moeten worden uitgebreid. Deze overwegingen hebben geleid tot de volgende onderzoeksvragen:

- Worden mest cellen in de spierbundels van de luchtwegen beïnvloed door standaard astma behandeling en is dit gerelateerd aan verbeteringen in longfunctie en reactie van luchtwegen op diepe inademing?
- Is het nuttig om nieuwe therapieën te ontwikkelen waarbij specifieke spierceleiwitten worden benaderd, zoals actine of desmine en kan daarmee bronchiale hyperreactiviteit worden verminderd?
- Is extracellulaire matrix in spierbundels in de luchtwegen verantwoordelijk voor bronchiale hyperreactiviteit?
- Hebben steroïden een acuut effect op luchtwegverwijding na diepe inademing *in vivo* door rek-geinduceerde verlenging van gladde spiercellen in de luchtwegen zoals is aangetoond *in vitro*?
- Kan passieve positieve druk inflatie gebruikt worden in de kliniek, zoals bij een acute astma exacerbatie?
- Zou passieve positieve druk inflatie ook verbeteringen kunnen geven bij andere longziektes, zoals COPD, of ziektes geassocieerd met een lager inademingsvolume?
- Remt tiotropium inderdaad allergie gedreven spiercelproliferatie en contractiliteit *in vivo* bij astma?
- Geven metingen van het temporale gedrag van luchtwegziekte inderdaad informatie over het beloop van de ziekte en het effect van behandeling?

10.8. Conclusie

Diepe inademing geeft een sterke fysiologische bescherming tegen luchtwegvernauwing wat verloren gaat bij patiënten met astma en andere longziekten, zoals COPD. Dit proefschrift heeft verder inzicht gegeven in het pathofysiologisch mechanisme dat hieraan ten grondslag ligt en heeft behandelopties getoond om dit proces te verbeteren.

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 21 maart 1978 te Apeldoorn. In 1996 werd het VWO-diploma gehaald aan het Veluws College te Apeldoorn en startte zij met de studie Geneeskunde aan de Universiteit Leiden. Tijdens deze studie deed zij haar wetenschappelijke stage bij de Raad van Bestuur (Prof. Dr. Buruma) van het Leids Universitair Medisch Centrum (LUMC). Het keuze-coschap werd gecombineerd met een onderzoek op de afdeling kinderoncologie (Dr. C.M. Zwaan) van het VU Medisch Centrum. Na het doorlopen van de co-schappen behaalde zij het artsexamen in juni 2003 en ving zij aan met het in dit proefschrift beschreven promotie-onderzoek (met subsidies van het Nederlands Astma Fonds en Boehringer Ingelheim) bij de afdeling Longziekten van het LUMC onder begeleiding van Prof. Dr. P.J. Sterk. Van september 2007 tot 2009 deed zij haar interne vooropleiding in het Sint Lucas Andreas Ziekenhuis te Amsterdam bij opleider Dr. C. Siegert. Daarna zette zij de opleiding tot longarts voort in het AMC bij opleider Prof. Dr. E.H.D. Bel.

The author of this thesis was born in Apeldoorn on March 21th 1978. In 1996, she graduated from secondary school at the 'Veluws College Apeldoorn", and started Medical School at the University of Leiden. During her study, she performed a scientific research project at the Board of Directory of the Leiden Univeristy Medical Center. In June 2003, following her medical training period, she obtained her medical degree and began working as a PhD fellow at the Department of Pulmonology at the LUMC. Her research project "towards restoring the physiological protection against airway narrowing in asthma", based on a grant by the Netherlands Asthma Foundation, was supervised by Prof. Dr. P.J. Sterk. In September 2007, she commenced her clinical training at the department of Internal Medicine at the Sint Lucas Andreas Hospital in Amsterdam (head: Dr. C. Siegert). In October 2009, she continued her clinical training to become a respiratory physician at the Department of Pulmonology of the Academic Medical Center Amsterdam (head: Prof. Dr. E.H.D. Bel).

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