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Effects of Asthma Combination Therapy on Extracellular Matrix Remodeling in Human Lung Fibroblasts

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Effects of Asthma Combination Therapy on Extracellular Matrix Remodeling in Human Lung Fibroblasts

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Till min syster,

mamma och pappa!

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II. **Lizbet Todorova**, Eylem Gürcan, Gunilla Westergren Thorsson and Anna Miller-Larsson. Extracellular Matrix Remodeling Induced by TGFβ1 in Human Lung Fibroblasts. Effects of Anti-Asthmatic Drugs: Budesonide and Formoterol. Submitted to *American Journal of Physiology - Lung Cellular and Molecular Physiology, 2007.*

III. **Lizbet Todorova,** Leif Bjermer, Anna Miller-Larsson, and Gunilla Westergren-Thorsson. Effects of Budesonide and Formoterol on Extracellular Matrix Production by Bronchial Fibroblasts from Healthy and Mild Asthmatics. *Manuscript in preparation, 2007.*

Additional peer-reviewed papers not included in the thesis

Lizbet Todorova, Sara Moses, Martin Bengtsson, Anna Hultgård-Nilsson, Mats Jönsson, Anders Malmström and Erik Eklund. Dermatan sulfate proteoglycans increase during phenotypic modulation of aortic smooth muscle cells. Submitted to *Archives of Biochemistry and Biophysics, 2007.*

Kerstin Tiedemann, Benny Olander, Erik Eklund, **Lizbet Todorova**, Martin Bengtsson, Marco Maccarana, Gunilla Westergren-Thorsson and Anders Malmström. Regulation of the Chondroitin/Dermatan Fine Structure by Transforming Growth Factor-β1 through Effects on Polymer Modification Enzymes. *Glycobiology. 2005 Dec;15(12):1277-85.*

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Abbreviations

αSMA	Alpha smooth muscle actin
BAL	Bronchoalveolar lavage
β2-AR	β2-Adrenoceptor
cAMP	Cyclic adenosine monophosphate
CS	Chondroitin sulfate
COPD	Chronic obstructive pulmonary disease
dpm	Disintegrations per minute
DS	Dermatan sulfate
DSPG	Dermatan sulfate proteoglycan
ECM	Extracellular matrix
ED-A	Alternatively spliced fibronectin (EIIIA)
FCS	Fetal calf serum
GAGs	Glycosaminoglycans
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response elements
HS	Heparan sulfate
IL	Interleukin
LABA	Long-acting β2-agonist
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
NF-kB	Nuclear factor-kappaB
PG	Proteoglycan
TGFβ	Transforming growth factor-beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Statement of thesis purpose

Extracellular matrix (ECM) remodeling is a crucial aspect of wound repair in all organs. It represents a dynamic process of ECM production and degradation as a reaction to an inflammatory insult, leading to either normal tissue restoration or, if disturbed, to fibrosis formation and structural tissue reorganization. In many lung disorders, including asthma, such structural reorganization is commonly known as airway remodeling, manifested in thickening of the airway walls, subepithelial fibrosis and impairment of lung function. Airway remodeling is regarded a consequence of either acute or persistent inflammation but may also precede inflammation. Resident cells of the lung, including fibroblasts, have the capacity to produce various ECM proteins, inflammatory mediators, and cytokines, perpetuating the inflammation in the airways of asthmatics. There is phenotypic heterogeneity among lung tissue fibroblasts, and myofibroblasts, being one of the subpopulations, are known to express features of smooth-muscle cells, with an increased capacity of ECM synthesis under the influence of pro-fibrotic mediators, such as transforming growth factor β1 (TGFβ1). An increased production of proteoglycans, collagens and other ECM molecules, may not only sequester and store inflammatory mediators, but also regulate many other cell-responses and sustain the process of airway remodeling. From clinical studies, combination treatment with an anti inflammatory glucocorticoid (GC) and long acting β2-agonist (LABA) has been shown to have complementary effects on improving lung function in general. However, airway remodeling of asthma appears rather resistant to current asthma treatment with inhaled GCs. This thesis was aimed to evaluate whether asthma combination treatment using a GC and LABA could have greater potential to affect ECM remodeling than the either drug alone. As fibroblasts are the main ECM producers within the lung, focus will be on lung fibroblasts of different origins, their ECM production and turnover, and their responses to the drugs under defined cell culture conditions.

Background

Overview of Asthma

The word "Asthma" originates from the Greek word ασθμα, which means "short breath" or "gasp for breath". Airway hyperresponsiveness, inflammation and mucus production are the principal components of the asthmatic response. Although these components are interrelated, they can exist at different times during the course of disease, and may require different therapeutic approaches. Persistent or not, inflammation and epithelial damage, initiate repair mechanisms which in asthmatics are usually disturbed, leading to fixed airflow obstruction and structural tissue reorganization, i.e. airway remodeling.
In the treatment of asthma, there have been many changes over the last 30 years. The bronchodilators, β2-agonists targeting bronchospasm, were widely used during the seventies followed by an increased use of inhaled corticosteroids during the eighties, targeting the inflammation. Today, as airway remodeling is recognized as a major hallmark of asthma (Figure 2), at some point most asthmatic patients need to combine both types of therapy to better control their asthma symptoms.

Acute inflammation in asthma

Symptomatic attacks of asthma may be caused by a number of factors, of which exposure to allergen is known to induce an inflammatory response. Acute inflammation in asthma is associated with bronchoconstriction, plasma exudation, vasodilatation and mucus hyper secretion. From experimentally induced allergic reactions it appears that the first response is an early phase reaction that usually changes into a late phase response. In this aspect, bronchial microcirculation has a pivotal role, as it promotes leakage with exudation of plasma caused by inflammatory mediators (eicosanoids and histamine). Extravasation of plasma with its active peptides and proteins (Figure 1) may cross the epithelium and reside in the airway lumen [1;2], creating changes in the subepithelial molecular environment. Although considered a first line defense mechanism, plasma exudate may compromise epithelial integrity and act as a pathogenic (pro-inflammatory) factor in asthma. The process of plasma exudation may also occur during an asthmatic

exacerbation. Indeed, increased levels of various serum proteins are found in bronchoalveolar lavage (BAL) fluid and induced sputum from asthmatics exposed to allergens or other stimuli [2-5], and an increased albumin level in BAL fluid was found to be the best predictor of prolonged bronchial inflammation [3].

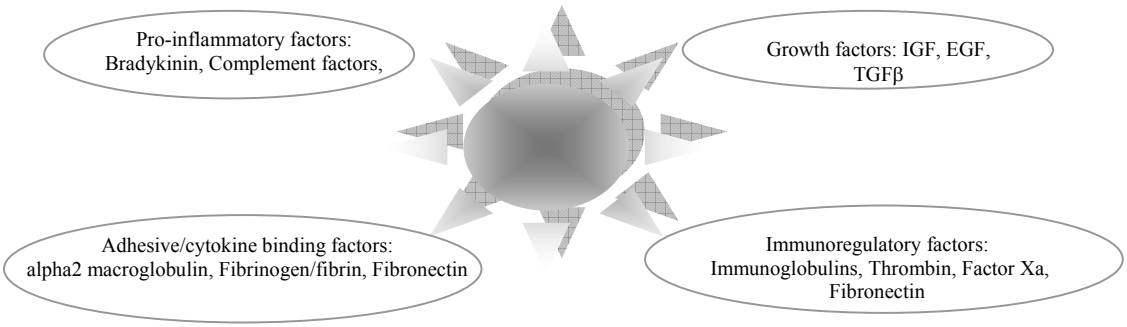

Figure 1. Content of bulk plasma exudate. Plasma derived peptides and proteins in plasma exudate are distributed in the airway mucosa. It represents a first line defense mechanism in airway mucosa [6], but can also perpetuate and sustain the airway inflammatory process [7]. Adapted from Greif L., 2003.

Chronic inflammation in asthma

The late phase response of an acute inflammation involves recruitment of inflammatory cells into the airways, and represents a chronic inflammatory condition. Asthma is usually regarded a chronic inflammatory disorder of the airway involving many different types of cells and cellular elements such as eosinophils, mast cells, macrophages, dendritic cells and T helper 2 (Th2) lymphocytes, and various cytokines and inflammatory mediators such as interleukins 4, 5, and 13 (IL-4, IL-5, IL-13) [8]. These mediators result in an increase in airway edema and mucus secretion, accumulating extracellular matrix (ECM) in addition to hypertrophy and hyperplasia of airway smooth muscle, and thickening of the bronchial wall (Figure 2). Features of such inflammation are evident even in young patients with intermittent asthma [9]. Though both acute and late phases of inflammation may contribute to airflow obstruction, it is the latter that causes

enhancement of bronchial hyperresponsiveness. Such airflow limitation is usually reversible, spontaneously or with treatment. However, if poorly controlled, and without adequate asthma therapy, it can be irreversible.

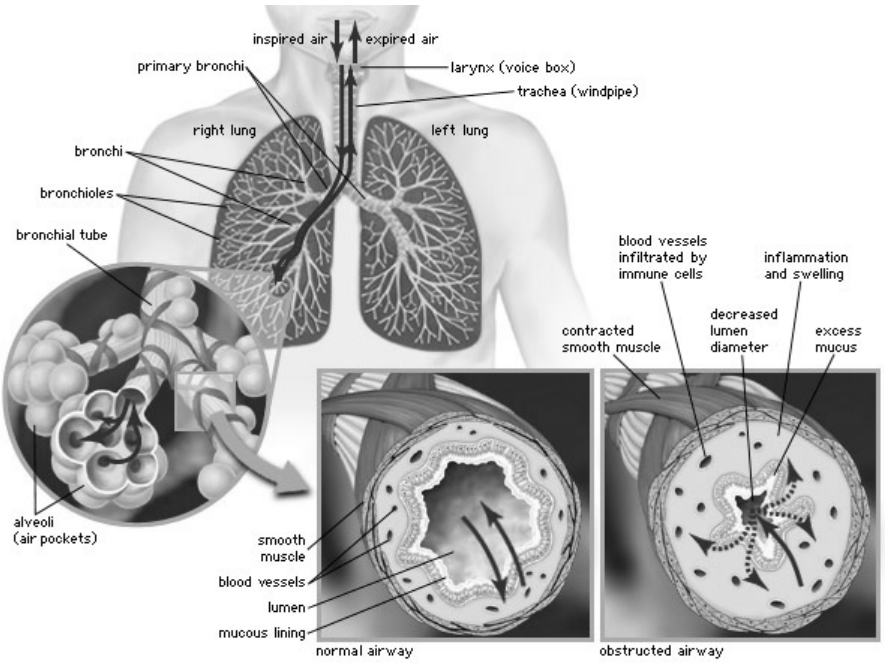

Figure 2. Normal (left) versus asthmatic (right) bronchial wall illustrating the thickening of the bronchial wall in asthmatic conditions as compared with normal conditions. Adapted from Encyclopedia Britannica.Inc.

Airway remodeling in asthma

In general, for asthmatic airways showing signs of both acute and chronic inflammatory reactions, the outcome is structural alterations within the airway, the so-called airway remodeling. These alterations are dynamic processes involving episodic attacks of acute inflammation on top of a persistent inflammation (Figure 3).

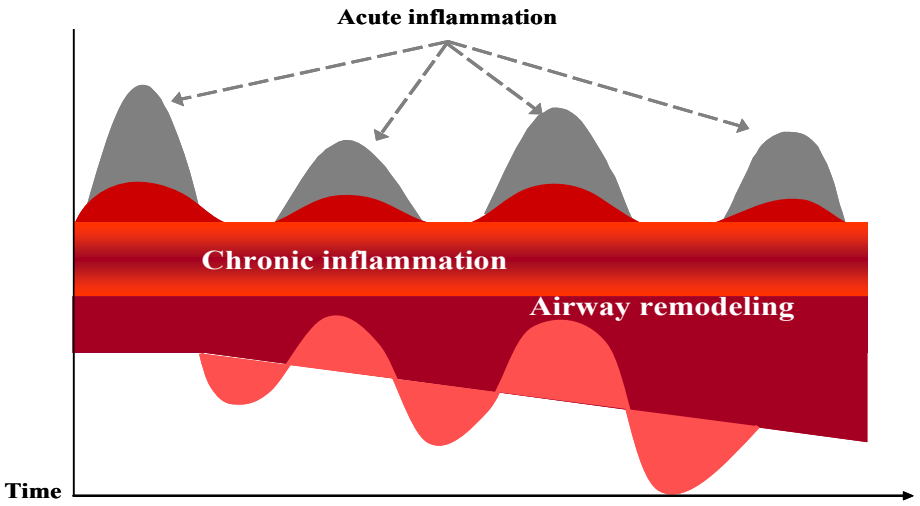

Figure 3. Dynamic and parallel processes of inflammation and remodeling in asthma. Chronic inflammation in asthma with episodic attacks is usually associated with acute inflammation on top of a persistent inflammation. If asthma remains uncontrolled or poorly controlled, the underlying persistent inflammation in combination with remodeling in the airways may reduce the extent of airway response to therapy. Modified from Miller-Larsson A., 2006.

The clinical and physiological consequences of such responses are incompletely understood. *In vivo* animal models indicate that these structural alterations have a more profound impact on bronchial hyperresponsiveness than the classical inflammation (Figure 4). The sites of airway remodeling include the epithelium and basal membranes, ECM, and small and large vessels. Some studies also illustrate that depending on the extent and location of structural changes throughout the various airway wall compartments, remodeling can enhance but also protect against excessive airway narrowing, despite the presence of acute inflammation [10].

However, there is controversy regarding its protective role since the airway remodeling can either precede or appear as a long-term consequence of inflammation [11].

Normally, the process of remodeling within the ECM is a critical aspect of wound repair in all organs [12;13]. It is a complex dynamic response to an inflammatory insult and tissue

injury. In asthma, while acute inflammation usually resolves with normal repair processes, with chronic inflammation the normal repair process is disturbed, resulting in profound changes in the composition, the content, and the organization of the cellular and molecular constituents of the airway wall and ECM [14].

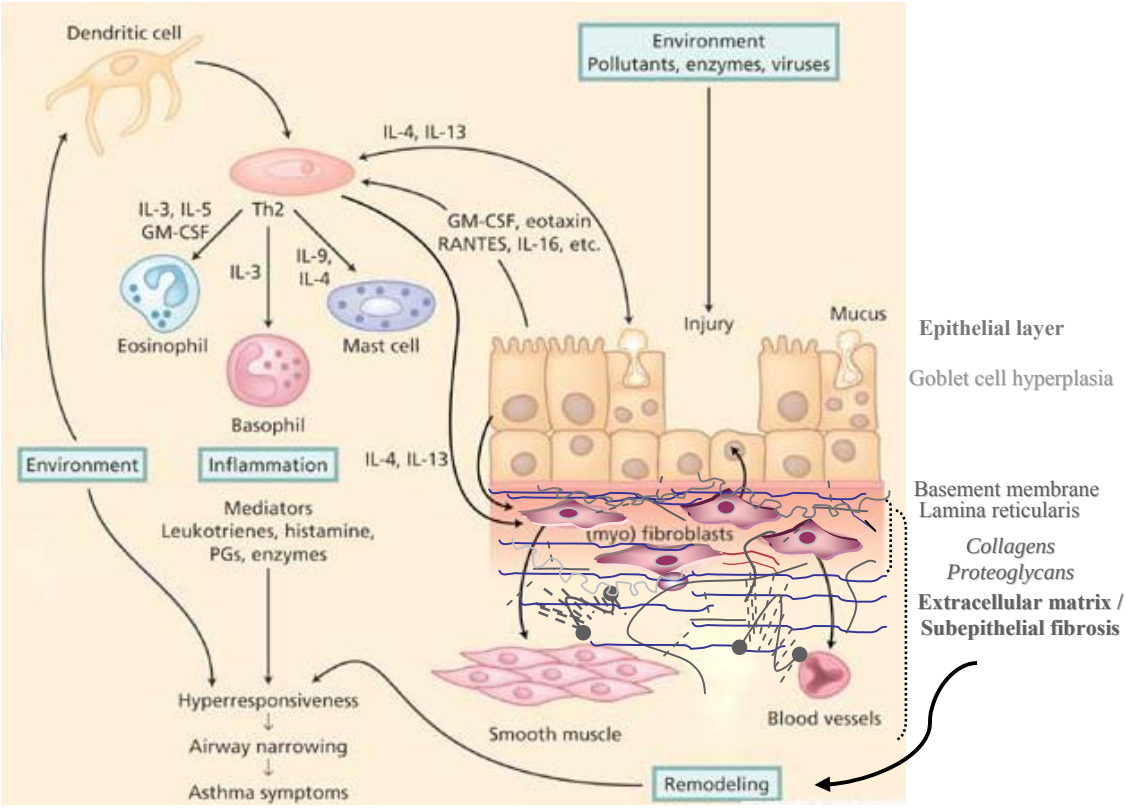

Figure 4. Subepithelial fibrosis formation. The pro-fibrotic mediators released from the epithelial cells after epithelial injury, act in concert with soluble mediators released from inflammatory cells and transduce signals into the extracellular matrix (ECM) inducing myofibroblasts differentiation and subsequent ECM synthesis. Adapted from ©Current Medicine.

The process of remodeling involves interactions of many components and growth factors such as transforming growth factor-beta (TGFβ), epithelial-derived growth factor (EGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as cytokines including IL-6, IL-11, IL-13, IL-5, and IL-9, that may in distinct or cooperative ways promote thickening of the airway wall *i.e.* smooth muscle hyperplasia, epithelial hypertrophy, and mucus gland and goblet cell hyperplasia [15] (Figure 4). Such structural reorganization is not

limited to patients with severe asthma. The degree of airway wall thickening however, may relate to the duration and severity of disease and the degree of airflow obstruction (i.e. airway hyperresponsiveness) [16,17].

The increased density of the subepithelial layer is usually accompanied and or sustained by shedding of the epithelial layer [18]. A damaged epithelium can release TGFβ, a potent pro-fibrotic factor that can inhibit the epithelial repair process, perpetuating injury responses down to the submucosal layer and ECM, where an increased number of activated fibroblasts (myofibroblasts) accumulate ECM components such as collagen and proteoglycans. In this concept of airway remodeling, much interest has focused on TGFβ as it can modulate the various aspects of airway remodeling (Figure 5). Numerous cells can produce TGFβ, but the major sources in the asthmatic airway are thought to be epithelium, fibroblasts, eosinophils, and airway macrophages. One aspect, that is central to airway remodeling, is the stimulatory effect of TGFβ on fibroblasts to increase the synthesis of ECM components [19]. This is also the basis for using TGFβ1 as a stimulator of lung fibroblasts in this thesis. A more detailed description of this cytokine will be given in the cytokine sections.

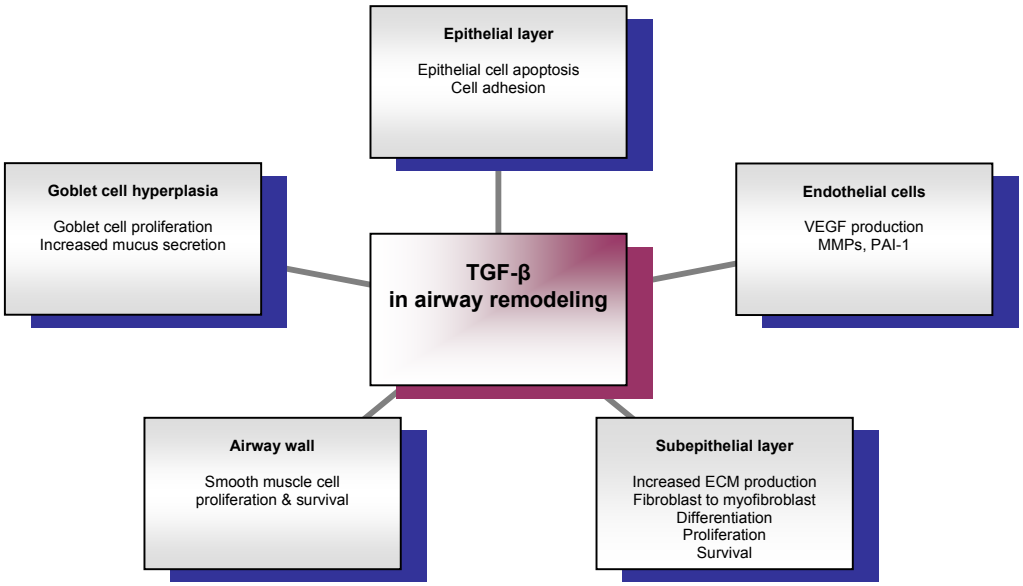

Figure 5. The various effects of TGFβ on the airway remodeling. Modified from Makinde T., 2007.

In addition, different environmental factors are known to have impact on the process of airway remodeling. By causing stress and repetitive injuries to the lung epithelium, an increased susceptibility of the airway epithelium to oxidative stress and an increased predisposition to retain airborne pathogens may lead to prolonged epithelial repair and hyperresponsiveness (Figure 4). Evidence suggests that the altered epithelium communicates with the underlying mesenchyme to create a microenvironment that propagates and amplifies the process of remodeling from the epithelial surface to the submucosal area where myofibroblasts play a central role [18;20] (Figure 4). It is suggested that in genetically susceptible individuals, exposure to different environmental factors results in a prolonged activity or reactivation of the airway epithelial mesenchymal trophic unit (EMTU). This trophic unit is central to the branching morphogenesis during foetal lung development, and can be reactivated in asthma, perpetuating airway remodeling [21]. Along with the increased epithelial susceptibility and prolonged repair, the EMTU response is believed to further complicate the asthmatic status, as mesenchymal cells (epithelial cells, fibroblasts and smooth muscle cells) proliferate and alter their function, ultimately leading to thickened and hyperresponsive airways (Figure 4). From animal studies, it is assumed that allergen exposure can repeatedly activate the EMTU. Simultaneous activation of epithelial and fibroblast cells is associated with an increased expression of the ECM component, tenascin [22].

From *in vitro* studies, lipid mediators have also been linked to asthma remodeling as leukotriene C4 increased collagenase expression by lung fibroblasts [23]. Prostaglandin E (PGE) is a prominent mediator that can inhibit fibroblast functions including proliferation and matrix production. As TGFβ increases fibroblast PGE production, a feedback control mechanism may regulate the remodeling within the ECM [24].

Endothelin is also a possible mediator as it shows synergistic action in combination with TGFβ and PDGF in inducing fibroblast proliferation and collagen production from asthmatic subjects [25].

In some asthmatics, it is not only the interplay of matrix molecules, growth factors and cytokines, but also a genetic predisposition that can sustain the chronic inflammatory response [26]. Among candidate genes are those that are more closely associated with

airway function, i.e. hyperresponsiveness and disease severity (*e.g.* treatment requirements), including the β2-adrenoceptor [27], and TGFβ [28]. Many cases of GC resistance may also be due to mutations or polymorphisms present in the GC receptor (GR) gene [29].

Recently, the identification of a disintegrin and metalloproteinase (*ADAM*) 33 as a major candidate gene for bronchial hyperresponsiveness in asthma [30], and the finding that polymorphisms in this gene are associated with lung function decline in asthmatics, suggest a possible role in airway remodeling [31].

Asthma pharmacotherapy

The purpose of asthma pharmacotherapy today is to optimize lung function, prevent acute episodes of asthma, maintain quality of life of the patient and avoid side effects of asthma medications. For most patients with asthma of different severities, inhalation therapy with bronchodilators (β2-agonists; relievers) and anti-inflammatory agents (GCs; controllers) forms the basis of treatment. Persistent inflammation and airway remodeling are two key features in asthma pathophysiology. While airway inflammation is well controlled by inhaled GCs, alone or in combination with LABAs, there is uncertainty regarding effects on the main features of airway remodeling. In particular, the effects of inhaled GCs on subepithelial fibrosis, a prominent feature of asthmatic airways, are controversial. Some studies have shown that subepithelial fibrosis is relatively resistant to inhaled GCs [32], while others have shown a reduction of subepithelial thickening in asthma [33]. GCs also prevent myofibroblastic differentiation [34]. Since it is now recognized that remodeling may occur earlier in the disease process than originally thought [21], clinical data from studies addressing this issue, indicate that the outcome may depend on how early in the disease process the anti-inflammatory therapy is started, and on the duration of treatment. There are indications that early use of inhaled GCs might limit some aspects of airway remodeling [35] and that, in particular, the reduction of subepithelial fibrosis may require longer treatment with inhaled GCs.

Therapy with β2-adrenoceptor agonists primarily targets acute bronchoconstriction of the airway, which is the other main feature of asthma besides inflammation. Some *in vitro* studies have also described anti-inflammatory effects of β2-agonists, but overall, β2-agonists are still not considered to have significant anti-inflammatory properties on their own. In regard to airway remodeling, one study [36] has suggested that therapy with LABAs could reduce the enhanced vascularisation of the airway mucosa as well as airway edema and airway wall thickness. Several studies since then have described that in structural resident cells of the lung, LABAs may exert some anti inflammatory and anti-remodeling effects, which are complemenatry, additive or even synergistic with the effects of GCs (Table 1).
At present, a therapy with a combination of an inhaled GC and a LABA is regarded as the most effective for patients whose symptoms are not fully controlled by monotherapy. From large clinical studies there is substantial evidence that combination therapy results in a better asthma control than higher doses of monotherapy. However, it is not yet known whether combination therapy is superior to therapy with an inhaled GC in controlling the major aspects of airway remodeling, including subepithelial fibrosis.

Anti-inflammatory glucocorticoids

Glucocorticoids, (also called corticosteroids, glucocorticosteroids or steroids) are the most effective anti-inflammatory therapy for many chronic inflammatory diseases. Budesonide (Pulmicort©) is one of several widely used inhaled GCs in asthma treatment; others are beclomethasone dipropionate (QVAR©), fluticasone propionate (Flovent©), triamcinolone (Azmacort©), flunisolide (Aerobid©), mometasone furoate (Asmanex©) and recently ciclesonide (Alvesco©). GCs are known to suppress multiple steps of the inflammatory pathways resulting in suppression of chronic inflammation. GCs have also been shown to have effect on some aspects of acute inflammation, such as inhibition of plasma exudation [37] and bronchial vasoconstriction [38].

To exert its physiological function, the GC diffuses across the cell membrane of the target cell and binds to the glucocorticoid receptor (GR) complex in the cytoplasm. The binding causes activation of the GR which dissociates from its complex and translocates

into the nucleus where it regulates gene transcription in several ways: (*1*) via binding to specific DNA sequences, thereby directly activating or repressing genes; (*2*) via interaction with other transcription factors; and (*3*) via modulating the mRNA stability. The first mechanism involves interactions of GR homodimers with the GC response elements (GRE) in the promoter region of GC-responsive genes to activate anti-inflammatory genes [39]. Inhibitors of nuclear factor-kappaB (NF)-κB and mitogen-activated protein kinase phosphatase (MKP-1) are some of the GC-induced anti-inflammatory genes, the latter being induced several fold to turn off the pro-inflammatory pathways of the mitogen-activated protein (MAP) kinases. However, high concentrations of GC are usually required for this response. GCs also bind to negative GRE and suppress transcription of GC responsive genes.

The second pathway, (also known as transrepression, not requiring binding to GRE), is believed to be the major one that the anti-inflammatory effects of GC are mediated through. This pathway mediates suppression of inflammatory genes that have been activated by pro-inflammatory transcription factors, such as NF-κB, and activator protein-1 (AP-1). This occurs via interactions between transcription factors and GR monomers, preventing binding of transcription factors to DNA and induction of gene transcription. Numerous immunoregulatory genes, whose expression is induced by pro-inflammatory mediators, contain binding sites for these transcription factors in their regulatory regions. Interactions between the GC and NF-κB or AP-1 may explain most of the anti-inflammatory and immunosuppressive activities of the GC. Once the inflammatory gene is switched off, the mRNA transcripts, encoding inflammatory proteins, including transcripts of various cytokines, adhesion proteins, and enzymes, decay at a rate determined by their stability [40].

Bronchodilatory β2-agonists

The β2-adrenoceptor agonists (also known as β2-adrenergic agonists or β2-agonists) are divided into two groups: short acting β2-agonist (SABA), and long acting β2-agonist (LABA). SABAs promote rapid but short lasting bronchodilation and are used mainly for initial treatment of bronchoconstriction during acute asthma episodes. Lately SABA is

most often used as an "as needed" complement to regular therapy with an inhaled GC. The reliance on SABAs, other than for acute relief of symptoms, is controversial, as regular long-term use may cause a partial loss of effectiveness and may mask airway inflammation. For regular consistent asthma therapy, LABAs, such as formoterol (Oxis©, Foradil©) and salmeterol (Serevent©) are used due to their long lasting bronchodilator effect (>12 h). Of the two, formoterol has a more rapid onset of action and can therefore be used as a acute reliever, giving fast as well as sustained relief of symptoms [41]. As with SABAs, reliance on regular use of LABAs is controversial, and recently LABAs have been recommended for use only together with an inhaled GC, in separate inhalers or in one inhaler containing both drugs.

The pharmacological effects of β2-agonists are exerted via binding to the β2-adrenoceptor (β2-AR), which is a cell surface G-protein coupled receptor. The main target for β2-agonist action is the airway smooth muscle cell, but the β2-AR is widely distributed also in vascular smooth muscle cells and bronchial epithelial cells, as well as, at lower density, in inflammatory cells infiltrating the lung and airways [42]. Following agonist-receptor complex formation, the α_s component of the associated G-protein dissociates and this results in activation of adenylyl cyclase which catalyzes the formation of intracellular cyclic adenosine monophosphate (cAMP). A subsequent activation of protein kinase A (PKA) and phosphorylation of myosin light chain kinase leads to inhibition of myosin-actin interactions and to smooth muscle relaxation and dilation of bronchial passages [43].

Combination asthma therapy with inhaled GC and LABA

Many clinical studies have shown improved asthma symptoms in patients using a GC and a LABA. In the very first study conducted in moderate asthmatics, it was shown that adding a LABA (salmeterol) to a standard dose of a GC resulted in a better airway function, fewer asthma symptoms and a reduced need for reliever medication [44]. More evidence on the benefits of adding a LABA to inhaled GC-therapy emerged from two large clinical trials where both mild and severe asthmatics were best controlled by the combination therapy (Figure 6). In the FACET study [45], performed in severe asthmatics,

the combination therapy with formoterol and either a low or high dose of budesonide was more effective in reduction of number of exacerbation than budesonide alone. In the OPTIMA study [46], performed in mild patients with persistent asthma, combination therapy with a low budesonide dose was more effective than a therapy with a double dose of budesonide alone.

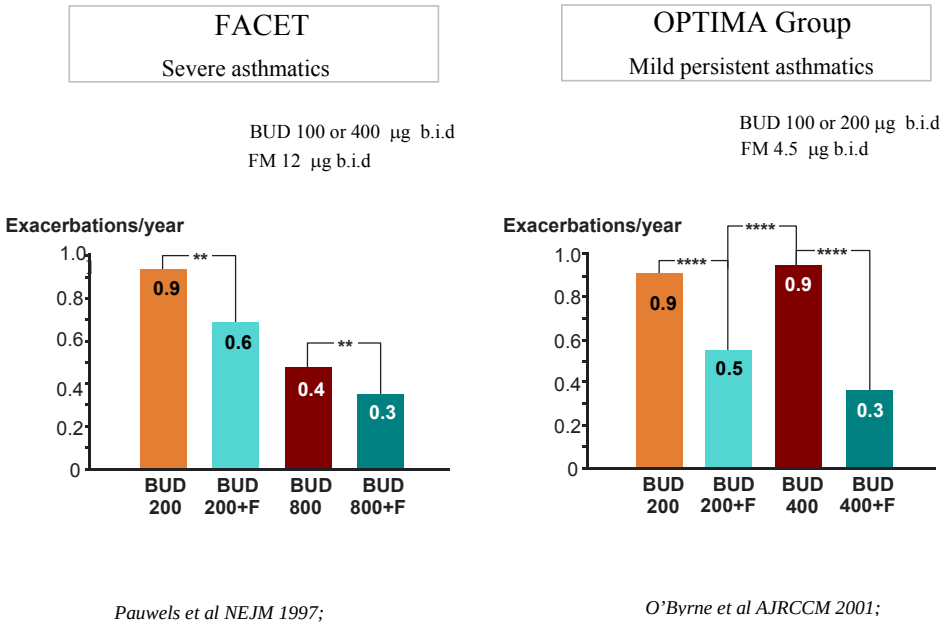

Figure 6. Asthma exacerbations are most successfully controlled by the combination therapy with inhaled GC and LABA (BUD, budesonide; FM, formoterol)

Combination therapy – mechanisms of action

In the lung, GCs and LABAs have been shown to have some complementary and additive effects on both inflammatory and structural resident cells within the airways [47-50] (Table 1). Some effects of LABAs, which may complement or add to the anti-inflammatory effects of inhaled GCs, are attenuation of allergen-induced plasma leakage in the airways and inhibition of histamine and leukotriene release from mast cells [42]. Increasing evidence suggests that GCs and β2-agonists interact with each other at molecular levels (for details see Figure 7) which may lead to synergistic effects of these drugs when used in combination [51].

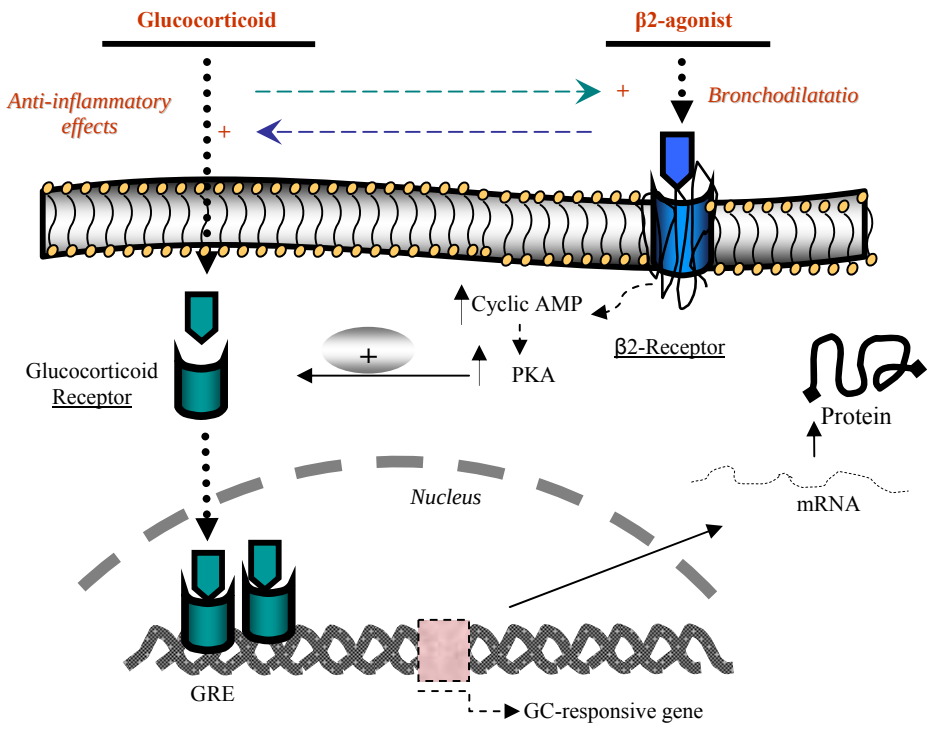

Figure 7. Interactions between GCs and LABAs on receptor and molecular level which may explain synerigistic effects when these drugs are applied in combination. GCs, in addition to their anti-inflammatory effects, also increase the number of β2-receptors; activated GC-receptors bind as a homodimer to the GC- responsive elements (GRE) in the promoter region on the β2-receptor gene resulting in increased synthesis of β2-receptors. On the other hand, β2-agonists, besides inducing direct bronchodilation, act on GC-receptors and increase their anti-inflammatory effect via increase of GC receptor nuclear translocation through cAMP and PKA-dependent pathway (Modified by Barnes P.J., 2002).

In vitro studies [52] and *in vivo* [53] have revealed that GCs enhance transcription of the β2-receptor gene, resulting in increased expression of β2-receptors at the cell surface. In addition, in *in vivo* models, GCs have been shown to prevent the downregulation of β2-receptors and desensitization that may occur after prolonged β2-agonist administration and during inflammatory conditions [54]. The GC-induced β2-receptor expression is in particular beneficial in cells that express only low levels of β2-AR, such as the mast cells and other inflammatory cells, in preserving and maintaining their response to β2-agonists.

There is also substantial evidence that β2-agonists may enhance the action of GCs via increased translocation of the GR into the cell nucleus. *In vitro* studies have demonstrated

that such translocation occurs in various types of human cells, such as lung fibroblasts [55], airway smooth muscle cells [56], bronchial epithelial cells and airway macrophages [57]. However, there is uncertainty regarding the bronchial epithelial cells [58], and thus, such an effect may be cell specific. The molecular basis for the GR nuclear translocation by β2-agonists is not yet completely elucidated. In some experiments, β2-agonists are themselves sufficient to translocate GR (ligand independent process) [55], while in others they seem to only promote GC-dependent translocation [57].

Table 1. Summary of in vitro and in vivo data on enhanced anti-inflammatory and remodeling effects of combination treatment with GC and LABA. Modified from Miller- Larsson A. and Selroos O., 2006)

• Inhibits superoxide production in eosinophils
•Inhibits number of vessels in the subepithelial layer
• Inhibits epithelial IL-8, GM-CSF and VEGF
• Decreases IL-8 and eotaxin in smooth muscle cells
• Decreases neutrophil elastase release
• Decreases number of eosinophils, neutrophils and mast cells
•Inhibits smooth muscle proliferation
•Inhibits migration of smooth muscle cells
•Decreases IL-8 in bronchoalveolar lavage
•Decreases ICAM expression in fibroblasts
•Decreases proteoglycan production in fibroblasts

Since the main focus in the thesis is on the GC and LABA and their effect on ECM remodeling aspects, other combination treatment options currently used in asthma, such as leukotriene receptor antagonists, IgE inhibitors, or anti-IL-5 will not be discussed.

Target cells in asthma, remodeling and treatment

It is becoming obvious that inflammatory processes may regulate airway remodeling through crosstalk between inflammatory and structural cells. Targeting such crosstalk may also have therapeutic applications since mechanisms that govern airway remodeling and collagen deposition are still not clear.

Inflammatory cells

During early and late phase of an inflammatory response, the ***mast cell*** is among the first cells to respond. It does so by degranulating and releasing mediators such as histamine, tryptase and prostaglandin. One of the mediators of the mast cell is tryptase, a serine protease that induces resident fibroblast and smooth muscle cell proliferation [59], suggesting a role for mast cells in airway remodeling. Mast cells are also a source of pro-inflammatory cytokines including IL-4, IL-5, IL-13, tumor necrosis factor-α (TNF-α) and GM-CSF, all contributing to the recruitment and action of another inflammatory cell, the eosinophil.

Under the influence of the various previously mentioned cytokines, ***eosinophils*** migrate from the bone marrow through the ECM of the airway. Activated eosinophils release pro-inflammatory cytokines such as IL-2, IL-4, IL-5, IL-13, and chemokines (RANTES, eotaxin) and are a major source of TGFβ [60]. All these mediators may in turn influence vascular permeability, mucus secretion, fibrosis, smooth muscle contraction, epithelial damage and inflammation [10]. Recent studies have implicated a role for eosinophil-derived TGFβ1 in bronchial hyperresponsiveness and the subepithelial deposition of ECM proteins [61].

Neutrophil infiltration is prominent mostly during the late phase response, as well as in more severe, persistent asthma [62], making its role less clear in milder forms of asthma. This short-lived and transient cell is rather associated with chronic obstructive pulmonary fibrosis (COPD) [63]. Recently, higher levels of TGFβ expression and release from

asthmatic neutrophils indicate that neutrophils may be involved in the airway remodeling process of asthmatic subjects.

Furthermore, during allergic inflammation in asthma, production of Th2 cytokines including IL-4, IL-5 and IL-13 is induced. These cytokines are responsible for both T-cell differentiation and eosinophil activation and recruitment [64]. Transgenic mice overexpressing these cytokines display features of airway hyperresponsiveness similar to those observed in asthma patients [65].

Currently, GCs are the most efficient treatment for controlling the inflammation within the airway (Table 2), especially the eosinophil mediated type.

Table 2 .Summary of some *in vitro* effects of GCs on various components of inflammation (Modified from Bergeron C. and Boulet L.P., 2006; Beckett P.A. and Howart P.H., 2003)

• Reduced number of mast cells, eosinophils, T-cells, by either oral or inhaled GC
• Decreased mRNA levels for GM-CSF, IL-4, IL-5, increased interferon-γ
• Decreased levels of exhaled NO and PC20
• Decreased NF-kB and AP1 in nasal epithelial cells upon rhinovirus infection
• Delayed reverse eosinophil apoptosis
• Inhibited production of the inflammatory leukotrienes
• Blocked cytokine (IL-1β) production, reduced number of circulating T cells
• Inhibited production of cytokines, e.g. IL-4, IL-5, TNF-α in macrophages
• Decreased local recruitment of eosinophils
• Increased sensitivity of β2 receptors

Resident structural cells

Epithelial layers form defense barriers between external milieu and underlying tissues that, for example in the lung, are frequently exposed to various challenges, some of which result in injury of the epithelial surface. The bronchial epithelium is thus a critical site in asthma development and other airway hyperresponsive diseases. Alterations in

airway epithelium integrity, or disorders in functions depending on the shedding of ***epithelial cells,*** may be the initial steps leading to airway hyperresponsiveness such as in asthma and COPD (see also Figure 4). According to some studies, in patients with asthma of varying severity, the greater the loss of surface epithelium in biopsy specimens, the greater the degree of airway responsiveness [66], suggesting that airway epithelial cells play a critical role in not only maintaining airway integrity and local microenvironment but also in mediating inflammation and airway hyperresponsiveness.
Indeed, epithelial cells can activate latent TGFβ1 and TGFβ3 through integrin mediated mechanisms. In vitro effects of TGFβ on epithelial cells include inhibition of proliferation, induction of epithelial motility, squamous differentiation and apoptosis [67]. Adhesion molecules besides integrins, such as cadherins, selectins and intercellular adhesion molecule-1 (ICAM-1) within the lung ECM play important roles in maintaining the integrity of the epithelium. However, the loss of epithelium is regarded as an uncertain determinant of extent of injury. There are variable reports on the loss of epithelium as seen in biopsies from individuals with mild asthma [68]. However, studies on primary cultures of human bronchial epithelial cells suggest that GCs may be very important for their protective role against epithelial injury [67].

Besides its primary function in contraction and relaxation of the airway, the ***smooth muscle cell*** is also recognized to be an active participant in inflammatory process of asthma. In biopsies from severe asthma, the distance of the muscle layer from the epithelial basement membrane is decreased when compared with biopsies obtained from patients with milder asthma or COPD [69]. The degree of such thickening of the airway wall may relate to the duration and severity of disease and to the degree of airway hyperresponsiveness [70]. However, smooth muscle cells within the ECM are multifunctional cells, capable of proliferation and synthesis of various pro-inflammatory factors [71]. They have a phenotypic potential to, upon injury and/or altered environment, de-differentiate and migrate towards the epithelium where they form new muscle of abnormal phenotype and abnormal function [72]. Observations from animal studies [73] suggest that after allergen challenge migratory cells with a contractile phenotype appear in greater numbers in the late response. Thus asthmatic subjects with repeated features of

a late response have increased numbers of migrating, contractile cells that may contribute to formation of the increased bronchial thickening observed in fatal asthma.
The proliferating smooth muscle cells have reduced contractile protein content [74] and they secrete various ECM components in response to inflammatory stimulus. Indeed, moderate asthmatic patients have relatively greater deposition of proteoglycan in the smooth muscle layer of the airway wall than do patients with more severe disease [75]. Recalling the hypothesis on the protective role of airway remodeling, it was proposed that such enhanced deposition of ECM could have a protecting role against excessive smooth muscle constriction [75]. Whether that alteration is in a positive or negative direction may depend on the specific layer of the airway wall where the proteoglycan accumulations occur. The increase of smooth muscle is a recognized consequence of hyperplasia and hypertrophy of the smooth muscle cells.
Suppression of these cells' growth by GC and prolonged exposure to LABA, suggests a potential to partly reverse the stimulus for increased muscle mass [76].

Although both epithelial and smooth muscle cells are capable of producing ECM and pro inflammatory mediators, it is the ***fibroblasts*** that carry the major effectors' role in the process of airway remodeling (see also Figure 4). The fibroblast and its capacity of synthesizing ECM components such as proteoglycans, and collagen I is one of the primary subjects highlighted in this thesis. It has been recognized that fibroblasts or distinct subpopulations thereof, perform functions that go further than that of structural elements. Besides their essential functions in ECM production, they can also act as ECM degrading enzyme producers, pro-inflammatory activators and modulators of the inflammatory response [77]. Fibroblasts from different anatomical locations are capable of expressing multiple regulatory molecules, including cytokines, growth factors, cell surface antigens and adhesion molecules. This enables them to influence their microenvironment. As the remodeling of the ECM is viewed as a critical component of the classical wound healing in response to injury, the fibroblast response and, in concert with pro inflammatory mediators such as TGFβ, will determine the outcome of remodeling events. Fibroblasts in granulation tissue have the capacity to adapt a migratory and proliferating phenotype, to exert tension on the ECM, develop stress, and

contract the ECM, thereby promoting wound closure [77;78] followed by regression of the fibroblast population and initiating ECM remodeling. This particular ability of fibroblasts to adapt a distinct morphological phenotype in granulation tissue was originally observed 1971 by Gabianni G[79], who also gave them the name myofibroblasts (Figure 8).

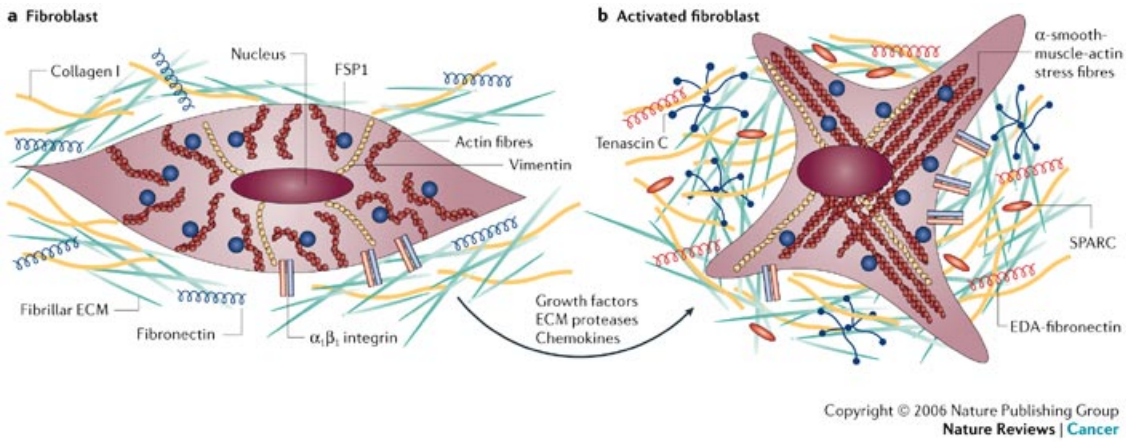

Figure 8. Differentiation of fibroblast into myofibroblast

Emergence of myofibroblasts

Early in the wound healing, the fibroblast that migrates to the site of injury is an activated but undifferentiated ECM producing fibroblast. As the repair process progresses, the fibroblast differentiates to a myofibroblast with features of both fibroblast and smooth muscle cell (Figure 8). Some researchers refer to these cells as smooth muscle-like cells or activated smooth muscle cells. It has also been proposed that within the lung, cells with the features of myofibroblasts in the mucosa, during certain circumstances, may represent de-differentiated smooth-muscle cells, arising from blocks of smooth muscle or even vascular smooth muscle, and should be referred to as fibromyocytes [72]. Regardless of the name, it is recognized that myofibroblasts may be recruited from resident cell populations, circulating precursors [80] and via epithelial-mesenchymal transition of epithelial cells (EMT) [81].

During the repair process, myofibroblasts are the responsible elements in creating a provisional matrix by synthesizing and secreting molecules necessary for the remodeling

of the ECM. Under pathological circumstances in various organs, the molecular phenotype shared by these activated fibroblasts, is the constitutive or transient expression of smooth muscle specific features [82;83]. Some examples of such cells include dermal myofibroblasts, vascular smooth muscle cells (SMC), lipocytes in the liver; mesangial cells in the kidney; and stromal myofibroblasts in different carcinomas [83]. In addition, the presence of myofibroblasts has been described in several fibrosis-related disorders [84].

The potential of the myofibroblasts to secrete and accumulate an aberrant ECM is likely due to an altered response to TGFβ, as mentioned previously; a crucial pro-fibrotic growth factor. Indeed, when lung fibroblasts are stimulated with TGFβ *in vitro*, proteoglycans and collagen are several-fold induced at both gene and protein level [85] (Paper II, III). Within the lung, regional fibroblast heterogeneity has been described [86]. In asthma of varying severity, a myofibroblast-like phenotype with migratory properties was found prominent in the distal part of the lung, while in the central airways the cells were more sensitive to TGFβ and IL-13, and thus more capable of synthesizing ECM than the distal phenotype. Regarding the increased proliferation rate, a correlation was observed between the thickness of the lamina reticularis and the number of subepithelial myofibroblasts [87;88]. The number of subepithelial myofibroblasts was significantly increased 24 h after a high-dose allergen challenge [73], suggesting that repeated allergen exposure with resulting increases in myofibroblasts may produce thickened lamina reticularis and smooth-muscle hyperplasia, which persist even when minimal airway inflammation is present. In addition, myofibroblasts grown from bronchial biopsies were capable of producing GM-CSF, IL-6, IL-8, and stem cell factor (SCF) [89] suggesting a role in the regulation of inflammatory cell recruitment and activation via complex cytokine network interactions. Resent *in vitro* experiments suggest that some GCs and LABAs can affect the contractile component smooth muscle actin (SMA) [90] highlighting myofibroblasts as a possible target cell for current asthma drugs.
Some of the effects of GCs on the different structural resident cells are summarized in Table 3.

Table 3. Summary of some *in vitro* effects of GCs on various structural resident cells (Modified from Beckett PA and Howart PH 2003)

•Inhibition of epithelial cell proliferation, goblet cell hyperplasia
•Decreased epithelial and fibroblast GM-CSF and Il-8 production
• Increased or decreased smooth muscle fibronectin production, depending on the GC
• Decreased or unaffected smooth muscle cytokine synthesis
• Reduced adhesion molecule expression by endothelial-, epithelial cells and fibroblasts.
• Decreased smooth muscle eotaxin release
• Decreased fibroblast TGFβ expression
• Increased or decreased fibroblast proliferation
• Decreased fibroblast collagen gene expression
•Decreased fibronectin mRNA in fibroblasts

The cytokine and growth factor network in asthma

The balance between pro- and anti-inflammatory cytokines is required for a proper resolution of lung inflammation and injury. TNF-α and IL-1β, which trigger the inflammation, must be downregulated to prevent further infiltration of inflammatory cells, a regulation step that is normally followed by apoptosis of the inflammatory cells. Dysregulation of these processes creates a loop of sustained cytokine production. There is a variety of pro-inflammatory cytokines and growth factors that mediate, sustain, and amplify both the inflammatory and the remodeling responses seen in the asthmatic subepithelial compartments. The "type 2 cytokine hypothesis of fibrosis" proposes that fibrosis occurs when cytokine balance shifts in a Th2 (type 2) direction [91]. Indeed, the Th2 phenotype of T-cells produces a panel of cytokines, including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and GM-CSF. However, in asthma, recurrent acute exacerbations generate “acute-on top of-chronic” inflammation (Figure 2), disturbing the existent cytokine

milieu by rapid increases in eosinophils and neutrophils and release of mediators to induce bronchoconstriction, airway edema, and mucus secretion.
The chronic type of asthma associated with subepithelial fibrosis is under the influence of a number of growth factors including EGF, TGFβ, IGF, PDGF, FGF and endothelin. Several of these are highly capable of inducing lung fibroblast differentiation [92], arguing against the statement that Th2 cytokines can induce fibrosis, independently of known pro-fibrotic mediators, such as TGFβ. For example, data from transgenic studies suggest that IL-13 mediates its fibrogenic effects in the lung and other organs by altering TGFβ cytokine action. TGFβ1, TGFβ2, IL-4 and IL-13 are all present in BAL fluid from asthmatics patients. While TGFβ1, TGFβ2 and IL-4 significantly increased α-SMA in fibroblasts, IL-4 caused corresponding increases in collagen III synthesis [93].

Increased angiogenesis is also a feature of the airway remodeling, associated with increased VEGF production. It was shown that both Th2 cytokines and TGFβ were able to induce airway smooth muscle release of VEGF, effects that were inhibited by interferon-γ and GC treatment [94]. Hence, the complexity of the cytokine and growth factor network in asthma appears rather to rely on mutual interaction.

TGFβ as a main mediator cytokine in asthma

The cytokine of particular interest in this thesis is TGFβ1. This multipotent cytokine has been extensively studied as it is involved in a whole range of biological functions, from cell growth to cell differentiation and fibrosis. Polymorphisms leading to increased TGFβ1 production have been linked to fibrosis [95]. Although TGFβ is essential for wound healing, overproduction of this cytokine can result in excessive deposition of scar tissue and fibrosis in multiple organs including the lung. Asthma is associated with fibrosis which is a general term applied to a large group of diseases characterized by excessive matrix deposition leading to organ dysregulation and failure.

Although many cells can produce TGFβ, the important sources in the asthmatic airway are, besides the fibroblasts, assumed to be epithelium, eosinophils, and airway macrophages (Table 4). Based on the findings that TGFβ1 levels are directly correlated to

the extent of lung fibrosis and to the thickness of the basement membrane, currently, it is believed to be the most important of the growth factors involved in the pulmonary fibrogenesis [60;96;97]. Its versatile effects, based on studies of both inflammatory and resident cells within the lung, are illustrated in Table 4. A paradox concerning TGFβ is that although tissue injury increases the production of TGFβ before the production of ECM increases, the exogenous TGFβ can still induce fibrosis independently of the tissue damage.
Increased basal levels of TGFβ1 have been reported in the BAL fluid of asthmatics [93;98]. The most pronounced differences between the TGFβ isoforms are the stage and site at which they are expressed in developing tissues, regenerating tissues, and in pathologic responses.

Almost all forms of the TGFβ subfamily (TGFβ1, TGFβ2 and TGFβ3) are released and localized at the cell surface as biologically inactive forms that are known also as latent TGFβ. The latent form is a complex of TGFβ, an N-terminal portion of the TGFβ precursor, and a specific binding protein known as latent TGFβ binding protein. Biologically active TGFβ results after dissociation from the complex.
The nature of the activation mechanism of latent- TGFβ *in vivo* is unclear. Also the mechanisms of TGFβ activation are still not known in detail. Extracellular activation of latent TGFβ1 can be achieved by several mechanisms, some of which involve: the urokinase plasminogen activator (uPA)/plasmin system, metalloproteinase (MMP)-2 [99], MMP-9 [100], integrins such as αvβ6 [101] and αvβ5, the later, being shown to contribute to autocrine TGFβ signaling and an increased myofibroblastic feature of fibroblasts from scleroderma patients [102].

TGFβ1 binding availability for cell surface receptors is potentiated extracellularly by highly sulfated, heparan sulfate proteoglycans (HSPG) [103]. Whereas certain proteoglycans, such as betaglycan and endoglin [104] facilitate TGFβ1 binding to its receptors, others, such as biglycan, fibromodulin and decorin, sequester TGFβ1 in the ECM [105;106]. This allows the factor to be stored in a biologically inactive form, and to be locally activated in an autocrine or paracrine way to control cellular responses. Indeed,

there are reports on an increased expression of TGFβ1 in the airway submucosa of asthmatics, which has been observed, despite a lack of difference in concurrent inflammatory cell infiltrate [107].

The most studied TGFβ receptors (TGFβR) (of the total 6, TGFβR1-6) are TGFβR1 (or ALK-5), the type 2 receptor, TGFβR2, and the type 3 receptor TGFβR3 or betaglycan, a heparan sulfate/chondroitin sulfate proteoglycan, which facilitates the binding of TGFβ to its receptor (for detailed TGFβ signaling pathway, see figure 9).

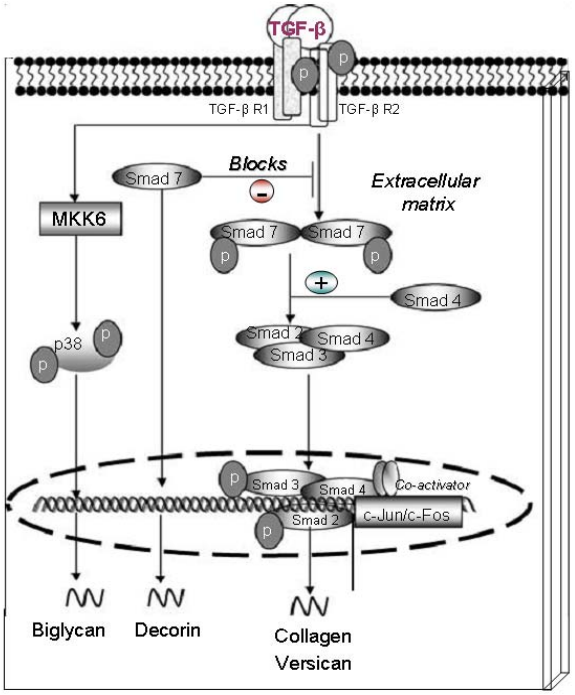

Figure 9. Signal Transduction Mediated by Transforming Growth Factor. In the ECM, TGFβ binds either to the type 3 TGFβ receptor (R3, betaglycan), which presents and enhances affinity to the type 2 receptor (R2), or directly to R2 on the cell membrane. The binding of TGFβ to R2 then leads to binding of the type 1 receptor (R1) to the complex and the phosphorylation of R1, which in turn phosphorylates the transcription factors Smad2 or Smad3. These then bind to Smad4, the common Smad, and the complex translocates to the nucleus where it interacts with various other transcription factors regulating the transcription of TGFβ–responsive genes and mediating the effects of TGFβ at the cellular level. Inhibitory Smad6 and Smad7 lack the region normally phosphorylated by R1 and thus interfere with the phosphorylation of Smad2 or Smad3 by R1. Adapted from Makinde T. 2007.

TGFβRs are expressed ubiquitously, and within the lung, TGFβR1 and 2 are found in macrophages, as well as in epithelial, vascular and airway smooth muscle, fibroblast and endothelial cells. Expression of TGFβR1 has been found downregulated in both mild and severe asthma [108] while of the isoforms, only TGFβ2 was different among the groups and increased in severe asthma. In addition, a decrease in the number of cells positive for TGFβR2 has been reported in the subepithelium of young asthmatics [109].

As shown in Figure 9, the Smad-family is the main but not the only signal transduction pathway for TGFβ. Other TGFβ superfamily members also signal via the Smad pathway. However, in asthma, TGFβ1 is regarded the main contributor to an increased Smad signaling. TGFβ Smad signaling is tightly controlled by MAP kinase signaling cascades. Although Smads do not themselves activate transcription [110] they are supposed to assist in the formation of a functional transcriptional complex on target promoters with other transcription factors.

Downstream of TGFβ signaling, several proteins are required, or act in concert with TGFβ to induce an optimal cellular response to TGFβ by either prolonging the wound healing response or enhancing the fibrotic response. Connective tissue growth factor, a recently described pro-fibrotic cytokine, is normally not expressed in fibroblasts unless cells are exposed to TGFβ, but is constitutively overexpressed in fibrosis [111]. Another protein is a splice variant of the ECM component fibronectin, ED-A domain-containing fibronectin, expressed during embryogenesis and de novo expressed during wound healing and fibrosis [19;112]. These proteins can both enhance the contractile phenotype of the fibroblast and prolong the induction of ECM accumulation.

Table 3. Inflammatory and structural cells that produce TGFβ and their response caused by TGF-β (from studies in human cell lines and tissue) (Adapted from Howell J.E. and McAnulty R.J., 2006)

Inflammatory cells that produce TGFβ and response vaused by TGFβ signaling in these cells
Macrophages / Monocytes Inhibits release of NOS, inhibit prostaglandin synthase production / Causes chemotaxis, enhances integrin and type IV collagenase expression
Mast cells / Eosinophils Causes chemotaxis and morphological changes / Enhances apoptosis, upregulates chemokine receptor, inhibits IL-5 and GM-CSF and eosinophil peroxidase release
T –cells / B-cells / Neutrophils/ Inhibits MMP-9 mRNA induction, modulates cell proliferation, inhibits apoptosis in T-cells / Inhibits proliferation and immunoglobulin secretion / Causes chemotaxis
Structural & Resident cells that produce TGFβ and **response caused by** TGFβ **signaling in these cells**
Epithelial cells Inhibits proliferation, enhances motility, stimulates cytoskeletal reorganisation, induces GM-CSF and reduces IL-8 production, induces squamous cell differentiation.
Fibroblasts and Myofibroblasts Causes chemotaxis Induces differentiation to myofibroblasts: α-Smooth muscle actin expression, formation of stress fibers and cytoskeletal reorganization. Synergizes with IL-13 to enhance eotaxin-1 release. Reduces MMP-1 mRNA, protein and activity, reduces MMP-3 protein and increases TIMP mRNA, protein and activity, induces mRNA and protein of collagen I and III and fibronectin. Stimulates collagen synthesis, decreases intracellular collagen degradation Decreases uPA and t-PA and increases PAI-1. Increases the expression of biglycan versican and heparan sulfate proteoglycan, and ED-A fibronectin. Enhances glycanation and fibronectin-binding of CD44 proteoglycan, down-regulates glypican and enhances the production of hyaluronan.
Smooth muscle cells Induces procollagen I, induces release of IL-8, COX-2 and PGE2 Synergistically enhances eotaxin release with IL-13 and IL-4 Reduces eotaxin-3 release and modulates proliferation
Endothelial cells Induces cytoskeletal reorganisation and endothelial permeability, stimulates Endothelin-1 production, inhibits proliferation, increases fibronectin and procollagen IV production.

The extracellular matrix and its role in asthma progression

All tissues and organs are comprised of cellular and extracellular compartments. Mutual interactions of these are crucial for proper physiological functions at organ, cellular, and subcellular level. The extracelular compartment includes the extracellular matrix (ECM) between the various cells and the basement membrane that underlies cells of both the airway epithelium and blood vessel endothelium. Due to its diverse nature and composition, ECM can serve many functions, such as providing support and anchorage for cells, segregating tissues from one another but also regulating intercellular communication and biological activity of nearby cells.

The ECM of the lung is produced mainly by fibroblast, creating a complex network of different combinations of collagens**,** proteoglycans (of special interest in this thesis), but also hyaluronan, fibronectin, and other adhesive glycoproteins (Figure 10).

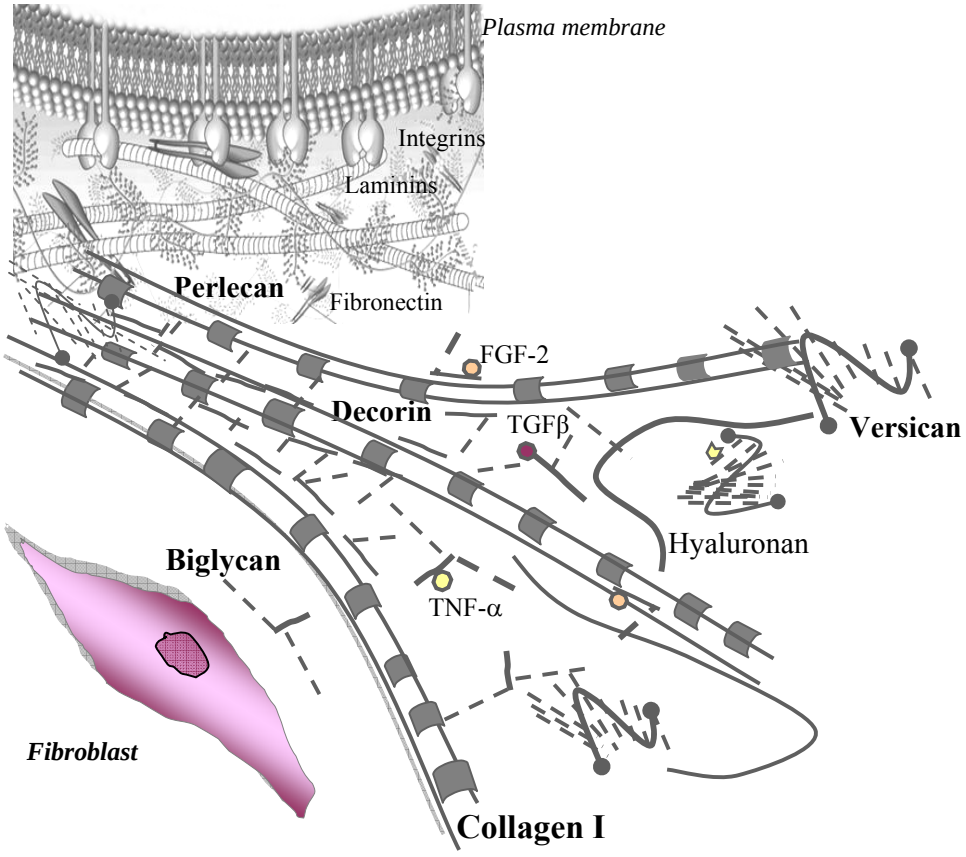

Figure.10. Schematic overview of the extracellular matrix (ECM) and its components' organization, produced by the resident structural cells. (Modified from Westergren-Thorsson G 1992)

The ECM is essential for processes like growth, wound healing and fibrosis. Under normal conditions, the ECM, being a highly organized and dynamic compartment, requires constant remodeling, a process regulated mainly by the family of matrix MMPs. Dysfunction in this regulation results in increased ECM accumulation as seen in asthmatics, and the fact that such deposition exists at specific sites in the airway wall suggests possible parallels in mechanism between asthma and other interstitial diseases. Systemic sclerosis (SS), idiopathic pulmonary fibrosis (IPF), but also COPD are some examples of diseases associated with an altered ECM composition and fibrosis formation. In the case of COPD, both fibrosis and massive tissue destruction (emphysema) can co-exist at the same time.

Collagens

The types of collagen associated with the airway remodeling are collagens I, III and V [113]. The main types of collagen normally found in connective tissues are the fibril forming collagens I, II, III, V, and IV, the latter a network forming basement membrane collagen. With exception of collagen II, and including collagen VI, all of these are essential for the lung ECM scaffold integrity.

Type I collagen, the most abundant protein in the interstitial compartment, together with collagen III, account for the majority of lung collagen (ratio 2:1), and have an important structural role by influencing numerous cellular responses. Excessive production and altered polymerization of type I collagen is a key feature of fibrotic disorders of the lung, [114] as well as the liver, kidney, skin and heart, often leading to severe organ dysfunction.

So far, about 28 distinct collagens have been identified, having in common a triple helical domain. They are the molecular scaffold in the body, providing strength and support for the connective tissues. Collagens are sub-categorized into different groups depending on their molecular structure, interaction, and type of polymerization. The procollagen molecule is composed of three polypeptides (pre-procollagen alpha chains) twisted in a helical fashion.

The process of fibril formation is tightly regulated and starts with synthesis of the pre-procollagen alpha chain on ribosomes. After translocation to the ER and Golgi, the polypeptide is co-translationally modified by hydroxylation and glycosylation. Assembly of three polypeptides to form procollagen occurs in the Golgi just before secretion of procollagen by exocytosis into the ECM. Both the C- and N-terminal extensions of the procollagen are processed extracellularly, an event that triggers the assembly of the mature protein into fibrils. Removal of the N- and C-propeptides from procollagens I, II, and III is catalyzed by the specific enzymes procollagen N- and C-proteinase. The two extension peptides from both ends of the procollagen molecule, C- and N- propeptides (PICP and PINP), are then released in equimolar concentrations into the circulation. The propeptide levels can therefore be measured in serum or cell culture medium (Paper II and III) as indicators of collagen production. Anomalies in the processing of the collagen propeptides can severely affect the aggregation of the molecules into fibrils, leading to defective connective tissues formation.

TGFβ1 is known to stimulate fibroblast collagen production and deposition. The synthesis of type I procollagen polypeptides is regulated by TGFβ partly by the TGFβ-response elements in the procollagen promoter, and via the Smad signaling pathway. GCs have been shown to diminish the synthesis of procollagen I without affecting the degradation of procollagen mRNAs [115]. The GC-mediated decreases of type I procollagen mRNAs in skin fibroblasts and lung fibroblasts have been known for more than a decade [116].

Despite these *in vitro* evidence of interaction between procollagen I, TGFβ and GCs, some *in vivo* studies show conflicting results on the ability of GCs to reduce collagen accumulation and diminishing thickening of the subepithelial layer. In addition, no significant differences have been found in the amount of submucosal collagen deposition and the number of TGFβ expressing cells when comparing asthmatics (mild, moderate, and severe) and normal control subjects [117], suggesting that although increased collagen deposition in the subepithelial layer is a characteristic of asthma, it may not be a functional read-out tool for asthma severity. Or perhaps, GCs might be effective in reducing submucosal collagen deposition when used for a long period of time and at a higher dose. The incapacity of inhaled GCs to inhibit TGFβ expression and subsequent

collagen accumulation may be responsible for the persistent fibrosis seen in this group of patients with severe asthma. Nevertheless, other factors must be considered; the complexity of collagen I synthesis, and processing and transactivation by TGFβ that could be dependent on additional regulatory elements and cross-talk between signaling pathways.

Proteoglycans

Proteoglycans constitute the other main class of macromolecules that make up the ECM. A straight forward description by Lander AD and Scott B. Selleck SB [118] was given on the subject of proteoglycans: **"**Proteoglycans: cell biologists have had a love-hate relationship with these molecules almost since their discovery. Their biochemical properties, dominated by heterogeneous and highly charged glycosaminoglycan (GAG) chains, can make purification challenging and structural analysis painful. Their ability to bind scores of growth factors, growth factor-binding proteins, extracellular proteases, protease inhibitors, ECM molecules, and other proteins takes the concept of molecular promiscuity to new heights. On top of this, they seem always to be underfoot, showing up on plasma membranes in hundreds of thousands of copies per cell and in extracellular matrices at milligram per milliliter concentrations" (cited with permission).

Proteoglycans are a superfamily of macromolecules ubiquitously expressed and with a multitude of functions in both physiological and pathological conditions. Proteoglycans consist of a core protein substituted with a varying number (from one to more than a hundred) glycosaminoglycan (GAG) side chains (Figure 11). The core protein component of proteoglycans is synthesized on ribosomes, and after translocation into the lumen of the RER, the main addition of GAGs to a link region in the core protein occurs in the Golgi. The GAG part of the proteoglycan is then modified by a series of epimerization and sulfation reactions that require numerous enzymes for its biosynthesis. This is the basis that generates vast structural diversity among them. Five main groups of GAGs are distinguished: chondroitin sulfate (CS) / dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronan. The proteoglycan is then either secreted into the ECM, transported to the cell membrane, or to intracellular compartments. Such complex

compositions of proteoglycans generate many unique functions of the individual proteoglycan that are attributed to either their protein core, or to the attached GAG chains. Proteoglycans have major roles in pathophysiological processes that occur in the ECM: they regulate ECM viscoelasticity; they maintain structure and function; they modulate the inflammatory response; and they influence tissue repair and remodeling [119;120].

Turnover of secreted proteoglycans usually involves enzymatic cleavage of the GAGs followed by intracellular degradation. Failure of proteoglycan degradation is characteristic of a group of genetic disorders, called mucopolysaccharidoses, where proteoglycans accumulate within the cell, due to inactivity of specific lysozomal enzymes that normally degrade GAGs. Depending on the type of proteoglycan that is not degraded, various disease symptoms can arise.

With the exception of keratan sulfate, all others are found in the lung ECM, produced by the resident structural cells. Proteoglycans carrying CS and DS GAGs are localized pericellulary, while proteoglycans with HS GAGs are mainly present in the basement membranes (Figure 10).

Apart from the nature of their GAG chains, proteoglycans can be categorized depending on their size. Examples of large proteoglycans are **versican**, **perlecan** and examples of small ones are the small leucine rich repeat proteoglycans (SLRPs) including **decorin**, and **biglycan**, respectively, all of which are also the main proteoglycans analyzed in this thesis (I, II, III). These proteoglycans constitute the ECM proteoglycans that are localized in different areas within the lung; versican resides in the lung ECM, perlecan in the vascular and epithelial basement membrane, decorin and biglycan in the ECM and in the epithelial basement membrane linked with collagen fibrils [120]. Beside ECM proteoglycans, there are also cell-surface associated proteoglycans and intracellular proteoglycans which will only briefly be described in the following sections.

Perhaps the main reason attention has been drawn to proteoglycans, in both inflammatory and fibrous disorders, is their interactions with numerous cytokines and growth factors, and their ability to create different interactions either by their GAGs or their core protein. Several studies have revealed that versican, biglycan, perlecan, and decorin are hallmarks of early and/or late progression of the ECM remodeling seen in asthma [120-124]. A positive correlation has been found between the deposition of versican and biglycan in the subepithelial layer of the airway wall and airway hyperresponsiveness in asthmatic patients [121]. Another significant correlation was found between a high degree of hyperresponsiveness and the larger amounts of proteoglycans produced by asthmatic bronchial fibroblast *in vitro* [123].

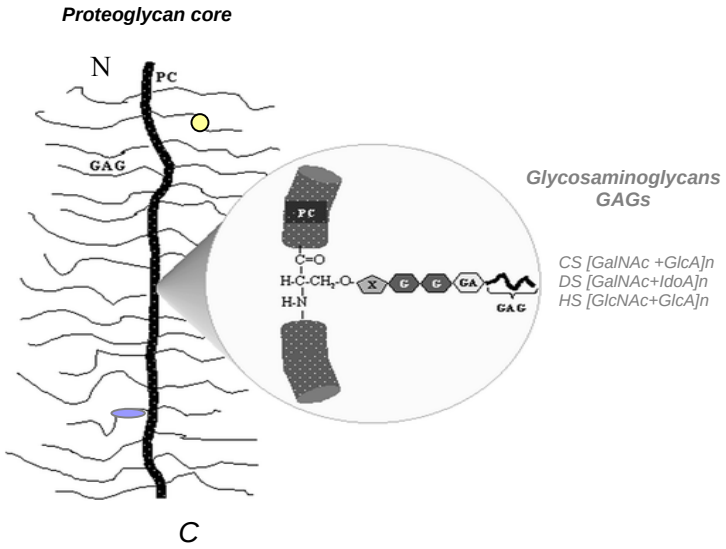

Figure 11. Schematic illustration of a proteoglycan with covalently attached GAGs, and the link region. GalNAc: N-acetyl galactosamine; GlcA: Glucuronic acid; GlcNAc: N-acetyl glucosamine; IdoA: Iduronic acid.

Versican is a CS/DS proteoglycan that belong to the hyalectan family having in common three structural domains: the N-terminal region, responsible for binding hyaluronan; a central domain carrying the GAG chains, and the C-terminal globular region that interacts with lectins on the cell surface, s well as other GAG and proteins such as tenascin. Versican consists of a core protein (200 kDa) and between 10 to 30 covalently attached CS GAG chains, giving this molecule a high degree of sulfation. Versican

molecules form a highly organized extracellular network. Deposition of versican is specific to both the early and late fibrotic airway remodeling process in lung diseases. In postmortem tissue from patients with severe asthma a marked deposition of versican is found in the airway wall ECM [120;125]. Versican is also significantly increased in atopic asthmatics and has been correlated with airway responsiveness [121]. Regardless of the nature of the driving inflammatory process, collagen I synthesis takes place in a versican-rich provisional matrix, surrounded by activated fibroblasts, suggesting that versican may influence the progression of the repair process following many different types of lung injury [126]. Versican is known to be upregulated by many cytokines including TGFβ, PDGF and EGF [85] (Paper II, III) and IL1β [127]. The numerous GAG chains of versican have been shown to bind various chemokines, and promote inflammatory cells infiltration by their associations with selectins on the surface on neutrophils [128]. In addition, versican has splice variants; some of which were shown to be differentially induced in bronchial SMCs [129].

Perlecan is mainly a basement membrane-associated proteoglycan, but is also found pericellularly [130], and belongs to the HS family of proteoglycans. Other members belonging to HSPG, also called "full-time" HSPGs are syndecans and glypicans found at the cell surface, agrin found pericellularly and the hybrid HSPG/collagen type XVIII, which together with betaglycan, CD44/epican, and testican are known as "part-time" HSPGs. Moreover, splice variants for perlecan, agrin, and type XVIII collagen protein cores, have also been reported [130]. These HSPGs are regarded as cellular extensions into the ECM, capable of coordinating numerous of protein- and cell signaling events within the ECM, and associated compartments such as the basement membranes.

Basement membranes are thin, cell-associated sheets distinguished from the ECM by their highly organized structure. These specialized forms of ECM are ubiquitously distributed in the body, and function not only as cell attachments, but also as regulators of diffusion, and growth factor presentation, maintenance of cell differentiation and cell migration. Within the lung, the basement membranes consist as three component layers: the laminas lucida, densa, and reticularis. In asthma, thickening of the lamina reticularis

is a characteristic feature of airway remodeling that can also be found in several other lung dissorders.

An important function for perlecan, among other HSPGs in this context, is its ability to store and regulate FGF-2 activity, a cytokine involved in both normal growth and thickening of the basement membrane. Co-localization of FGF-2 and HSPG in epithelial and endothelial basement membranes in mild asthmatics suggests a cooperative and vital role in airway remodeling in asthma [131]. In addition, during fibrosis formation, the apoptosis of endothelial cells triggers the proteolysis of ECM with subsequent release of a C-terminal fragment of perlecan which in turn inhibits apoptosis of fibroblasts [132]. Perlecan production is increased in BAL fluid fibroblasts, and in bronchial fibroblasts perlecan production is negatively correlated to airway hyperresponsiveness in asthmatics [123]. Perlecan is also the predominating proteoglycan in the basement membrane. With a >400 kDa core protein, complex domain structure and three attached HS GAGs, each of them averaging >300 KDa, this proteoglycan likely harbors multifunctional properties yet to be shown. Via binding to other classical constituents of the basement membrane such as collagen IV, laminin, nidogen, entactin, bamacan, and other perlecan molecules, this proteoglycan is proposed to have a stabilizing effect on the basement membranes. A hybrid variant of perlecan with DS chains has also been reported.

Various functions of HS GAG chains, including binding to several growth factors besides FGF-2, are being revealed. For example, HSPGs bind TGFβ1 and TGFβ2 [103], while free GAGs may protect TGFβ1 from proteolytic degradation [133]. As mentioned previously, cell surface HSPGs, such as betaglycan, may act as co-receptors that facilitate TGFβ interaction with its type II receptor [134] and may also regulate TGFβ availability by controlling the deposition of latent TGFβ into the ECM in association with fibronectin [135]. HSPGs have also been implicated to contribute to VEGF accumulation in the ECM [136]. These examples reflect the multifunctional involvement of HSPGs in both normal and pathological conditions.

Of the cell-surface associated HSPGs, the best characterized are the syndecans and glypicans. The syndecans are integral membrane proteins with a single transmembrane and a cytoplasmic domain, thought to interact with the actin cytoskeleton. This

proteoglycan may also appear as a hybrid variant, possessing both HS and CS GAGs. *In vivo* model studies indicate that upon insult, syndecan-1 proteoglycan is shedded as a result of the innate host response to tissue injury and inflammation [137]. However, the reason and mechanism behind this are not fully understood. HS chains of syndecan-1 ectodomains may possess anti-inflammatory properties, and attenuate allergic lung inflammation via suppression of Th2 cell recruitment to the lung [138].
Glypicans possess an extracellular region with GAG attachment sites, and a C-terminal glycosylphosphatidylinositol (GPI) anchor by which they are linked to the outer plasma membrane. Glypican family members are selectively expressed on different cell types and recognized for involvement in growth factor signaling and transport of various inflammatory mediators. It is also suggested that glypican, along with syndecan and perlecan, may have an important role in regulating the biological activity of FGF-2 via their HS GAG chains. Other examples of cell-associated proteoglycan members are thrombomoduline and CD44.

Biglycan and Decorin are members of the family of small leucine-rich proteoglycans (SLRPs) that reside in the ECM. Other members in this family are asporin, fibromodulin, lumican, PRELP, osteoadherin, keratocan and mimecan. They all have core proteins with leucine-rich repeats usually occupying more than 70% of their core proteins. The molecular size of their core protein varies between 36 and 40 kDa. Biglycan contains two CS/DS GAG chains attached near its N-terminus making it different from decorin that only has one GAG chain. These proteoglycans are secreted from the cells after synthesis and are found throughout the ECM. They both have profound functions, not only as integral components of the ECM but also as main regulatory molecules by their ability to bind several cytokines and growth factors, in particular TGFβ [105;139]. Beside the modulation of cytokine activities both biglycan and decorin are directly involved in cell signaling, thereby regulating proliferation and apoptosis of various cell types *in vitro* and *in vivo* [140].

Although these are very small molecules, as compared to HSPG, they can effect the whole net structure and composition of the ECM, due to their cross-linking ability

between collagen fibrils [141;142]. Decorin in particular, depending on the circumstances, is suggested as a major determinant of whether the collagen fibrils will appear tightly or loosely attached to each other. Biglycan deficient mice have decreased capacity to produce marrow stromal cells, reduced response to TGFβ, reduced collagen synthesis and relatively more apoptosis [142]. It has been proposed that SLRP can coordinately regulate fibrillar organization. Decorin-deficient tissues were shown to contain more biglycan than control tissues, suggesting a possible compensatory action for biglycan in the absence of decorin [143]. Such effect of biglycan was though absent regarding lung mechanics in decorin deficient mice [141]. However, the exact impact of decorin on the collagen network in asthma and other lung disorders such as COPD is controversial and rather complex. For example, in asthmatic airways, a reduced amount of decorin is found in the bronchial mucosa [144], and in fatal asthma less decorin is also found in the external area of small airways [125]. In the thickened airways, along with sustained collagen, this will imply that less decorin may cause loosening of the collagen and emphysema, leading to loss of lung elastic recoil. In this aspect, highly induced levels of decorin would then imply stiffening of local collagens and greater thickening of the airway wall. Regarding the ability of biglycan and decorin, in regulating and sequestering TGFβ, there is also conflicting reports, on decorin in particular and whether it has an anti-fibrotic action [145] or a pro-fibrotic role [106;146;147].

Considering the wide distribution and multiple roles of proteoglycans in inflammatory and fibrotic disorders, attempts have been made to decrease an accumulating reactive ECM by use of GCs. In studies of early osteoarthritis, levels of increased aggrecan and versican were not consistently decreased by GC treatment except at high concentrations [148]. However, in other cells, such as skin fibroblast, while decorin levels were increased, biglycan expression was decreased or unaffected at clinically relevant doses of GC [149]. These inconsistent findings suggest that effects of corticosteroids on ECM-related genes may be dose-dependent, cell specific to certain genes and that there might be other factors that will determine the final effect.

Other ECM proteins

There are several other proteins whose functions are essential for the organization, maintenance and function of the ECM. Some of these, such as hyaluronan, fibronectin, tenascin and elastin are all associated with the airway remodeling in asthma.

Hyaluronan is the simplest of the GAGs, regarding the structure. This negatively charged polysaccharide is found in the ECM, without being attached to a core protein. Hyaluronan has been assigned various physiological functions in the ECM partly due to its ability to bind water. Whereas other GAGs are synthesized inside the cell and released into the ECM, hyaluronan is spun out from the cell surface by an enzyme complex embedded in the plasma membrane. Under physiologic conditions, hyaluronan exists as a high-molecular-weight polymer, but after tissue injury it undergoes a dynamic regulation resulting in accumulation of lower molecular weight species [150]. The major receptor for hyaluronan, CD44, also functions to clear hyaluronan fragments that are produced after lung injury. Failure to clear hyaluronan fragments has been shown to sustain inflammation [151]. In addition, elevated levels of hyaluronan have been reported in asthmatic BAL fluid and the low-molecular hyaluronan fragments have been implicated in morphological changes in eosinophils with subsequently increased synthesis of ICAM-1 and TGFβ [152]. The high-molecular-mass hyaluronan expressed on the cell surface of alveolar epithelial cells have been shown to interact with Toll-like receptors 2 and 4, providing a homeostatic signal to protect the distal lung from injury and promote repair. Thus, restoring high-molecular- hyaluronan fragments to the lung in the context of lung injury may promote repair and restoration of lung function [153]. Fibronectin is important for organizing the ECM, but also for guiding cell migration and cell adhesion. One of the splice variants of fibronectin, ED-A fibronectin has been implicated in myofibroblast differentiation by TGFβ [78] which in turn may be dependent on cell adhesion and integrin signaling [154].

Another ECM molecules that are also found elevated in severe as well as atopic asthmatic is tenascin [155], important component for the cell movement, and laminins [156], that are crucial for the organization of the basement membrane.

A network of elastic fibers in the ECM gives the surrounding tissue elasticity. The main component of elastic fibers is elastin, and deposition of it has been found in children with severe asthma, along with thickened reticular basement membrane, hypertrophied airway smooth muscle, and damaged airway epithelium [157].

Metalloproteinases

There are two groups of enzymes that are required, involved, and of importance for the integrity of the ECM and a balanced turnover of its components. These are the metalloproteinases (MMPs) and their negative regulators, the tissue inhibitors of MMP, (TIMPs). The large family of MMPs at present includes 26 members. They are classified based on their substrate preferences, despite overlapping substrate specificity (Table 5). There are five main subgroups of MMPs consisting of collagenases (such as MMP-1) that degrade fibrillar forms of ECM collagen; the gelatinases (MMP-2 and -9) that are more specific for denatured collagens and collagen type IV of the basement membrane; the stromelysins (such as MMP-3) which primarily cleave non-collagen components of the ECM such as proteoglycans, fibronectin, and laminin. These are also the main MMPs investigated in this thesis. Additional subgroups include membrane-type MMPs found on the surface of many cell types and matrilysins, metalloelastase, and other MMPs with less defined characteristics [158;159].

Table 5. Classification of the matrix metalloproteinase family and substrate specificity (Modified from Jarjour NN and Kelly EA, 2003).

Group	*Descriptive name*	*Abbreviation name*	*Principal substrates*
Collagenases	Interstitial collagenase	**MMP-1**	Fibrillar collagen types I, II, III gelatin, proteoglycan core protein
	Neutrophil collagenase	MMP-8	As above
	Collagenase-3	MMP-13	As above and fibrillin
	Collagenase-4	MMP-18	As above
Stromelysins	Stromelysin-1	**MMP-3**	Proteoglycans, laminin, fibronectin, casein, collagen II, III, IV, V, IX, X, XI, elastin, gelatin, fibrillin, pro-MMP-1, pro-MMP-8, -9, -13, proteoglycan core, tenascin, vitronectin

	Stromelysin-2	MMP-10	As above
	Matrilysin	MMP-7	Collagen IV, fibronectin, gelatin, laminin, nidogen, pro-MMP-1, proteoglycan core protein
Gelatinases	Gelatinase A (72 kDa)	**MMP-2**	Gelatins, bFGF, collagens I, IV, V, VII, X, XI, elastin, entactin, fibrillin, fibronectin, galectin-2, gelatin, laminin, proteoglycan core protein of cartilage
	Gelatinase B (92 kDa)	**MMP-9**	Gelatins, Collagens I, IV, V, elastin, fibrillin, galectin-3, proteoglycan core protein
Membrane-type (MT)	MT1-MMP	MMP-14	Collagen, I, fibrillin, fibronectin, gelatin, laminin-1, pro-MMP-2, -13, proteoglycan core protein of cartilage, tenascin
	MT2-MMP	MMP-15	Fibronectin, laminin, pro-MMP-2, -13, tenascin
	MT3-MMP	MMP-16	Collagen III, fibronectin, gelatin, pro-MMP-2
	MT4-MMP MT5-MMP	MMP-17 MMP-21	Currently unknown
Others	Stromelysin-3	MMP-11	Serine protease inhibitor Alpha-1-antiprotease
	Metalloelastase	MMP-12	Elastin, Collagen IV, fibrillin, fibronectin, laminin, vitronectin

The MMPs are a group of zinc- and calcium- dependent enzymes capable of degrading almost all components of the ECM, including basement membranes, growth factors, chemokines, proteinases, and cell surface proteins, such as adhesion molecules [158]. With such broad specificity, these enzymes are involved in a number of physiological processes, including embryogenesis, normal tissue remodeling, wound healing, angiogenesis, but also under pathological conditions, including asthma[158].

As in all tissues, expression of MMPs in the lung is a highly regulated process. These enzymes are tightly regulated at multiple levels, including gene transcription, activation of the latent enzyme, and inactivation by specific inhibitors. With the exception of MMP-2, a constitutively produced MMP, gene transcription of MMPs can be induced by various cytokines and growth factors [160], including pro-inflammatory mediators such as Il-1β and TNF-α. Once secreted, in order to become active, MMPs requires removal of the pro-peptide domain that blocks access to their catalytic site. Proteases such as trypsin,

plasmin, plasminogen activators, and elastase as well as other MMPs can participate in this process [161], both *in vivo* and *in vitro*. TIMPs are the tissue specific inhibitors of MMPs that bind non-covalently in a 1:1 molar ratio with MMPs. There are currently four known TIMPs (TIMP-1 to 4). In addition to their MMP-inhibitory activity, TIMPs are implicated to affect cell growth by both inducing apoptotic cell death and, under some conditions, by stimulating cellular proliferation [162].

There is emerging evidence supporting the view that an imbalance between the MMPs and TIMPs in injured lungs may promote altered composition, deposition, and organization of the ECM components, favoring fibrosis. In the airways, MMPs are secreted by both the inflammatory and the structural resident cells [163;164]. Both MMPs and TIMP-1 have been found in exaggerated quantities in sputum, BAL fluid, and biopsies from patients with asthma [159]. In particular, the ratio between MMP-9 and TIMP-1 is considered crucial for ECM remodeling of asthmatic airways [165].

Asthma exacerbations are characterized by increased **MMP-9** activity, and this MMP is also the most frequently reported in asthma. Such increased activity may be related to exaggerated airway inflammation and airway remodeling. In addition, MMP-9 was increased in bronchial biopsies of asthmatics [88], where MMP-9 immunoreactivity in the subepithelial layer was associated with asthma severity [166]. The level and activity of MMP-9 was also increased in sputum of asthmatics [167;168], in BAL fluid [169], and further enhanced during asthma exacerbations [170].

MMP-2 is another gelatinase that mainly degrades type IV collagen, a major component of the airway subepithelial basement membrane (Table 5). MMP-2, although regarded as a constitutively expressed MMP, has also been implicated in ECM turnover. Increased MMP-2 levels are reported in both BAL fluid and induced sputum from asthmatics [167;170], however at much lower levels than MMP-9.

The collagenase **MMP-1** is able to initiate breakdown of the fibrillar collagen types I, II, and III (Table5). Active MMP-1 is capable of hydrolyzing type I collagen, however, it degrades collagen III to a higher degree as it hydrolyzes type III collagen 10-fold faster

than type I collagen [171]. MMP-1 is mainly secreted by local fibroblasts and macrophages. In sputum cells from mild asthmatics, increased mRNA transcripts for MMP-1 have been related to FEV_1 reduction in asthmatics [172].

MMP-3 belongs to the stromelysins, which are thought of as the main regulators of proteoglycans, and a range of ECM proteins including collagen IV, V, fibronectin, laminin, and elastin [173] (Table 5). MMP-3 immunoreactivity have been detected in both mild and severe asthmatics and without significant difference in the extent of immunoreactivity between the groups [163]. Others have reported increased levels of MMP-3 in severe asthmatics [174]. There are reports on MMP-3 localization in the submucosal matrix of patients with chronic asthma [163] but the MMP-3 immunoreactivity did not seem correlated with inflammatory cell number. Such findings suggest that it may be derived from resident rather than inflammatory cells, and thus, of particular importance for ECM. Indeed, in cartilage, inverse correlation has been reported between MMP-3 and the sulfation ratio in proteoglycan, implying that changes in the composition of ECM correlates with increased MMP-3 [175].

While MMPs are recognized as mediators of airway inflammation and remodeling, anti-iflammatory actions have also been reported from murine models [176]. The differential tissue distribution along with the overlapping substrate specificity and possible compensatory mechanism, make the MMP function and regulation rather complex and far from clear in asthma. Recently, Wenzel and colleagues reported an increase in MMP-9 activity in the subepithelial basement membrane that is accompanied by higher TGFβ activity [166]. This co-localization in the ECM, in particular during inflammation and fibrotic processes suggests a close association between MMP-9 and TGFβ. For example, MMP-9 is involved in activating latent TGFβ [100] and such interactions in e.g. COPD, depending on relative activity of each component in the ECM or other compartments, may result in either destructive or fibrotic events respectively. Furthermore, MMP-9 is constitutively produced in fibrocytes, a circulating progenitor cell and an important source of active MMP-9 involved in angiogenesis in the very early phases of wound healing [177].

As for the MMPs, TIMP-1 levels in the airways of asthmatics, have also been shown to be elevated, favoring airway fibrosis [168;169] however, it may also protect airway tissue by inhibiting activity of various MMPs. Especially the high TIMP-1 levels and the low MMP-9/TIMP-1 ratio in sputum from asthmatics is associated with airway obstruction [168] and with structural airway changes in severe asthmatics [178].

In some studies, asthma treatment with inhaled GCs downregulates the MMP-9 activity and upregulates TIMP-1 expression; that would imply a decrease of the subepithelial collagen deposition [33]. Other studies have reported that inhaled GCs do not affect BAL fluid MMP-9 or TIMP-1 levels or MMP-9 activity. In reports not obtaining any effects of GCs on MMP levels or activity it has been concluded that lack of effect is due to a low baseline inflammatory activity in the subjects [179]. On the other hand, both an increase of TIMP-1 [180] and a decrease [181] by inhaled GCs have been reported.

In addition to the MMP superfamily there are also cell surface transmembrane proteins known as ADAMs (a disintegrin and metalloproteinase). Although they posses a metalloproteinase-like activity, the ADAMs are distinguished from membrane type (MT) MMPs by the presence of a disintegrin-like domain with unique properties to promote adhesion of ADAMs to cell surface integrins, thus facilitating cell-cell and cell-matrix interactions. There are currently more than 30 known cell surface ADAMs. Polymorphisms in the gene for ADAM 33 have been associated with lung function decline in asthmatics, suggesting a possible role in airway remodeling [31].

Present investigation

The main purpose of this thesis was to investigate whether anti-asthma treatment with the combination of a GC, budesonide, and a LABA, formoterol, under defined conditions, would affect the activity or ECM production and turnover of lung fibroblasts from different origins. (For study design and methods used, see figure 12).

The following aims were undertaken:

1. Establishing an *in vitro* model resembling an early exudative environment by exposure of human embryonic lung fibroblasts (HFL-1) to 10% serum. Will budesonide and formoterol affect the HFL-1 and their total as well as individual proteoglycan production? If so, how are the effects mediated?

2. Establishing a more chronic and fibrotic environment by exposure of HFL-1 fibroblasts to TGFβ1. Do the effects of budesonide and formoterol on proteoglycan production differ under these conditions compared with 10% serum exposure? Do these drugs affect the production of collagen I in the presence of TGFβ1? Does the effect of the drugs in the presence of TGFβ1 involve altered activity of MMPs and/or their inhibitor TIMP-1?

3. Establishing primary fibroblast cultures derived from adult lung biopsies. Are there major differences between ECM production and turnover in fibroblasts from mild asthmatic patients compared to healthy subjects at baseline and in the presence of TGF1β? Is there any correlation between ECM production at baseline (cells exposed to 0.4% serum) and patient lung function? Will the fibroblasts from the healthy and asthmatic subjects respond to the drug treatment in a similar way and to a similar degree? Are the drug effects similar to the effects in HFL-1 fibroblasts?

Study design and the various methods used.

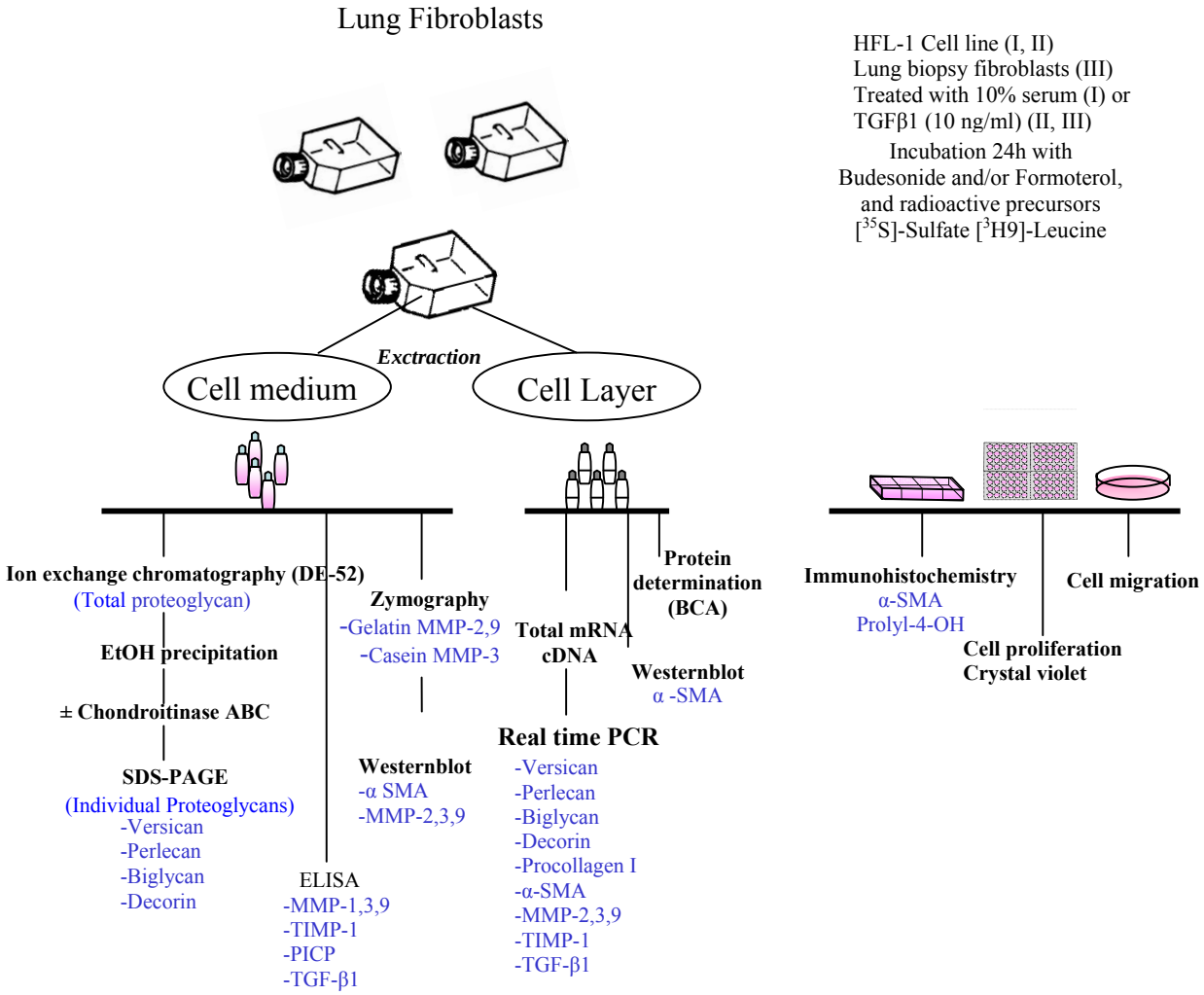

Figure 12. Study design. For details on methods and performance see Papers I, II, III.

Lung Fibroblast Proteoglycan Production Induced by Serum is Inhibited by Budesonide and Formoterol

Paper I

Our main issue in this study was whether budesonide and formoterol would affect the fibroblast proteoglycan production *in vitro*. We used 10% donor calf serum as a cell activator, and this induced both HFL-1 cell proliferation and proteoglycan production (2.5–5-fold). Treatment with budesonide alone decreased the total proteoglycan production only to some extent, and while formoterol had no inhibitory effects, when combined, these drugs synergistically reduced the total proteoglycan production. To exclude secondary mediated effects, a proliferation assay was performed and under a time period of 72 h, no marked effects of the drugs were seen. We then found that the synergistic effect of the drug combination was dependent on functional GR and β-adrenoceptor.

Next, we investigated whether budesonide and formoterol have any effects on the individual proteoglycans; versican, perlecan, biglycan and decorin (Figure 13).

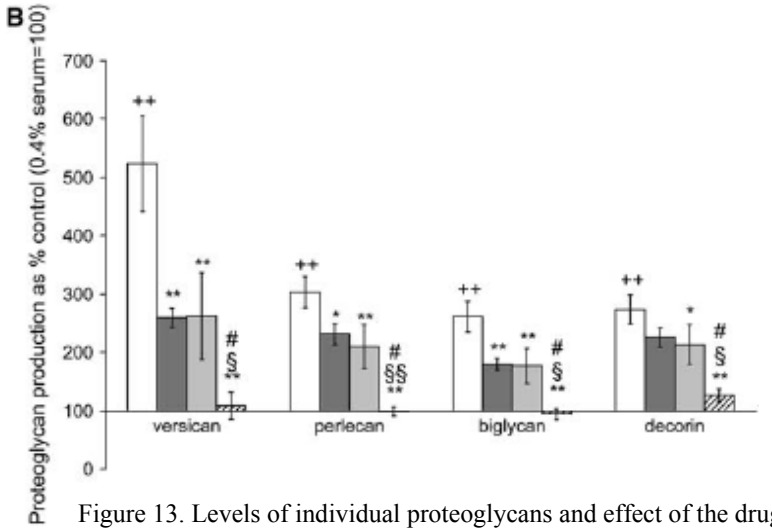

Figure 13. Levels of individual proteoglycans and effect of the drugs in 10% serum conditions. Open bars represent 10% serum (with drug vehicle). Budesonide (dark gray bars), Formoterol (light gray bars), their combination (hatched bars).

Both budesonide and formoterol alone had similar inhibitory effects on production of specific proteoglycans, but an enhanced and complete inhibition was seen with the drug

in combination. At mRNA level, formoterol did not have any effects on the individual proteoglycans except for reduction of versican expression. However, budesonide, alone and in combination with formoterol, significantly counteracted both serum-induced increase of versican mRNA as well as the decrease seen for decorin mRNA. It was concluded that *in vitro,* under conditions resembling early inflammation, the complementary action of budesonide and formoterol in combination reduced serum-induced proteoglycan production primarily at post-transcriptional level and without affecting fibroblast proliferation.

Extracellular matrix remodeling in human lung fibroblasts stimulated by TGFβ1. Effects of Anti-Asthmatic Drugs: Budesonide and Formoterol.

Paper II

How are TGFβ1-activated fibroblasts affected by budesonide and formoterol? TGFβ1 is a known inducer of fibroblast differentiation and synthesis of ECM components. In this study, TGFβ1 stimulation of HFL-1 resulted in shift of phenotype from an elongated, spindle like shape with long cytoplasmic projections in 0.4% serum, into a more rounded form with a more compact cytoplasm and with more well-defined stress fibers in the presence of TGFβ1 (Figure 14). The 2.5-fold increase of proteoglycan production, induced by TGFβ1, was reduced in a concentration dependent manner by budesonide but not by formoterol. From a pharmacological point of view, in the combination of the drugs used in a 100:1 budesonide:formoterol ratio (which approximates the clinical dose ratio), the addition of formoterol reduced the proteoglycan

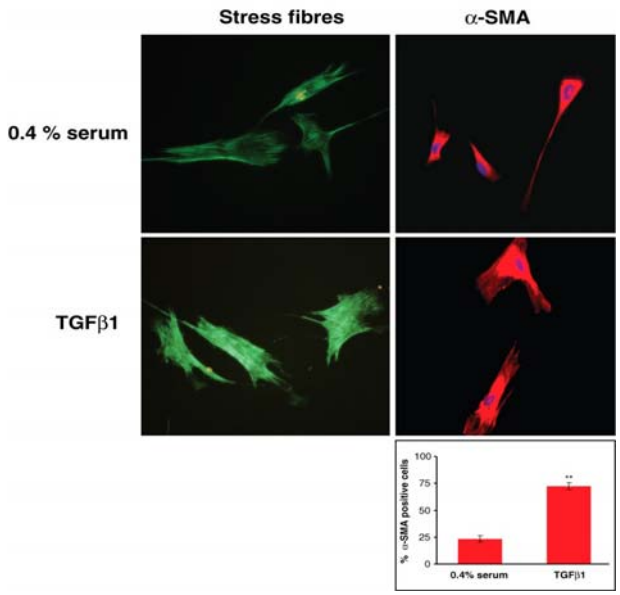

Figure 14. Fibroblast (myo) fibroblast transition in the presence of TGFβ1.

production to a level similar that achieved by budesonide at a 10-fold higher concentration (Figure 15).

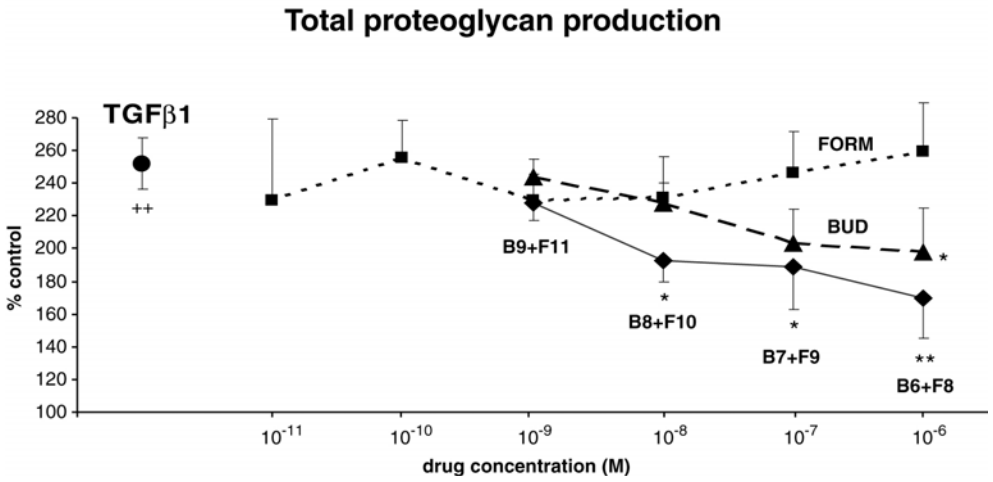

Figure 15. Dose response curves of budesonide and formoterol on total proteoglycan production in the presence of TGFβ1.

We then speculated that MMPs, known ECM degradation responsible enzymes, could be involved or even contribute to the observed effect on the proteoglycans. We chose the gelatinases MMP-2, MMP-9 and the stromelysin MMP-3 whose many target substrates include collagen I and proteoglycans. We found that TGFβ1 enhanced the production level and activity of all components measured; the various proteoglycans (Figure 16), PICP (a marker for ongoing collagen I synthesis), TIMP-1 protein, as well as the activities of MMP-3 and MMP-9 and MMP-2.

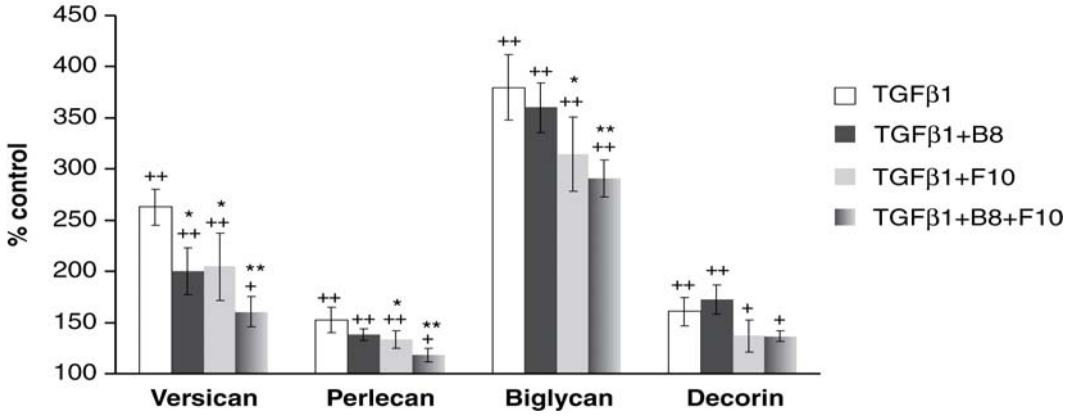

Figure 16. Levels of individual proteoglycans and effect of the drugs in the presence of TGFβ1.

Regarding the effects of the drugs, we found that the individual proteoglycan productions, in the presence of TGFβ1, were differentially regulated. Both budesonide and formoterol on their own, significantly reduced versican (40%) and perlecan (30%) (Figure 16). These effects were even greater when the drugs were applied together (63-64%). In contrast, biglycan and decorin productions were not affected by budesonide, but they were both reduced by formoterol (23-39%) and by the drug combination (32-39%). These additive effects of formoterol were not seen at mRNA level, but in combination with budesonide both versican and biglycan mRNA were decreased (49-50%). Regarding mRNA levels for MMP-2, only budesonide was able to decrease it. mRNA levels for proMMP-3 appeared completely suppressed by TGFβ1, and while significantly increased by the drug combination at mRNA level, the active enzyme was barely affected. On the other hand, the MMP-9 activity was markedly reduced, so the overall effects in this study suggest that complementary effects of budesonide and formoterol can more efficiently abolish ECM remodeling. These effects appear posttranscriptionally mediated, and may involve dysregulated MMP-9/TIMP-1 activities.

Extracellular matrix production by bronchial fibroblasts from healthy and mild asthmatics. Effects of budesonide and formoterol.

Paper III

In this study we used the same approach as in Paper II, but here, the effects of the drugs were investigated on bronchial biopsy-derived fibroblasts from 8 mild asthmatics and 5 healthy subjects. We started by characterizing *in vitro* baseline (0.4 % serum) and TGFβ1-induced production of the individual proteoglycans. We then proceeded with PICP, and investigated production and activity of various MMPs and TIMP-1. We found that in fibroblasts from asthmatic subjects: *1)* baseline PICP was negatively correlated with the patients lung function (measured as FEV1), *2)* PICP in the presence of TGFβ1 was decreased (by 58%), owing to additive actions of the drug combination (Figure 17D), *3)* biglycan production was reduced by nearly 40% by the drug combination, although the total proteoglycan production in the presence of TGFβ1 was rather resistant to the drugs.

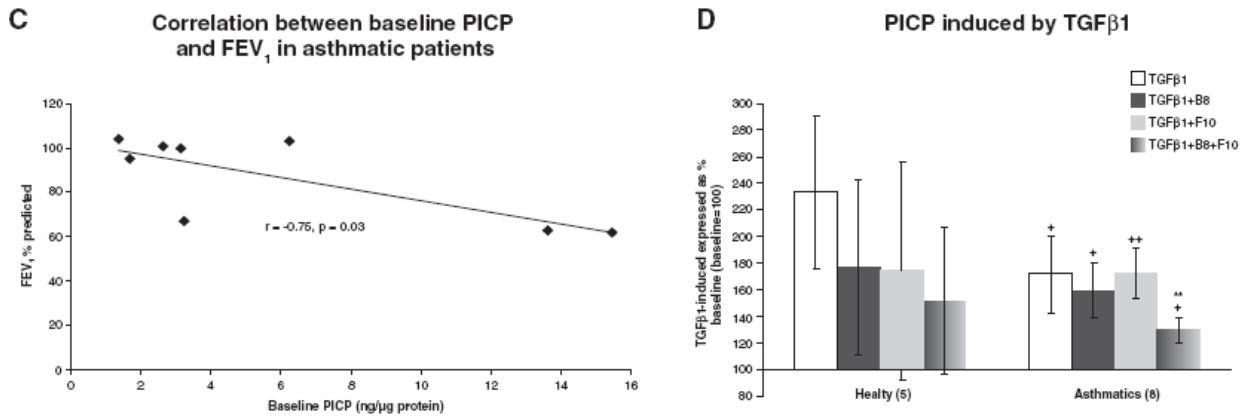

Figure 17. Procollagen Type I C-peptide (PICP) levels at baseline (0.4% serum)(C) and in presence of TGFβ1 (D).

In the presence of TGFβ1 the total proteoglycan production increased in cells from both healthy and asthmatic subjects. In healthy cells, the total proteoglycan production was almost completely reduced (84%) by the combination of the drugs but not by either drug alone. Regarding effects on the individual proteoglycans from the healthy subjects, a marked reduction (>60%) by the drug combination was also seen on the production of versican, perlecan and biglycan (Figure 18), which (with exception for decorin) are in agreement with the findings in HFL-1 cells in Paper II.

In fibroblast from asthmatic subjects, effect of the drugs was limited to biglycan and decorin (Figure 18). In general, TGFβ is known to induce biglycan while often suppressing levels of decorin. Indeed, in majority of the asthmatics subjects (5 of 8 subjects), we found that TGFβ1 markedly suppressed decorin production (see also Figure 3C in Paper III). In these patients treatment with the drugs, either alone or in combination, efficiently reversed and brought back the decorin production to the baseline. Regarding biglycan, treatment with the combination was most efficient in reducing biglycan levels. Thus, these drugs most likely have an important effect on the collagen network formation as both biglycan and decorin are involved in fibrillation and organization of the collagen network in the ECM.

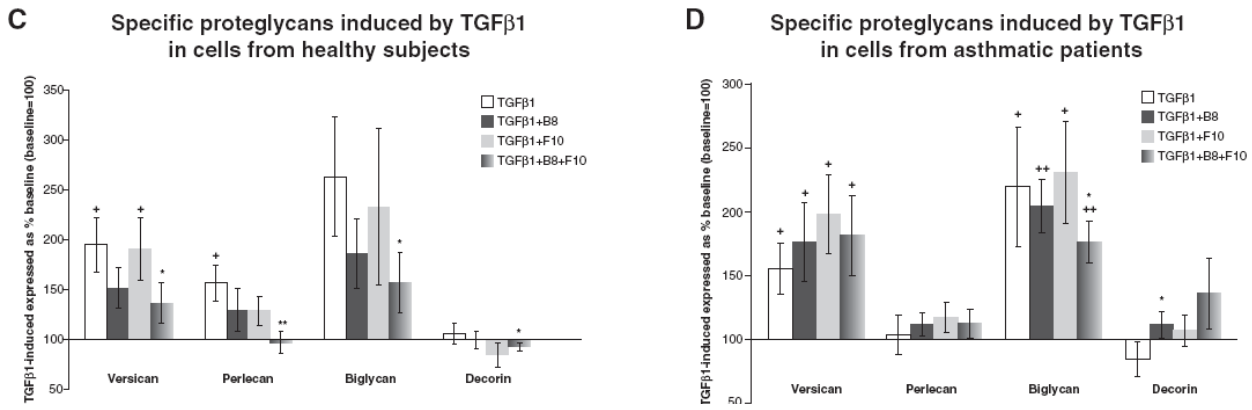

Figure 18. Levels of individual proteoglycans from healthy (B) and asthmatics (C) and effects of the drugs in the presence of TGFβ1.

Notably, an interesting finding is a high and positive correlation between baseline MMP-9 activity and patient lung function. In addition however, there was no correlation of baseline MMP-9 activity to the levels of procollagen I or the individual proteoglycans. This may indicate that high MMP-9 activity contributes positively and improves lung function. In particular, those asthmatics whose MMP-9 activity appeared suppressed in the presence of TGFβ1 (see also Figure 5C, D in Paper III) could benefit from the combination treatment as it enhances their MMP-9 activity, which in turn could counteract an accumulating ECM.

We also found a negative correlation of baseline activities for MMP-3 and MMP-2 to the patient lung function, and a positive correlation to PICP levels and the individual proteoglycans. Depending of whether it is the collagen or proteoglycan accumulation that takes place first in the ECM, high levels of MMP could alter the composition ECM. On the other hand, an active ECM degradation along with active ECM synthesis could also reflect a dynamic repair and turnover process, considering the trend of higher levels of total proteoglycans that was observed after drug treatment in the asthmatics subjects (see also Figure 1C Paper III). Nevertheless, studies with a larger number of subjects, with less heterogeneity if possible, would increase our chances of elucidating the effects observed.

Protein levels of TIMP-1, the main inhibitor of MMPs, at baseline in the asthmatic subjects, was found positively and highly correlated with the levels of PICP, versican and total proteoglycan content. High TIMP-1 levels might promote accumulation of ECM, and may contribute to airway fibrosis. In healthy subjects, the protein level of TIMP-1 appeared induced by TGFβ1. This induction was brought back to baseline by the drug combination. A similar finding on the effects of the combination was observed in Paper II. The difference, between the healthy subjects and HFL-1, was that only in the healthy subjects were TIMP-1 levels significantly reduced by the drugs when not combined. On the other hand, in asthmatics, TIMP-1 levels were unaffected by either TGFβ1 or the drugs, and considering the findings of others that a high MMP-9/TIMP-1 ratio may be beneficial in asthma our results suggest a beneficial effect of the combination therapy.

Thus, the main conclusion of this paper is that by the reduction of collagen I synthesis and biglycan production in fibroblasts from asthmatic subjects activated by TGFβ1, the combination of budesonide and formoterol may have beneficial effects in asthmatic airways, counteracting enhanced ECM synthesis and deposition.

General discussion

By culturing fibroblasts under specified conditions, such as incubating in serum or in the presence of a pro-fibrotic cytokine, such as TGFβ1, *in vivo* phenotypic plasticity of lung tissue in health and disease may be mimicked *in vitro*. The results presented in this thesis on fibroblasts originating from embryonic and adult human lung tissue show that various aspects of the airway remodeling process *in vivo* can be addressed in *in vitro* investigations; for example fibroblast proliferation, migration and ECM production.
In the healthy lung, resident fibroblasts are embedded in their local ECM and have low proliferation rate and activity. In asthmatic lungs, however, there is an ongoing chronic inflammation that periodically, for example during disease exacerbations, becomes acute and results in an excessive plasma exudation into the ECM. This leads to activation of resident lung fibroblasts by various plasma components, including serum and growth factors, resulting in an increased proliferation rate and ECM production. Plasma exudation was shown to be well correlated to symptoms in airway disease [182]. It is therefore relevant to explore how anti-asthma drugs affect fibroblast responses under such conditions; especially the synthesis of proteoglycans and collagen, whose production in asthmatic patients is negatively correlated with lung function [121;123;183].
In the studies performed on embryonic lung fibroblasts we found that both 10% serum (Paper I) and TGFβ1 (Paper II) caused a markedly increased proteoglycan production. Furthermore, both serum-induced and TGFβ1-induced versican, perlecan, and biglycan synthesis were efficiently reduced by a GC, budesonide, and a LABA, formoterol. Besides additive effects on these main and individual proteoglycans, synergistic effect of the drugs on the total proteoglycan production was observed (Paper I) where formoterol had no effect on its own. The inhibitory effects of formoterol on specific proteoglycans were somewhat surprising, as β2-agonists are not considered to have significant anti-inflammatory effects *in vivo*.
Furthermore, TGFβ1 has been shown to decrease β2-adrenoceptor mRNA and receptor number in tracheal smooth muscle cells [184], which would lessen the effects of $ß_2$-agonists, but this does not seem to occur in lung fibroblasts used in our studies, as the effects of formoterol were rather similar in serum and in the presence of TGFβ1. Similarly,

regarding the GC-receptor, we found that TGFβ1 significantly increased its transcript levels in the embryonic fibroblasts (unpublished observations Todorova L.), which should increase sensitivity to GCs. Again, the effects of budesonide on proteoglycan production were similar in both serum- and TGFβ1-stimulated fibroblasts. In addition, and importantly, we found that the effects of budesonide and formoterol on serum-induced proteoglycan production were dependent on functional GRs and β-adrenoceptors. In Paper II, there was also a significant increase of PICP by TGFβ1, but levels of PICP were not affected by the drugs; in fact, levels of PICP were further increased after concurrent treatment with budesonide alone, but interestingly, not in combination with formoterol. Thus, besides additive and synergistic effects of drugs on proteoglycan production, there might be a type of compensatory effect of these drugs, suggesting that possible harmful effect of one drug is compensated by addition of the other one. Hence our findings suggest that the anti-asthmatic drugs, budesonide and formoterol have beneficial effects on resident active fibroblasts and their ECM production and accumulation, in particular when used in combination.

Here it is, of course, important to consider to what extent the results obtained from an *in vitro* study can be translated to *in vivo* conditions? The complex nature of lung associated disorders, such as asthma, requires investigations *in vivo*, in lung tissue but also on resident lung cells *in vitro*, in parallel to evaluations from clinical studies. In this context, the strength of an *in vitro* model is that it allows direct assessments of cell specific behavior under defined conditions. In Papers I and II, a commercial cell line of embryonic lung origin (HFL-1) was used. But how relevant is the embryonic origin? Results of many studies indicate that these cells can be considered as highly relevant and suitable since they retain an active fibroblast phenotype during a limited number of passages. Malmström et al.[185], have performed thorough proteomic analyses of this cell line identifying 600 gene products. Furthermore, primary results in our group indicate that HFL-1 could resemble the phenotype of the fibroblast localized in the peripheral lung (unpublished data, Hallgren O.), being more active and mobile than the fibroblast from the central part of the lung. Similar types of fibroblasts have also been found in BAL fluid from patients with mild asthma but not in healthy controls [186]. Indeed, there is

a great heterogeneity of the fibroblasts described that exist within the lung and, is this then associated with the great heterogeneity of airway inflammation and the degree of remodeling within the large and small airway in the asthmatic lung? And most importantly, how will it affect the response to therapy? It certainly requires studies *in vitro* addressing the effects of drugs *ex vivo* on cells derived from diseased lung tissue. In Paper III, we used bronchial biopsy-derived fibroblasts from the central airways of mild asthmatic patients and healthy subjects. We found great heterogeneity between fibroblasts from both healthy and mild asthmatics regarding their baseline (i.e. culturing the cells with only 0.4% serum) production of ECM and MMP molecules and their response to TGFβ1. This is especially notable for the fibroblasts obtained from the patients with mild asthma, showing that there are large differences between the biopsy-derived fibroblasts that could be related to their state of disease. Similar observations have also been described from proteomic studies where larger heterogeneity was seen between patients than within the lung of the same patient [187].

However, it is obvious that there are also several limitations of *in vitro* models using primary cells, including that cultured cells do not fully maintain and reflect the range of intercellular communications present *in vivo*. There are reports illustrating that in the earliest stages of fibroblast growth *in vitro*, the patterns of type I procollagen and fibronectin biosynthesis, by cells in primary culture, closely correspond with those found in the intact lung. However, with prolonged culture, the distinctive phenotypes of cells from e.g. healthy as well as fibrotic (bleomycin treated) lungs change steadily. With prolonged time in culture, the biosynthetic profiles of both cell phenotypes were nearly identical [188]. Such findings stress the importance of using primary cultures in early passages.

When studying bronchial biopsy-derived fibroblasts from the central airways of mild asthmatic patients and healthy subjects (III), one important observation during the early passages of these cells was the great variability in proliferation rate and in cell morphology (studied with light microscopy) as compared to HFL-1 cells. One could

expect that in culture, fibroblast from asthmatic subjects would behave differently, but the fibroblast from the healthy donors as well, appeared rather heterogeneous.
However, both at baseline, (i.e. culturing the cells with only 0.4% serum) and in the presence of TGFβ1, the fibroblasts from asthmatic subjects tended to produce, with exception of decorin, higher levels of versican, perlecan and biglycan, than the healthy ones.
Treatment of the fibroblasts from healthy subjects with the drugs in combination resulted in a decreased production of all four proteoglycans, owing to additive action of the drugs, as neither budesonide nor formoterol had significant effects when applied alone.
On the other hand, treatment of fibroblasts from asthmatic subjects with the drugs did not affect the production of either versican or perlecan, the latter being non responsive to TGFβ1. However, there was a significant decrease of biglycan by the drugs in combination, although to a lower degree than the effects on biglycan from in fibroblasts from healthy subjects.

Of notice, regarding the decorin production, the majority of fibroblasts from asthmatic subjects had suppressed levels of decorin in the presence of TGFβ1. Although TGF β1 is known to negatively regulate decorin, we found that in these cells, interestingly, budesonide was able to reverse the TGFβ1-supressed decorin production.

In general, the responses to both TGFβ1 and to the drugs differed between the fibroblasts from healthy and asthmatic subjects. While the effects of the drugs obtained in the fibroblast from healthy subjects were similar to those obtained in HFL-1 (with the exception of decorin), fibroblasts from the asthmatics were less sensitive to TGFβ1. Perhaps the limited drug-effects on the fibroblasts from asthmatics were due to a lessened response to TGFβ1 and an altered fibroblast differentiation?

When considering the overall results on proteoglycan production in the different cell phenotypes under different conditions, and the effects of the drugs, there is apparently differential regulation of these molecules with variable response to the drugs. Our findings emphasize the importance of simulating a defined microenvironment when

investigating the biochemical and pharmacological responsiveness of lung fibroblasts. It is tempting to speculate that the inflammatory responses that typically precede chronic fibrotic events are controlled by a subset of resident fibroblasts, and that another subset of fibroblasts is perhaps responsible for the extensive ECM production, which characterize fibrosis. However, a more likely explanation for the partial lack of effect of the drugs is to be found in the group of asthmatic patients included in this study. Although they were all classified as mild asthmatics (responding to inhaled GCs) there were three patients who differed from the remaining five, by low FEV predicted (<70%) and a higher hyperreactivity to metacholine (PD 20<0.05µg) (see table 1, Paper III), indicating that these three patients have a more persistent asthma. Definition of mild asthma includes both intermittent and persistent mild asthma according to the Global Initiative for Asthma (GINA) classification (http://www.ginasthma.com/Guidelineitem.asp), suggesting that inflammation and airway remodeling in this class of patients may appear prominent, of varying intensity, or nonspecific. Indeed, in the group of three patients that appeared more persistent asthmatics, two of the patients responded rather differentially from the others by having higher production at baseline, and lower TGFβ and drug responses. These two patients therefore, enhanced experimental variations and decreased statistical power of the study in Paper III.

Still, according to the results of this thesis, of the proteoglycans investigated, biglycan was one of the proteoglycans that was induced in both serum and TGFβ1 and that responded to treatment with the drugs (I, II, III). A possible target for remodeling? Indeed, several other studies have connected biglycan with early inflammation and remodeling, by showing greater increase of biglycan in moderate than severe asthmatics [75]. Biglycan interacts with known pro-fibrotic cytokines, such as TGFβ1 and TNFα [105;139]. In addition, the core protein of biglycan has been shown to bind collagens and fibronectin; hence, biglycan may modulate collagen fiber assembly, cell migration and cell adhesion during fibrogenic processes [146;189]. Recently, a pro-inflammatory feature was described for biglycan mediated via signaling via Toll-like receptors [190]. Considering the fact that proteoglycans are not just structural components of the ECM, but play an important role in processes associated with deposition of collagens, cell adhesion, regulation of cytokines and growth factors, biglycan may have a profound role during

both inflammation and remodeling processes in asthma. It is then highly relevant that this proteoglycan, whose synthesis is induced by pro-inflammatory cytokines such as TGFβ1, is significantly reduced by the combination of budesonide and formoterol (Papers I, II, III).

Another important component of the ECM, besides proteoglycans, is procollagen I. Levels for PICP, a marker for ongoing collagen I synthesis, have been found inversely correlated with lung function in asthmatics, and increased upon exacerbation [191], indicating that collagen synthesis increases during asthma exacerbation in parallel with various inflammatory mediators. We found that in asthmatic subjects, baseline levels of PICP were also negatively correlated with patient lung function (Paper III). Furthermore, our results are in agreement with the concept that during remodeling processes, collagen I synthesis takes place in versican rich ECM that is also required for a normal cell proliferation [192]. We found a higher level of versican production in the asthmatic subjects (paper III), which may enhance the synthesis of collagen I. However, in our study, the drug combination significantly decreased ongoing collagen I synthesis, without affecting the levels of versican.

Furthermore, in the fibroblasts of asthmatic subjects, there were smaller amounts of biglycan and decorin, both of them associated with collagen fibrillogenesis. Formation and deposition of insoluble collagen is not only affected by the rate of synthesis of soluble procollagen but also by the process of proper collagen fibrillation, which, if disturbed, may form a scar. There are indications that a proper fibrillation process may require optimal levels of decorin and possibly of the structurally related biglycan. Thus, considering our results in Paper III on the asthmatic subjects, where the drug combination significantly reduced PICP and biglycan while normalizing the decreased decorin levels, suggests that the combination of the drugs may counteract an increased deposition of collagen and ECM in the subepithelium of asthmatic airways.

One of the major findings of Paper III was a positive correlation between baseline MMP-9 activity in the fibroblasts from asthmatic subjects and their lung function. It is recognized that the subepithelial fibrosis in asthma may occur due to a disproportion

between ECM synthesis and its degradation by MMPs. These degradative enzymes target the majority of the ECM molecules, including collagens and proteoglycans. Intriguingly, the baseline activities of MMP-2 and MMP-3 in fibroblast from asthmatic subjects were found negatively correlated to the asthmatics lung function. However, the balance of synthesis and degradation of the ECM is not only linked to the activity of MMPs but also to their endogenous inhibitor TIMP-1. A high MMP-9/TIMP-1 ratio is often regarded as beneficial in asthma. Our results seem to support this notion, as besides positive correlation between MMP-9 activity and patient lung function, there was a positive correlation between baseline TIMP-1 protein and the levels of PICP, versican and total PG, suggesting that high TIMP-1 levels may promote airway fibrosis in asthmatic airways.

Perhaps when investigating actions of MMPs, more potent inducers should be used such as IL-1β and TNF-α. TGF-β is generally considered to inhibit the action of MMPs while increasing that of TIMP, favoring ECM accumulation and fibrosis. In our experiments on fibroblast from asthmatic subjects, no such effects were seen on TIMP-1 levels. But, in the fibroblasts from healthy subjects, TGFβ1 increased TIMP-1 levels, which were significantly reduced by the drugs. In either case, the decrease of TIMP- levels does not necessarily imply decreased activity. Using reverse zymography will be much more revealing on the actual activity of TIMP and the effects of the drugs. However, our results clearly reflect the complex regulatory mechanisms behind.

One could argue that when working with primary cultures stimulated with, as in our case with TGFβ, or other known cytokines and growth factors, it would be difficult to interpret cellular and pharmacological responses. Yet, different culturing conditions are equally necessary. Regarding the different cell conditions in our study design, it would be relevant and highly interesting to investigate the effects of the drugs at baseline conditions in 0.4% serum. It is however possible that the biopsy derived fibroblasts in particular, would not survive for a longer period of incubations and treatment at such a low serum condition. In a limited set of experiments we did perform 24 h incubations of HFL-1 with the drugs and 0.4% serum. There were significant reductions by budesonide and formoterol on the total proteoglycan production. As the drugs are also able to reduce

the proteoglycan production in the presence of TGFβ1, it raises uncertainty of whether the effects are solely on TGFβ1-induced responses or at baseline levels. Using a neutralizing antibody against TGFβ1, it might reveal whether the effect on proteoglycans is merely mediated via the action of budesonide/formoterol or if the effects on baseline responses are affected by endogenous TGFβ1. To add some complexity, there are also conflicting reports on whether GCs can affect the activity and levels of TGFβ1. A neutralizing effect of a cytokine such as TGFβ would also have impact on other aspects, perhaps compensatory mechanisms between the extracellular components.

Taken together, considering the complex pathophysiology of asthma, and despite recognized limitations of *in vitro* studies, cell-based studies remain an important and complementary tool for studying specific cellular and pharmacological responses in lung resident cells. Investigating cell responses during early and late inflammatory and remodeling processes linked to different parts of the lung may account for and have specific implications for asthma therapy.

Future perspectives

Regarding our findings on different baseline synthesis of ECM components and activities of MMPs in fibroblast from mild asthmatics subjects (with emphasis on the more severe asthmatic subjects) as well as their correlation with patient lung function, it would be relevant to investigate the effects of the drugs at baseline conditions on fibroblasts from mild versus severe asthmatics. How different is their ECM composition and how will that have impact on treatment responses. How will the fibroblast population in the more proximal (peripheral) lung be affected by the combination? This would also be relevant in patients with COPD.

It would also be relevant to, besides TGFβ1, use other pro-fibrotic cytokines as cell activators and investigate the effects of drugs on both baseline and cytokine-induced responses. Regarding large heterogeneity in biopsy derived cells and patient asthmatic phenotypes, the future study should involve a greater number of patients to increase statistical power.

The major finding in this thesis, that the combination of the drugs in the majority of asthmatic patients, significantly reduced enhanced fibroblast synthesis of collagen I and biglycan as well as normalized the decreased levels of decorin, warrants future studies on the role of decorin and biglycan on proper fibrillation of collagen and collagen deposition in the subepithelium of asthmatic airways. In addition, TGFβ1 is known to affect both the proteoglycan core protein and the GAG fine structure of these molecules [193]. Considering very recent data on DS- deficient tissue displaying thicker and altered fibrillogenesis (unpublished observation, Malmström A.) it is also of relevance for future studies to explore whether treatment with budesonide and formoterol will affect the C-5 epimerase enzyme activity and the formation of DS proteoglycans.

Further, the increased production of versican in fibroblasts from asthmatic patients and its resistance to the effects of drugs requires study on the role of versican in airway remodeling in asthma and its possible impact on relative steroid insensitivity in a subgroup of severe asthmatics.

The next interesting issue to address in future studies is the role of various MMPs and TIMPs on ECM remodeling in asthmatic airways. In the light of our findings on baseline

MMP-9 and its positive correlation to patient lung function, as well as the positive correlation of TIMP-1 to procollagen I, versican and total proteoglycan, a possible beneficial role of a high MMP-9/TIMP-1 ratio would be relevant to investigate. The specific matter here is whether high TIMP-1 levels are beneficial or harmful. Measuring the PICP and MMP-3 levels in blood or BAL fluid respectively could also be a possible read-out.
Perhaps the response to treatment of different fibroblast populations could answer the question whether asthma treatment affects both central and peripheral airways. It also raises the next question on the possible recruitment of mesenchymal progenitor cells, the fibrocytes. Do they appear early and what is their role in the ECM? Will they be sensitive to treatment? What effect will other combination therapies have?

Regarding the airway remodeling, some studies suggest that depending on the extent and location of structural changes throughout the various airway wall compartments, remodeling can enhance but also protect against excessive airway narrowing, despite inflammation. Is then the remodeling process a normal response to injury, or is the response itself abnormal? Within this context, how are the ECM components involved in other aspects of remodeling such as angiogenesis? If TGFβ and VEGF are considerate major modulators of processes such as ECM remodeling and angiogenesis respectively, then the interaction of these two factors could complicate the process of remodeling in asthma. In COPD for example, TGFβ and VEGF represents a molecular link between inflammatory cell infiltrations at sites of injury contributing to airway remodeling in COPD. Decorin could be of importance in this context as this molecule is required for a proper fibrilogenesis and maintenance of the ECM integrity, considering the reduced decorin found in the bronchial mucosa [144], and in the external area of small airways in fatal asthma [125].
It would also be of relevance to study if the lung fibroblasts have a more immunomodulatory role, in particular as we in preliminary studies have seen that they express receptors such as CXCR1 and 2 (unpublished observations Westergren-Thorsson G.). Both of these molecules are linked to the IL-8 axis and thereby also to the neutrophils, cells that appear to have more central role in patients with severe asthma and

airway remodeling. Interestingly, there are reports about polymorphisms in those molecules that can be linked to airway remodeling under more severe circumstances. It would be of interest to look further into the role of innate immunity especially Toll-like receptor 4 which interacts with biglycan, to study if this system is affected in patients with more severe asthma and whether peptides containing biglycan motifs could modulate innate immunity through the Toll-like receptor 4 axis, but perhaps also the CXCR1 system.

Popularized summary in Swedish

(Populärvetenskaplig sammanfattning på svenska)

Generellt kännetecknas astma av en inflammation i luftvägarna. Om den lämnas obehandlad kan den leda till hyperreaktivitet i luftvägarna med begränsning av luftflödet och svullnad i slemhinnorna som följd. Dessa är de kliniska symptomen men vad som åtföljer inflammationen är en specifik typ av strukturella förändringar i lungvävnaden kallad fibrosbildning. Termen som används för att beskriva en sådan omstrukturering av de grundläggande komponenterna i lungvävnaden är luftvägsremodulering. I astma är balansen mellan nybildning och nedbrytning av lungvävnaden störd vilket resulterar i att vävnaden blir mer kompakt och mindre elastiskt med en försämrad lungfunktion som följd.

Dagens forskning har till större del fokuserats kring att förstå den inflammatoriska fasen av sjukdomen. Detta har resulterat i olika terapiformer som på ett tillfredsställande sätt kan hämma denna process. Tyvärr är dessa behandlingsformer mindre effektiva i att motverka fibrosbildningen under sjukdomen även om den kan kopplas till både den akuta och den mer kroniska inflammationsprocessen. Genom bronkoskopi har man upptäckt att de strukturella förändringarna kan uppstå redan innan eller mycket tidigt i sjukdomsförloppet. Därför är det av stor betydelse att förstå dessa förlopp och att tidigt kunna diagnostisera och behandla patienten.

Denna avhandling har fokuserat på att studera hur kombinationsbehandling bestående av en anti-inflammatorisk glukokortikoid, budesonide, och en luftvägsvidgande långtidsverkande β2-agonist, formoterol, kan påverka den cell som är viktigast för bindvävstillverkningen, nämligen fibroblasten.

Fibroblasten är en s.k. strukturell cell i lungvävnaden som har en viktig roll i att bibehålla en välorganiserad struktur i lungvävnaden. Den finns inbäddad i en samling av molekyler som utgör själva grunden i vävnaden och som kallas för extracellulär matrix. I tidigare studier har vi sett att sammansättningen och strukturen av komponenter i extracellulär matrix varierar mycket i ett tidigt stadium av astma jämfört med ett senare stadium av sjukdomen.

Som modellsystem har vi använt oss av både en typ av embryonala fibroblaster och av fibroblaster etablerade från små vävnadsbiopsier av slemhinnan i lungan från patienter med mild astma eller från friska frivilliga. Dessa fibroblast-cellkulturer har vi låtit växa i fysiologisk näring tills de etablerat sitt eget extracellulär matrix. Sedan har vi ändrat omgivningen för dem för att efterlikna dels den akuta och dels den mer kroniska typen av inflammation, varpå substanserna budesonide och formoterol har tillsatts antingen var för sig eller tillsammans. De olika systemen har sedan jämförts avseende effekter på fibroblasternas produktion av proteoglykaner och

kollagen. Dessa molekuler bildar tillsammans ett strukturellt nätverk kring fibroblasterna. Proteoglykanerna binder till kollagen och kan styra tätheten på kollagenfibrerna och därmed deras elasticitet vilken har stor betydelse för lungans funktion. Proteoglykaner binder även till inflammatoriska signaleringsmolekyler, s.k. cytokiner, och tillväxtfaktorer och fungerar därmed som en upplagringsplats för dessa molekyler. En del cytokiner har viktig betydelse i början av astmautvecklingen eftersom de påverkar struktur och sammansättning av extracellulär matrix. Detta i sin tur påverkar celldelning, vidhäftning och förflyttning av inflammatoriska celler. Andra cytokiner har direkt effekt på fibroblasterna och kan omvandla dem till s.k. myofibroblaster som liknar glatta muskelceller och har ökad förmåga att producera och ackumulera extracellulära komponenter som t ex proteoglykaner och kollagener.

För en normal bindvävsomsättning krävs enzymer som kan bryta ned proteoglykaner och kollagen, s.k. metalloproteaser, och även enzymer som kan hämma metalloproteaserna. En obalans i denna reglering kan vara en stor bidragande faktor till fibrosbildning såsom i astma, men också till massiv vävnadsnedbrytning såsom i andra lungrelaterade sjukdomar som kronisk obstruktiv lungsjukdom (KOL).

Resultatet av studierna i denna avhandling visar att kombinationsterapin genom att påverka ackumuleringen av extracellulära komponenter kan hämma vävnadsproduktion i både den akuta men även i den kroniska modellen. Viktigt är att poängtera att effekten inte uppnås via en effekt på fibroblastens celldelning och tillväxt utan är en direkt additiv och komplementerande påverkan av substanserna på syntesen. Genom att jämföra fibroblasters bindvävsomsättning från normala och astmatiska patienter, upptäcktes flera likheter men också skillnader vilka sannolikt återspeglar olika svårhetsgrad av astma hos patienterna. Våra resultat talar också för vikten av att inleda kombinationsterapi tidigt i astmaförloppet.

Sammanfattningsvis visar denna avhandling att kombinationsterapi är en terapiform att föredra om man vill påverka inte bara inflammationen utan även de strukturella förändringar som sker vid astma. Baserat på resultaten som kommit fram i denna avhandling kan förhoppningsvis fortsatta studier leda till bättre förståelse för andra lungsjukdomar där patologisk bindvävsomlagring sker, såsom vid KOL.

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