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# Relationship between matrix production by bronchial fibroblasts and lung function and AHR in asthma

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**Running head:** Matrix production by lung fibroblasts in asthma

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## ABSTRACT

The reasons for enhanced deposition of extracellular matrix in the airways of asthmatic patients and the subsequent consequences on lung function are uncertain. Here, we investigated the synthesis of procollagen I and proteoglycans, the activity of various metalloproteinases (MMPs) and the production of their inhibitor TIMP-1 in biopsy-derived bronchial fibroblasts from eight patients with stable mild-to-moderate asthma, and how they are related to patients' lung function and airway hyperactivity (AHR).

Following 24-h fibroblast incubation in 0.4% serum, procollagen I carboxyterminal propeptide (PICP), TIMP-1 and MMP-1 in cell media were analysed by ELISA, MMP-2, MMP-3, MMP-9 by zymography and total proteoglycan production by [$^{35}$S]-sulphate-incorporation/ion chromatography.

Patient´s $FEV_1$% predicted and methacholine log $PD_{20}$ negatively correlated with PICP synthesized by patients' bronchial fibroblasts (r=-0.74 and r=-0.71, respectively). PICP and proteoglycan amounts positively correlated ($0.8 \leq r \leq 0.9$) with MMP-2 and MMP-3 activity. A positive correlation (r=0.75) was also found between proteoglycan production and TIMP-1. There was no correlation between MMP-9 activity and PICP or proteoglycan production. MMP-9 activity positively correlated with patients' $FEV_1$% predicted (r=0.97) and methacholine log $PD_{20}$ (r=0.86), whereas negative associations ($-0.6 \leq r \leq -0.7$) were observed for MMP-2 and MMP-3.

In stable mild-to-moderate asthma, increased procollagen I synthesis and activity of MMP-2 and MMP-3 in bronchial fibroblasts may negatively affect patients' lung function and AHR. In contrast, MMP-9 activity was not associated with procollagen or proteoglycan production, or worsening of patients' lung function. An enhanced production of procollagen I and proteoglycans might be a result of a negative feedback from their degradation by MMP-2 and MMP-3.


**Key words:** Procollagen I, proteoglycan, metalloproteinase, TIMP-1, asthma, airway remodeling

## INTRODUCTION

A prominent feature of airway remodeling in asthma is an enhanced deposition of extracellular matrix (ECM) throughout the airway wall. Increased concentration of various collagens, proteoglycans and fibronectin are observed in the subepithelial lamina reticularis, in the deeper submucosa area and around the smooth muscle layer (reviewed in Ref. 1). Such structural changes occur early in asthmatic children and may be responsible for the greatest loss of lung function early in the course of asthma [2].

Increased deposition of ECM is determined by synthesis of ECM components and their degradation. Fibroblasts are the main cells responsible for ECM production and turnover in the airways and lung tissue although other resident cells, such as airway epithelial cells and smooth muscle cells may be involved [3, 4]. A greater number of fibroblasts were found in the submucosal layer of the airways of asthmatic patients than in healthy subjects [5], and the increased number of fibroblasts also correlated to thickening of the subepithelial basement membrane [6]. The main enzymes responsible for ECM degradation are the matrix metalloproteinases (MMPs), which are produced by lung fibroblasts and target almost all components of the ECM [7]. Paradoxically, along with the increased ECM deposition in the airways of asthmatic patients, the concentration and activity of various MMPs are also enhanced compared with healthy subjects. The major MMPs increased in the airways of asthmatic patients are gelatinases MMP-9 and MMP-2 [8, 9], stromelysin MMP-3 [10] and collagenase MMP-1 [11]. The MMP-1, MMP-2 and MMP-9 are key regulators of collagens while MMP-3 is considered as a main regulator of proteoglycans; however, all these MMPs have broad substrate specificity and participate in turnover of various ECM molecules. The activity of MMPs is in turn regulated by the tissue inhibitors of metalloproteinase (TIMPs), especially TIMP-1, which is the most common and targets all active MMPs by non-covalent binding in a 1:1 molar ratio [12]. In the airways of asthmatic patients, TIMP-1 concentrations remain either unchanged [9], even after allergen challenge [13], or are enhanced [14-17], often even exceeding molar concentration of MMPs [16-18].

The reasons for the excessive ECM deposition in the airways of asthmatic patients, and its consequences for lung function and airway hyperreactivity, are uncertain. Despite increased ECM accumulation, an enhanced concentration and activity of MMPs in the asthmatic airways are generally considered as detrimental while TIMP-1 increase is seen as beneficial, although a relationship between metalloproteolytic activity in the airways and lung function of asthmatic patients is not very well studied and results are inconsistent. The excessive ECM deposition could be a result of an increased synthesis of ECM and/or attenuated degradation of ECM. On the other hand, an enhanced ECM degradation, especially when long-lasting, could via defensive negative feedback result in an over-repair where TIMP-1 might play a role.

To address these issues, the present study has investigated the synthesis of procollagen I and total proteoglycans by bronchial fibroblasts derived from biopsies obtained from stable patients with mild-to-moderate asthma and from age-matched healthy volunteers. The activity of the MMP-9, MMP-3, MMP-2 and the production of MMP-1 and TIMP-1 protein were also measured. We have then investigated whether, and how, the synthesis of procollagen I and of proteoglycans, as well as the activity of MMPs and the production of TIMP-1, are related to patients' lung function, measured as forced expiratory volume in 1 second and expressed as per cent of the predicted normal value ($FEV_1$%), and to airway hyperreactivity measured by methacholine challenge.

## MATERIALS and METHODS

### Subjects

Eight patients (4 female and 4 male) suffering from asthma and bronchial hyperresponsiveness were included in the study. The patients were 25–55-year-old (25–39 years for 7 of 8 patients), non-smokers, with stable mild-to-moderate asthma according to GINA guidelines (www.ginaasthma.org), with confirmed airway hyperresponsiveness to methacholine. A positive methacholine challenge test was defined as a fall in $FEV_1 \geq 20\%$ ($PD_{20}$) on a cumulative dose of methacholine < 2000 µg, measured by a tidal volume triggered technique (Automatic Provocation System, Erich Jaeger GmbH). Of the 8 patients, 5 had an $FEV_1$% predicted of around 100% (included the oldest, 55-year-old man) while 3 had values in the range of 62–67%; these 3 patients also had the lowest $PD_{20}$ values (<0.05 µg) (Table 1). All patients were atopic, with skin prick tests confirming sensitization towards perennial allergens, were free of infections during the six weeks before bronchoscopy, and were without corticosteroid treatment for six months prior to the study.

Five age-matched control subjects (4 male and 1 female, 23–41 years of age) were included in the study. These were non-asthmatic, non-allergic healthy volunteers without respiratory symptoms and a negative methacholine challenge test ($PD_{20}$ >2000 µg).

The study was reviewed and approved by the Swedish Research Ethical Committee (No LU339-00) and informed consent was obtained from all participants.

### Fibroblast outgrowth from biopsies

Bronchial biopsies were collected from the right lung of each subject. Primary fibroblast-like cells were established (as previously described in Ref. 19) from the biopsies (one for each subject) that did not show any differences in size, vascular or muscle content. Briefly, the

biopsies were cut into small pieces and were allowed to adhere to cell-culture plastic before the addition of Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% fetal Clone III (Hyclone, UT), 1% L-glutamine, 0.5% gentamicin and 5 µg/ml amphotericin. Biopsies were cultured until outgrowth of cells with morphology typical of a fibroblast, including a spindle-like shape and several protrusions [20, 21]. To ensure that true fibroblasts were cultured, markers for fibroblasts and smooth muscle cells were used. The cells were seeded in 4-well glass chamber slides (Nalge Nunc International, Rochester, NY, USA) for 48 h, fixed with 4% paraformaldehyde. Antibodies against vimentin in dilution to 1 µg/ml (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α-smooth muscle actin diluted 1:200 (Dako, Glostrup, Denmark) and prolyl 4-hydroxylase in dilution to 1 µg/ml (Acris Antibodies, Hiddenhausen, Germany) were used as positive controls while a smooth muscle cell marker SM22 in dilution to 2.5 µg/ml (Abcam, Cambridge, UK) was used as a negative control. Corresponding Alexa fluorochrome (Molecular Probes, Eugene, OR, USA) in 1% BSA and 5% goat serum (Vector Laboratories Inc., Burlingame, CA, USA) were used as secondary antibodies. Nuclei were visualized with 4'-6-diamidino-2-phenylindole staining (Invitrogen Corp., Carlsbad, CA, USA). Isolated primary fibroblasts were used in passages 4–6 for further experiments (cells were split 1:2 at expansions).

## Study Design

Experiments were performed in biopsy-derived bronchial primary fibroblasts cultured in 6-well plates in Earle's minimal essential medium (Sigma-Aldrich, Irvine, UK) containing 10% (v/v) Fetal Clone III serum, (Hyclone, Logan, UT, US), 1% L-glutamine, 0.5% gentamicin and 5 µg/ml amphotericin at 37ºC in a humidified 5% $CO_2$ and 95% atmosphere. Prior to the experiments, fibroblasts were starved for 2 h in DMEM supplemented with 1% donor calf serum and 2 mM L-glutamine. Subsequently, cells were incubated with 0.4% serum for 24 h in a $MgSO_4$-poor DMEM (Gibco BRL, Paisley, UK); the last 22 h in the presence of 50 µCi/ml [$^{35}$S]-sulphate (PerkinElmer Life Science, Boston, MA, USA). After incubation, the cell medium was collected and the concentration of procollagen I carboxyterminal propeptide (PICP), proteoglycans, MMP-

1 and TIMP-1 and the activity of MMP-9, MMP-3, MMP-2 were determined as described below. All experiments with cells from each individual were performed in homogenous, confluent cultures, repeated two or three times, and mean value was calculated for each patient.

### Immunoassay for PICP, MMP-1 and TIMP-1

Concentrations of PICP in the culture media were measured by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's method (Takara Biochemicals Co., Osaka, Japan). Concentrations of MMP-1 and TIMP-1 (both free and complexed with MMPs) were analyzed by ELISA (Amersham, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Data obtained were related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

### Radiographic analysis of proteoglycans by anion-exchange chromatography

Proteoglycans produced and released into the cell culture medium were isolated, purified and quantified by [$^{35}$S]-sulphate incorporation into the proteoglycan glycosaminoglycan chains, as previously described [22]. Briefly, collected culture media were applied to anion-exchange DEAE cellulose columns. After washing out the unincorporated radioactive precursors, proteoglycans were eluted, analyzed for [$^{35}$S]-sulphate activity, quantified by liquid scintillation counting and related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

### Zymography for MMP-9, MMP-2 and MMP-3

Presence and activity of proteolytic enzymes were analyzed using Novex polyacrylamide zymogram gels and a prestained molecular-weight marker BluePlus2 Prestained Standard (Invitrogen, Stockholm, Sweden), as described elsewhere [23]. Briefly, the gelatinase activity of MMP-9 and MMP-2 in the collected and concentrated conditioned media was determined by

preactivating equal volumes of each sample with 1 mM 4-aminophenyl mercuric acetate (APMA) for 24–72 h (a longer time period was required for MMP-9), and run on 10% zymogram under non-denaturing conditions.

To determine the caseinolytic activity of MMP-3, all samples were preactivated with 5 µg/ml trypsin for 30 min at 37°C. The activation was terminated by 2 mM phenyl-methylsulphon-fluoride and samples were run on 4–16% zymogram (prestained Blue Casein) at 125 V for 2 h at 4°C [23].

The gels were scanned and the gelatinolytic and caseinolytic band areas of activity were quantified by densitometric analysis of an inverted display using transformation tool program (BioRad Quantity One Software from Bio-Rad Laboratories Inc., Hercules, CA, USA), and presented as optical density (OD) related to the total protein content in the corresponding cell layer using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

**Western immunoblotting of MMP-9, MMP-2 and MMP-3**

Identification of the different MMPs was confirmed by Western blot. Briefly, equal amounts of preactivated samples were subjected to 10% Tris-Glycine gels for MMP-2 and MMP-9, or 4–12% for MMP-3 (Invitrogen, Stockholm, Sweden) and by electro-blotting transferred to a PVDF-P Immobilon membrane (Millipore, Bedford, MA, USA) [23]. The following primary antibodies were used: MMP-2 mouse anti-human monoclonal IgG/K, MMP-9 rabbit anti-mouse (Gelatinase B) polyclonal full length, and rabbit anti-MMP-3 (Chemicon,Temecula, CA, USA); all were diluted 1:1000 in TrisHCl, 150 mM NaCl, pH 7.5, 0.05% Tween with 1% BSA. Polyclonal secondary antibodies used were rabbit anti-mouse-HRP for MMP-2, swine anti-rabbit-HRP for MMP-9, and rabbit anti-mouse-HRP for MMP-3; again all were diluted 1:1000 (DakoCytomation, Glostrup, Denmark). Bands were visualized after development with ECL Western Blotting Detection Reagents (Amersham GE Healthcare, Cardiff, UK).

## Statistical Analysis

Data are shown as values for individual subjects. An outlier test was performed for each analysis to identify extreme data points. Data points lying outside 10 times standard deviation from mean value of the remaining points were excluded from the analysis in question. The correlation analysis was performed by testing the significance of Pearson correlation coefficients (r) with t-test of the regression. The mean values for asthmatic patients and healthy volunteers were compared by unpaired, two-tailed independent t-test for small samples. Differences were considered statistically significant at $p<0.05$. All analyses were performed using Astute Software 1.5 (DDU Software, Leeds, UK).

## RESULTS

### Production of PICP and proteoglycans

The synthesis of PICP and the total proteoglycans were on average 2.3-fold and 1.4-fold higher, respectively, in bronchial fibroblasts from asthmatic patients than from healthy volunteers; however, this difference was not statistically significant. The amount of PICP produced by fibroblasts from asthmatic patients was negatively correlated to patient $FEV_1$% predicted (r= -0.74, p=0.03; Fig. 1A) and to methacholine log $PD_{20}$ (r= -0.71, p=0.048; Fig. 1B). The negative associations between the total amount of proteoglycans and patient $FEV_1$% predicted or log $PD_{20}$ were not statistically significant (r= -0.52, p=0.19 and r= -0.53, p=0.18, respectively). There was a strong and positive correlation between the amounts of PICP and proteoglycans both in fibroblasts from patients (r=0.93, p=0.001; Fig. 1C) and in those from healthy volunteers (r=0.89, p=0.041).

### MMP-9

Gelatin zymography showed enzymatic activity of MMP-9 in fibroblast medium as an 82-kDa band (Fig 2A**)**, while the 92-kDa band of the pro-form and complexed forms around 200-kDa were often not visible. Very high MMP-9 activity was obtained for one patient (Pt 8) who had a $FEV_1$ of 63% predicted value. This activity was 6–30 times higher than those of the other asthmatic patients. This data point was identified as an outlier – lying more than 10 times standard deviation (actually more than 14 times) from the mean value of the remaining patients – and thus was excluded from further MMP-9 analysis. MMP-9 activity was 1.6-fold lower in fibroblasts from asthmatic patients than in those from healthy volunteers but this difference did not reach statistical significance (p=0.14).

There was a very strong and significant positive correlation between fibroblast MMP-9 activity and patient $FEV_1$% predicted (r=0.97, p=0.0004; Fig. 2B) and methacholine log $PD_{20}$ (r=0.86, p=0.01; Fig 2C). There was no significant correlation between MMP-9 activity and amount of

PICP or proteoglycans in fibroblasts from patients (r= -0.52, p=0.23 and r= -0.27, p=0.56, respectively) or fibroblasts from healthy volunteers (r= -0.46, p=0.44 and r= -0.43, p=0.47, respectively).

**MMP-2**

Gelatin zymography showed enzymatic activity of MMP-2 in fibroblast medium as a 62-kDa band, a pro-form at 72-kDa and high complexes band around 100 kDa (Fig. 2A). As for MMP-9, fibroblasts from Pt 8 showed extremely high MMP-2 activity that was 7–50 times higher than that of the other asthmatic patients. This data point was identified as an outlier (lying more than 10 times standard deviation, actually more than 22 times, from the mean value of the remaining patients) and thus was excluded from further MMP-2 analysis. MMP-2 activity was similar in fibroblasts from asthmatic patients and healthy volunteers and approximately 10 times higher than MMP-9 activity.

There was a trend for a negative association between MMP-2 activity and patient $FEV_1$% predicted (r= -0.60, p=0.15; Fig. 3A) and methacholine log $PD_{20}$ (r= -0.69, p=0.09; Fig. 3B). At the same time, the MMP-2 activity was strongly and positively correlated with both the amount of PICP (r=0.94, p=0.001; Fig. 3C) and proteoglycans (r=0.89, p=0.007; Fig. 3D) in patients' fibroblasts. In contrast, in fibroblasts from healthy volunteers there was no correlation between MMP-2 activity and PICP or proteoglycan synthesis (r= -0.39, p=0.51 and r= -0.10, p=0.89, respectively).

**MMP-3**

Casein zymography showed enzymatic activity of MMP-3 in fibroblast medium as an active band at 45 kDa, pro-form double band at 57/59 kDa and complexed form around 100 kDa (Fig. 4A). MMP-3 activity was 2.5-fold higher in fibroblasts from asthmatic patients than in fibroblasts from healthy volunteers, but this difference was not statistically significant. There was a negative

association, with borderline statistical significance, between the MMP-3 activity and patient $FEV_1$% predicted (r= -0.70, p=0.052; Fig. 4B) as well as methacholine log $PD_{20}$ (r= -0.67, p=0.07; Fig. 4C). The MMP-3 activity was strongly and positively correlated with both the amount of PICP (r = 0.89, p = 0.003; Fig. 4D) and proteoglycans (r=0.76, p=0.03; Fig. 4E) in patients' fibroblasts. In contrast, in fibroblasts from healthy volunteers there was no correlation between MMP-3 activity and PICP or proteoglycan production (r= -0.47, p=0.42 and r= -0.16, p=0.79, respectively).

**MMP-1**

MMP-1 protein levels were 3.7-fold higher in fibroblasts from asthmatic patients than those from healthy volunteers, but this difference did not reach statistical significance. The MMP-1 amount produced by lung fibroblasts did not correlate with patient $FEV_1$% predicted (r=0.26, p=0.54) or methacholine log $PD_{20}$ (r=0.52, p=0.19). Furthermore, the MMP-1 level did not correlate with the amount of PICP (r= -0.06, p=0.88) or proteoglycans (r= -0.04, p=0.93) in patients' fibroblasts or fibroblasts from healthy volunteers (r= -0.21, p=0.73 and r= -0.34, p=0.58, respectively).

**TIMP-1**

The level of TIMP-1 protein was 1.5-fold higher in fibroblasts from asthmatic patients than in those from healthy volunteers but this difference was not statistically significant. There was no correlation between the amount of TIMP-1 produced by fibroblasts and patient $FEV_1$% predicted (r= -0.12, p=0.77) or methacholine log $PD_{20}$ (r= -0.29, p=0.49). TIMP-1 levels correlated positively with the PICP levels in fibroblasts from healthy volunteers (r=0.96, p=0.008) and from asthmatic patients, although the latter did not reach statistical significance (r=0.55, p=0.16; Fig. 5A). TIMP-1 levels also correlated positively with the total amount of proteoglycans both in fibroblasts from healthy volunteers (r=0.84, p=0.07) and in those from asthmatic patients (r=0.75, p=0.03; Fig. 5B).

There were no significant correlations between TIMP-1 and the activity of MMP-9 (r=0.16, p=0.72), MMP-2 (r=0.63, p=0.13), MMP-3 (r=0.43, p=0.29) or MMP-1 protein (r= -0.10, p=0.81) in patients' fibroblasts or fibroblasts from healthy volunteers (r= -0.41, p=0.49 for MMP-9; r= -0.46, p=0.43 for MMP-2; r= -0.59, p=0.30 for MMP-3; r= -0.39, p=0.52 for MMP-1).

## DISCUSSION

The present study shows that a decrease in lung function and an increase in airway hyperreactivity to methacholine in patients with stable, mild-to-moderate asthma are associated with increased procollagen I synthesis by biopsy-derived bronchial fibroblasts. Production of both procollagen I and proteoglycans was strongly and positively correlated with the activity of MMP-2 and MMP-3 in asthmatic patients' fibroblasts but not in fibroblasts from age-matched healthy volunteers. In contrast, MMP-9 activity was not associated with either procollagen I or proteoglycan production in either fibroblasts from asthmatic patients or healthy volunteers. Furthermore, MMP-9 activity was strongly and positively correlated with patient $FEV_1$% predicted and methacholine log $PD_{20}$ whereas MMP-2 and MMP-3 activities were negatively associated.

Increased levels of PICP have been reported in sputum of asthmatic patients [24] and also in the present study fibroblasts from asthmatic patients had more than 2-fold higher levels of PICP compared to healthy volunteers, although this difference did not reach statistical significance. PICP levels reflect ongoing procollagen I synthesis and have been proposed as a new marker of inflammation and airway remodeling in asthma. Indeed, in the present study, PICP produced by bronchial fibroblasts from asthmatic patients were negatively correlated with patients' lung function assessed by $FEV_1$ and with methacholine log $PD_{20}$. This finding supports the hypothesis that increased synthesis of collagen I in the airways of asthmatic patients is clinically important and plays a role in a decrease of lung function and in an increase of airway hyperreactivity.

An increased deposition of specific proteoglycans in the subepithelial layer in atopic, mild asthmatic patients was reported in several studies [25–27] and in the present study there was a trend for a higher production of the total amount of proteoglycans by bronchial fibroblasts from asthmatic patients than those from healthy volunteers. Altered proteoglycan deposition within the lungs may disturb the fine-tuned ECM organization and function, and may influence the deposition of other ECM molecules such as collagen and fibronectin [28, 29]. In the present

study, a negative association between the total amount of proteoglycans produced by bronchial fibroblasts from asthmatic patients and patients' lung function, or methacholine log $PD_{20}$, was not statistically significant. Previous studies, however, have shown that bronchial fibroblasts from subjects with hyperresponsive airways produced more proteoglycans (total amount) than those from healthy subjects [30], and that some specific proteoglycans were correlated to low airway hyperreactivity in asthmatic patients [26, 30].

The production of PICP and proteoglycans by bronchial fibroblasts from asthmatic patients in the present study was strongly and positively correlated with the activity of MMP-2 and MMP-3 whereas there were no such correlations in fibroblasts from healthy volunteers. In contrast, MMP-9 activity was not correlated to PICP or proteoglycan synthesis, either in fibroblasts from asthmatic patients or in those from healthy volunteers, despite MMP-9 sharing a broad spectrum of substrate specificity with MMP-2. Nevertheless, MMP-9 does not degrade the two main collagen types, I and III, deposited under subepithelial basement membrane and submucosa in the airways of asthmatic patients, while MMP-2 degrades them both, and MMP-3 degrades collagen type III [7]. This seems to support the hypothesis that degradation of collagens by MMP-2 and MMP-3 may lead via negative feedback mechanisms to increased synthesis and over-repair manifested as an excessive collagen deposition. No significant findings on MMP-1 in the present study may be due to the fact that only the level of MMP-1 protein but not its activity was measured (zymography assay for MMP-1 was not commercially available).

Since deposition of ECM is the net result of ECM synthesis and degradation, an imbalance between various MMPs and their main inhibitor TIMP-1 can influence ECM airway remodeling in asthma [31]. The TIMP-1 protects airway tissue against activity of various MMPs but it may also prevent proper repair and promote airway fibrosis. Indeed, sputum TIMP-1 levels in mild stable asthmatic patients were shown to be positively correlated with airway wall thickness while MMP-9 levels were inversely correlated [18]. Furthermore, high levels of TIMP-1 tended to increase with asthma severity [16], and TIMP-1 levels correlated negatively while the MMP-

9/TIMP-1 ratio correlated positively with $FEV_1$% predicted values [17, 32], although no such correlations were observed elsewhere [33]. In the present study, there was a trend for higher TIMP-1 levels in patients' bronchial fibroblasts than those from healthy volunteers; however, they did not correlate with patients' FEV1% predicted or methacholine log $PD_{20}$. On the other hand, TIMP-1 positively and strongly correlated with the production of proteoglycans and procollagen I in fibroblasts from both healthy and asthmatic individuals, although in asthmatic patients' fibroblasts the correlation with procollagen I synthesis did not reach statistical significance. Thus, TIMP-1 may promote synthesis and deposition of proteoglycans and procollagen I in the airways of both healthy individuals and asthmatic patients although its role in enhanced collagen deposition in asthmatic patients needs confirmation in a larger study. Interestingly, although there were no statistically significant correlations between TIMP-1 and the activity of MMPs, negative associations between TIMP-1 and activity of all MMPs investigated were seen in fibroblasts from healthy volunteers while positive associations in fibroblasts from asthmatic patients were observed. This suggests a disturbed relationship between the activities of MMPs and production of TIMP-1 by bronchial fibroblasts in asthma patients and advocates for a scenario where increased MMP activity results in over-production of TIMP-1 and subsequent over-repair of ECM.

In contrast to MMP-2 and MMP-3, the activity of MMP-9 produced by bronchial fibroblasts was not associated with worsening of patients' lung function and airway hyperreactivity in the present study; on the contrary, the activity of MMP-9 was positively and strongly correlated with patient $FEV_1$% predicted and methacholine log $PD_{20}$. Based on this finding, it is tempting to speculate that MMP-9 may have a beneficial role in asthma. However, one needs to be cautious because only 8 patients were investigated in this study, including only 3 patients with low $FEV_1$% predicted and low methacholine log $PD_{20}$. Therefore, additional studies with more patients across a wider spectrum of asthma severities are warranted to further evaluate this finding. Relatively few studies have investigated a relationship between airway MMP-9 and lung function in asthma, and results are inconsistent. Asthmatic patients showing positive immunostaining for MMP-9 in

subepithelial basement membranes had significantly lower $FEV_1$ values than asthmatic patients with negative staining [34]. Likewise, after allergen challenge in patients with mild asthma, there was a positive correlation between sputum MMP-9 and a maximal fall in $FEV_1$ both in the acute phase [13] and in the late phase [35]. On the other hand, in stable asthma of varying severities, no correlation between sputum MMP-9 and $FEV_1$ values was observed [17, 32, 33].

In the present study, the activity of MMP-9 in bronchial fibroblasts from each asthmatic patient was approximately 10-times lower than that of MMP-2. This is in contrast to much higher levels of MMP-9 than MMP-2 being detected in the airways of moderate-severe asthmatic patients, both during asthma attacks and remissions [9, 10] as well as in stable asthma [16]. On the other hand, during remissions in moderate-severe asthma, MMP-9 levels were only slightly higher than those in healthy subjects [9]. Furthermore, in patients with mild asthma only low MMP-9 levels were detected [10], and MMP-9 levels or activity were often not significantly different from control subjects [15, 34, this study] but were increased after allergen challenge [13, 15]. These, and other findings (reviewed in Ref. 36), suggest that the major source of MMP-9 in acute/severe asthma are inflammatory cells, while MMP-9 production by structural cells, including bronchial fibroblasts, epithelial cells and smooth muscle cells, may determine the MMP-9 levels during stable disease.

Besides ECM degradation and remodeling, mechanisms responsible for the effects of MMPs on lung function and airway hyperreactivity in asthma may also involve other effects crucial in asthma pathology. For example, one of the major factors for increased airway hyperreactivity in asthma is hyperplasia of airway smooth muscle cells, and MMP-2 was shown to act as a permissive factor for proliferation of these cells [37]. Furthermore, it is well known that MMPs also cleave non-ECM proteins and by that may activate or deactivate various cytokines, chemokines and their cell receptors, modulating inflammation in the airways. In this context, an important role of MMPs seems to be regulation of chemokine gradients and thus traffic of inflammatory cells where, however, ECM degradation such as proteolysis of proteoglycans,

which bind and regulate chemokine function, may be involved. Studies in MMP-9-deficient mice strongly suggest that MMP-9 is a dominant airway MMP that facilitates egression of inflammatory cells from lung tissue into airway lumen and thus promotes resolution of inflammation [38, 39]. MMP-9 also promotes migration of regenerating epithelial cells implied in reepithelialization of the denuded airway mucosa and wound closure after injury [40, 41]. A protective role of MMP-9 was demonstrated in ozone-induced airway inflammation in mice [42] and, most recently, MMP-9 was shown to exert anti-microbial function [43], which may be essential in prevention of asthma exacerbations.

In conclusion, the present study shows that in patients with stable mild-to-moderate asthma, increased synthesis of procollagen I and activity of MMP-2 and MMP-3 in bronchial fibroblasts may negatively affect patients' lung function and airway hyperreactivity. In contrast, MMP-9 activity was not associated with increased procollagen I or proteoglycan production, or worsening of patients' lung function and airway hyperreactivity. Since the synthesis of procollagen I and proteoglycans by bronchial fibroblasts from asthmatic patients positively and strongly correlated with the activity of MMP-2 and MMP-3, while no such correlations were observed in fibroblasts from healthy volunteers, an enhanced synthesis and deposition of these ECM molecules in the airways of asthmatic patients might be a result of negative feedback from ECM degradation by these enzymes.

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## CONFLICT OF INTEREST STATEMENT

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## FIGURE LEGENDS

**Figure 1. Procollagen I carboxyterminal propeptide (PICP) synthesized by bronchial fibroblasts from 8 asthmatic patients.** PICP was measured by ELISA and normalized to the total protein content in the corresponding cell layer (ng/μg protein). **A and B**: Relation between PICP amount and patient $FEV_1$% predicted (A), and methacholine log $PD_{20}$ (B). **C:** Relation between PICP amount and total proteoglycan (PG) production measured as [$^{35}$S]-sulphate incorporation and normalized to the total protein content in the corresponding cell layer (dpm/μg protein, dpm=disintegrations/min). Symbols represent individual patients Pt 1=open triangle, Pt 2=star, Pt 3=closed diamond, Pt 4=open diamond, Pt 5=closed square, Pt 6=open circle, Pt 7=open square, Pt 8=closed triangle. Correlation analyses were performed using Pearson correlation coefficients (r) with t-test of the regression.

**Figure 2. Activity of MMP-9 in conditioned media of bronchial fibroblasts from 7 asthmatic patients**. Symbol for each individual is shown as in Fig. 1; Pt 8 as an outlier is excluded. **A:** Representative gelatin zymogram and Western blot (WB) revealing both the presence and enzymatic activity of MMP-9 and MMP-2 in conditioned media. For MMP-9, the pro-form enzyme was measured as a band at 92 kDa, the active enzyme at 82 kDa, and high molecular complexes around 200 kDa. MMP-9 activity at 82 kDa was quantified by densitometric analysis of an inverted display and presented as optical density (OD) normalized to the total protein content (μg) in the corresponding cell layer. **A and B**: Relation between MMP-9 activity and $FEV_1$% predicted (A), and methacholine log $PD_{20}$ (B). Correlation analyses were performed using Pearson correlation coefficients (r) with t-test of the regression.

**Figure 3. Activity of MMP-2 in conditioned media of bronchial fibroblasts from 7 asthmatic patients**. Symbol for each individual is shown as in Fig. 1; Pt 8 as an outlier is excluded. Representative gelatin zymogram and Western blot (WB) are shown in Fig. 2A where the MMP-2 pro-form enzyme was measured as a band at 72 kDa, the active enzyme at 62 kDa and high

molecular complexes around 100 kDa. MMP-2 activity at 62 kDa was quantified by densitometric analysis of an inverted display and presented as optical density (OD) normalized to the total protein content (μg) in the corresponding cell. **A and B**: Relation between MMP-2 activity and $FEV_1$% predicted (A), and methacholine log $PD_{20}$ (B). **C and D**: Relation between MMP-2 activity and procollagen I carboxyterminal propeptide (PICP) amount (C), and total proteoglycan (PG) amount (D) (dpm=disintegrations/min). Correlation analyses were performed using Pearson correlation coefficients (r) with t-test of the regression.

**Figure 4**. **Activity of MMP-3 in conditioned media of bronchial fibroblasts from 8 asthmatic patients**. Symbol for each individual is shown as in Fig. 1. **A:** Representative casein zymogram and Western blot (WB) revealing both the presence and enzymatic activity of MMP-3 in conditioned media. The pro-form enzyme was measured as a double band at 57/59 kDa, the active enzyme at 45 kDa, and complex form around 100 kDa. MMP-3 activity at 45 kDa was quantified by densitometric analysis of an inverted display and presented as optical density (OD) normalized to the total protein content (μg) in the corresponding cell layer. **A and B**: Relation between MMP-3 activity and $FEV_1$% predicted (A), and methacholine log $PD_{20}$ (B). **C and D**: Relation between MMP-3 activity and procollagen I carboxyterminal propeptide (PICP) amount (C), and total proteoglycan (PG) amount (D) (dpm=disintegrations/min). Correlation analyses were performed using Pearson correlation coefficients (r) with t-test of the regression.

**Figure 5**. **TIMP-1 protein production by bronchial fibroblasts from 8 asthmatic patients**. TIMP-1 was determined by ELISA and normalized to the total protein content in the corresponding cell layer (ng/μg protein). Symbol for each individual is shown as in Fig. 1. **A and B**: Relation between TIMP-1 and procollagen I carboxyterminal propeptide (PICP) amount (A), and total proteoglycan (PG) amount (B) (dpm=disintegrations/min). Correlation analyses were performed using Pearson correlation coefficients (r) with t-test of the regression.

**Table 1. Baseline characteristics of patients with asthma included in the study**

| | **Sex** | **Age** (yr) | **$FEV_1$** (% pred) | **$PD_{20}$** (μg) |
|---|---|---|---|---|
| **Pt 1** | F | 28 | 101 | 60 |
| **Pt 2** | F | 31 | 104 | 15 |
| **Pt 3** | M | 30 | 67 | <0.05 |
| **Pt 4** | F | 25 | 95 | 20 |
| **Pt 5** | M | 35 | 62 | <0.05 |
| **Pt 6** | M | 55 | 103 | 75 |
| **Pt 7** | M | 26 | 100 | 826 |
| **Pt 8** | F | 39 | 63 | <0.05 |

Inclusion criteria for asthmatic patients are described in the Methods. Definition of abbreviations: F=female, M=male, $PD_{20}$ (μg) to methacholine stimulation; % pred=% predicted.