Bronchoalveolar matrix metalloproteinase 9 relates to restrictive lung function impairment in systemic sclerosis

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Systemic sclerosis; Bronchoalveolar lavage; MMP-9; Interstitial lung disease

Summary
Systemic sclerosis (SSc) is frequently associated with interstitial lung disease (ILD) often leading to lung fibrosis. In this study we investigated whether matrix metalloproteinase 9 (MMP-9) and its natural inhibitor: the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), would be associated with remodelling in ILD in SSc.

Levels of total MMP-9, pro-MMP-9 and TIMP-1 were measured in bronchoalveolar lavage (BAL) fluid from nine SSc patients with ILD, seven SSc patients without ILD and 16 age- and sex-matched healthy controls.

Total MMP-9 and pro-MMP-9 levels were significantly elevated in SSc patients with ILD, compared to levels in SSc patients without ILD and healthy controls. In SSc patients with ILD calculated active MMP-9 levels were significantly higher than in SSc patients without ILD and tended to be higher than in healthy controls. TIMP-1 levels were elevated in both patient groups compared to healthy controls. Total-, pro- and active MMP-9 levels as well as pro-MMP-TIMP-1 and active MMP-9/TIMP-1 ratios were inversely associated with total lung capacity.

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1Contribution equal to first authorship.

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Introduction

Interstitial lung disease (ILD) has been found in 75% of systemic sclerosis (SSc) cases at autopsy. ILD may develop in both limited (lcSSc) and diffuse cutaneous scleroderma (dcSSc) and is associated with decreased survival. Extensive ILD is, however, far more common in dcSSc and often associated with the presence of anti scleroderma-70 antibodies (anti Scl-70 Ab). The pathogenesis of ILD in association with SSc is unclear and the current treatment unspecific.

The histopathology of ILD in SSc is often characterised by remodelling and thickening of alveolar septal interstitiae with lose or dense fibrosis, foci of tissue degradation with honeycombing and lymphocyte infiltration. The histology correlates to the findings by high resolution computed tomography (HRCT) of the lungs, and ILD in SSc is conveniently diagnosed by this method, which permits imaging of the lung parenchyma in detail. Apart from reported elevated levels of neutrophil elastase and mast cell tryptase in bronchoalveolar lavage (BAL) in SSc, it is unclear what enzymes may be involved in extracellular matrix (ECM) degradation and remodelling in ILD in SSc.

The matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases that have the ability to degrade all major connective tissue matrices. MMP-9, a type IV collagenase, also called gelatinase B because of its ability to break down gelatin, has substrate specificity for a variety of ECM constituents such as native collagens IV and V, gelatin, elastin and vitronectin and has been observed to play an important role in lung tissue remodelling in an experimental model of lung fibrosis. In man, increased MMP-9 production has been found in several lung diseases, characterised by the degradation and remodelling of lung interstitial tissue and airways. As collagen IV is an important constituent of alveolar and bronchial basement membranes, MMP-9 is considered to play an important role in the development of the histopathologic changes found in idiopathic lung fibrosis, emphysema, chronic obstructive lung disease (COPD), the acute respiratory distress syndrome (ARDS) and asthma. Extracellular control of MMP-9 activity is mainly accomplished by the tissue inhibitor of metalloproteinases 1 (TIMP-1).

MMP-9 is produced constitutively by neutrophils and eosinophils, stored in granules and immediately released in the pro-form upon chemokine stimulation. In mast cells, monocytes and lymphocytes, MMP-9 synthesis is induced by inflammatory mediators and released with certain latency.

Neutrophils are incapable of TIMP-1 production, but eosinophils, mast cells, monocytes/macrophages and fibroblasts synthesise TIMP-1 upon cytokine stimulation. Complexed as well as unbound TIMP-1 is susceptible to the degradation by serine proteases released from activated neutrophils and mast cells. Excess unbound TIMP-1 may play a role in the generation of fibrosis, promoting fibroblast proliferation.

Since an increased number of neutrophils and mast cells, is the hallmark of BAL fluid changes in ILD in SSc, and as eosinophils and mast cell numbers often are elevated, an increased airway secretion of MMP-9 and TIMP-1 was anticipated. Based on these considerations, we hypothesised that MMP-9 and TIMP-1 may play essential roles in ECM remodelling in ILD in SSc. To investigate this, we measured levels of MMP-9 and TIMP-1 in BAL fluid from SSc patients with signs of ILD on HRCT, in SSc patients with normal HRCT and in healthy controls and related these levels to lung function.

Patients and methods

The characteristics of the patients and controls are given in Table 1. Sixteen consecutive patients (13 women and 3 men) with SSc according to the American College of Rheumatology (formerly the American Rheumatism Association) criteria were studied. Twelve of the patients (11 women and one man) had lcSSc and four (two women and two men) had dcSSc. Three of the patients with lcSSc (all female) also fulfilled the Alarcon-Segovia and Cardiel criteria for mixed connective tissue disease (MCTD). At the time of investigation, six patients with lcSSc had anti-centromere antibodies, one with lcSSc and two with dcSSc had anti-Scl-70 Ab and all three, who fulfilled the criteria for MCTD, had high-titer anti-RNP antibodies.

Signs of ILD on HRCT of the lungs were found in nine of the 16 SSc patients, that is in six of the 12 patients (50%) with lcSSc and in three of the four patients (75%) with dcSSc. Anti-Scl-70 antibodies were present in three of the seven SSc patients with signs of ILD on HRCT and in none of the seven SSc patients with normal HRCT. The patients had never smoked, except for two patients with signs of ILD on HRCT and two SSc patients with normal HRCT, who were ex-smokers but had not smoked for the last three years prior to bronchoscopy. The age of the SSc patients with signs of ILD on HRCT tended to be higher than that of the SSc patients with normal HRCT (62.0 (13.0) (median (interquartile range)) vs. 53.0 (14.0); P = 0.06). The disease duration tended to be shorter in SSc patients with signs of ILD on HRCT compared to SSc with normal HRCT (6.0 (9.3) vs. 14.0 (20.8); P = 0.09). Table 1. Three SSc patients with signs of ILD on HRCT and three SSc patients with normal HRCT were medicated with corticosteroids. Sixteen age- and sex-matched healthy subjects (who had never smoked) randomly selected from within the age cohort in the population register of the county...
of Västerbotten, Sweden, served as controls. The study was approved by the Ethics committee of Umeå University and informed consent was obtained from all study subjects.

Bronchoscopies

Fiberoptic bronchoscopy (Olympus BF type IT200, Tokyo, Japan) with BAL was conducted as previously outlined. In summary, 3 \( /C2 \) 60 ml of sterile phosphate buffered saline (PBS), pH 7.3, at 37\( ^\circ \)C was instilled into the segmental bronchus of the middle lobe of the right lung. The immediately recovered aspirate was collected into a siliconised container placed on ice.

Processing of lavage fluid and cells

The aspirated BAL fluid was passed through a nylon filter (pore diameter 100\( \mu \)m, Syntab Product AB, Malmö, Sweden) and centrifuged at 400 \( g \) for 15 min to remove mucus and cellular components. Supernatants were separated from cell pellets and the cell-free fluid divided into aliquots and stored at \(-80^\circ\) C prior to analysis. Cell pellets derived from BAL were re-suspended in PBS to give a final concentration of 10\( ^6 \) cells/ml and total and differential leukocyte counts performed. The total number of cells in the lavage fluid was counted in a Bürker chamber. Cytocentrifuged specimens with 5 \( /C2 \) 10\(^4 \) non-epithelial cells per slide were prepared using a Cytospin 3 (Shandon Southern Instruments Inc., Sewikly, PA, USA) 1000 rpm (96g) for 5 min. Cell differential counts were conducted on slides stained according to May-Grünwald Giemsa and 400 cells per slide were counted. Mast cells were counted in at least 10 visual fields at \( \times 160 \) magnification on slides stained with acid toluidine blue and counter stained with Mayer’s acid haematoxylin.

### MMP-9

Cleavage of pro-MMP-9 near or at residue 87 results in the active enzyme with a mass of approximately 82 kDa. Levels of total-MMP-9 (active+pro-MMP-9) were measured by means of an ELISA assay (R&D Systems, Oxon, UK). The detection limit for total MMP-9 was 0.156 ng/ml.

Levels of pro-MMP-9 were analysed using an ELISA method (Amersham Pharmacia, Buckinghamshire, UK). The values recorded included free and complexed forms. For the pro-MMP-9 ELISA the detection limit was 0.06 ng/ml.

Active MMP-9 values were calculated as the difference between concentrations of total MMP-9 and pro-MMP-9.

### TIMP-1

Levels of TIMP-1 were measured using an ELISA method (Amersham Pharmacia, Buckinghamshire, UK). The values recorded included free and MMP-bound forms. For the TIMP-1 ELISA the detection limit was 1.25 ng/ml.

### Myeloperoxidase (MPO)

MPO is released from activated neutrophils and is therefore considered a marker of neutrophil activation. MPO was measured by a radio-immuno assay (RIA) (Pharmacia AB, Uppsala, Sweden). The detection limit was 8 \( \mu \)g/L.

### Methyl-histamine

Histamine is synthesised by mast cells and basophils and stored in secretory granules. Released histamine disappears within minutes due to methylation via histamine-\( N \)-methyltransferase. Methyl-histamine was measured by RIA (Pharmacia AB, Uppsala, Sweden). The detection limit was 0.1 \( \mu \)g/L.

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**Table 1** Clinical characteristics of patients with systemic sclerosis and the control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SSc without ILD</th>
<th>SSc with ILD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(( N = 7 ))</td>
<td>(( N = 9 ))</td>
<td>(( N = 16 ))</td>
</tr>
<tr>
<td>Age, years</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>Females/males</td>
<td>6/1</td>
<td>7/2</td>
<td>13/3</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>14.0 (20.8)</td>
<td>6.0 (9.3)</td>
<td></td>
</tr>
<tr>
<td>Limited/diffuse cutaneous SSc</td>
<td>6/1</td>
<td>6/3</td>
<td></td>
</tr>
<tr>
<td>Skin score</td>
<td>1.0 (2.0)</td>
<td>1.0 (4.0)</td>
<td></td>
</tr>
<tr>
<td>Previous scleroderma renal crisis</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Oesophagus involvement</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Anticentromere-/Anti-scleroderma 70/Anti-ribonucleoprotein antibodies</td>
<td>4/0/1</td>
<td>2/3/2</td>
<td></td>
</tr>
<tr>
<td>FEV(_1)</td>
<td>118.0 (24.9)</td>
<td>99.1 (29.1)</td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>121.0 (16.3)</td>
<td>97.5 (23.0)</td>
<td></td>
</tr>
<tr>
<td>TLC</td>
<td>117.0 (8.3)</td>
<td>90.7 (11.6)**</td>
<td></td>
</tr>
<tr>
<td>DL(_CO)</td>
<td>88.9 (26.9)</td>
<td>66.5 (31.6)</td>
<td></td>
</tr>
</tbody>
</table>

FEV\(_1\), forced expired volume during the first second; VC, vital capacity; TLC, total lung capacity; DL\(_CO\), diffusion capacity for carbon monoxide. Lung functions are expressed in % of predicted.

\( **P = 0.0095. \)
Eosinophilic cationic protein (ECP)

ECP is a strikingly basic protein localised in the eosinophil granule matrix and a member of the ribonuclease gene superfamily. ECP was measured by RIA (Pharmacia AB, Uppsala, Sweden). The detection limit was 1 μg/L.

Lung function assessments

Lung volumes, dynamic spirometry and diffusing capacity of the lung for carbon monoxide (DLCO) were measured according to standard procedures (Master Spirometer and Master Pro Transfer; Jaeger, Würzburg, Germany).

HRCT of the lungs

HRCT of the lungs was performed using a Philips Tomoscan LX Single slice scanner (MA, USA). Scans were performed at full inspiration in the supine position with 120 kV, 175 mAmp including contiguous scans throughout the lungs with 10 mm thickness followed by scans with 1.5 mm thickness with a slice spacing of 30 mm. Ground glass opacities, reticulate pattern fibrosis, non-septal and/or subpleural lines and honeycombing were considered signs of ILD.4

Statistical analysis

Results are reported as median and interquartile range (IQR). Comparisons between the three groups (SSc patients with ILD, SSc patients without ILD and controls) were analysed with a non-parametric ANOVA (Kruskal–Wallis test). A significant difference was considered at the 5% level. If a significant change was found in a parameter using the Kruskal–Wallis test, post-hoc analyses were performed using Mann–Whitney U-test. The Mann–Whitney non-parametric test was also used to compare lung function variables between SSc patients with and without ILD. Correlation analyses were performed using the non-parametric Spearman’s rank correlation test. P values less than 0.05 were considered significant.

Results

Lung function and gas transfer

The nine patients with signs of ILD on HRCT had significantly reduced total lung capacity (TLC), as an indicator of restrictive lung function deterioration, compared with patients without ILD (P = 0.01). Vital capacity (VC) showed a tendency towards a decrease in SSc patients with ILD, although not significant (P = 0.057). Neither FEV1 nor DLCO differed between the groups (Table 1).

HRCT findings

Of the nine patients with signs of ILD on HRCT two had ground glass changes as well as honeycombing, of these two patients, one had ground glass changes confined to the lower parts of the lower lobes while the other had ground glass changes in the lower parts of all lobes. Three patients had reticular pattern lung fibrosis with mild honeycombing in the lower parts of the lower lobes and the remaining four had reticular pattern lung fibrosis in the lower parts of the lower lobes.

Total and differential cell counts in BAL fluid

The neutrophil is a key cell in airway inflammation in SSc and although the Kruskal–Wallis test only showed a tendency towards a significant difference in neutrophil numbers between the three groups (P = 0.085), post-hoc analyses were carried out. The number of neutrophils was slightly elevated in SSc patients with signs of ILD on HRCT compared to SSc patients with normal HRCT (P = 0.04) and compared to controls (P = 0.049). The number of eosinophils was significantly higher in the nine SSc patients with signs of ILD on HRCT compared to healthy controls (P = 0.007). The number of mast cells was significantly elevated in both SSc patients with signs of ILD on HRCT and in SSc patients with normal HRCT compared to healthy controls (P = 0.009 and 0.008, respectively). There was no significant difference in the volumes of BAL fluid recovered from the SSc patients and the normal controls (Table 2).

Levels of total MMP-9, Pro-MMP-9; calculated levels of active MMP-9, TIMP-1; pro-MMP-9/TIMP-1 and active MMP-9/TIMP-1 ratios in BAL fluid

Total MMP-9 and pro-MMP-9 levels were significantly increased in BAL fluid from the nine SSc patients with signs of ILD on HRCT compared to levels in healthy controls (P = 0.012 and 0.03, respectively) and compared to levels in SSc patients with normal HRCT (P = 0.0008 for both). In SSc patients with ILD, calculated active MMP-9 levels were significantly higher than in SSc patients without ILD (P = 0.0012) and tended to be higher than in healthy controls (P = 0.057). In SSc patients without ILD calculated active MMP-9 levels were significantly lower than in healthy controls (P = 0.0093). TIMP-1 levels were significantly higher in both the nine SSc patients with signs of ILD on HRCT (P = 0.02) and the seven SSc patients with normal HRCT (P = 0.048) compared to controls. The pro-MMP-9/TIMP-1 ratio was higher in SSc patients with signs of ILD on HRCT, compared to SSc patients with normal HRCT (P = 0.005), but did not differ significantly from the ratio found in healthy controls (P = 0.3). In SSc patients with ILD the active MMP-9/TIMP-1 ratio was significantly higher than in SSc patients without ILD (P = 0.0070), but did not differ from healthy controls (P = 0.808). In SSc patients without ILD the active MMP-9/TIMP-1 ratio was significantly lower than in healthy controls (P = 0.0013) (Table 3).

MPO, ECP and M-histamine in BAL fluid

As a sign of neutrophil activation, MPO levels were significantly elevated in BAL fluid from SSc with signs of ILD on HRCT compared to both healthy controls (P = 0.004) and SSc patients with normal HRCT (P = 0.009). ECP levels showed a tendency towards an increase in SSc with signs of ILD on HRCT both compared to SSc with normal HRCT and to healthy controls (P = 0.03 in both; Kruskal–Wallis test.
Methyl-histamine was only detectable in three SSc patients with signs of ILD on HRCT and in no SSc patient with normal HRCT or any of the controls (data not shown) (Table 3).

Correlation analyses

For the total group of 16 SSc patients with and without ILD, the levels of total MMP-9, pro-MMP-9 and active MMP-9 were significantly associated with the number of neutrophils \((r_s = 0.74; P = 0.006 \text{ and } r_s = 0.550; P = 0.03, r_s = 0.711; P = 0.008, \text{ respectively})\). Eosinophil numbers tended to non-significantly correlate with total MMP-9 \((r_s = 0.494; P = 0.07)\) and pro-MMP-9 \((r_s = 0.458; P = 0.09)\) and correlated significantly to levels of active MMP-9 \((r_s = 0.544; P = 0.04)\).

Levels of TIMP-1 showed a trend towards a significant association with the number of mast cells \((r_s = 0.52; P = 0.051)\).

As regards the relationship between metalloprotease/antiprotease and restrictive lung function changes, the levels of total-MMP-9, pro-MMP-9 and the pro-MMP-9/TIMP-1 ratio were all negatively associated with TLC \((r_s = -0.68; P = 0.01, r_s = -0.58; P = 0.02 \text{ and } r_s = -0.56; P = 0.03, \text{ respectively})\).

### Table 2  
Cellularity of bronchoalveolar lavage fluid in 16 patients with systemic sclerosis and 16 healthy controls.  

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SSc without ILD</th>
<th>P vs. controls</th>
<th>SSc with ILD</th>
<th>P vs. controls</th>
<th>P comparison between SSc groups</th>
<th>P Kruskal–Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 16</td>
<td>N = 7</td>
<td></td>
<td>N = 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells (\times 10^6/\text{ml})</td>
<td>4.2 (2.75)</td>
<td>3.6 (3.7)</td>
<td>0.6</td>
<td>5.3 (7.8)</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3006</td>
</tr>
<tr>
<td>Alveolar macrophages (\times 10^6/\text{ml})</td>
<td>3.3 (2.6)</td>
<td>2.2 (3.0)</td>
<td>0.9</td>
<td>4.0 (9.5)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3708</td>
</tr>
<tr>
<td>Lymphocytes (\times 10^6/\text{ml})</td>
<td>0.7 (0.5)</td>
<td>0.7 (0.5)</td>
<td>0.6</td>
<td>0.8 (0.8)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8274</td>
</tr>
<tr>
<td>Neutrophils (\times 10^6/\text{ml})</td>
<td>0.04 (0.1)</td>
<td>0.01 (0.1)</td>
<td>0.3</td>
<td>0.1 (0.2)</td>
<td>0.049</td>
<td>0.04</td>
<td>0.0854</td>
</tr>
<tr>
<td>Eosinophils (\times 10^6/\text{ml})</td>
<td>0.007 (0.02)</td>
<td>0.03 (0.1)</td>
<td>0.1</td>
<td>0.1 (0.2)</td>
<td>0.007</td>
<td>0.08</td>
<td>0.0142</td>
</tr>
<tr>
<td>Mast cells (\times 10^6/\text{ml})</td>
<td>0.0005 (0.008)</td>
<td>0.0015 (0.016)</td>
<td>0.034</td>
<td>0.004 (0.017)</td>
<td>0.0033 (0.017)</td>
<td>0.4</td>
<td>0.0086</td>
</tr>
</tbody>
</table>

SSc, systemic sclerosis; ILD, interstitial lung disease; IQR, interquartile range.

### Table 3  
Soluble components in bronchoalveolar lavage fluid from 16 patients with systemic sclerosis and 16 healthy controls.  

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SSc without ILD</th>
<th>P vs. controls</th>
<th>SSc with ILD</th>
<th>P vs. controls</th>
<th>P comparison between SSc groups</th>
<th>P Kruskal–Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 16</td>
<td>N = 7</td>
<td></td>
<td>N = 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECP (\mu\text{g}/\text{L})</td>
<td>0.9 (0.5)</td>
<td>1.0 (0.06)</td>
<td>0.8</td>
<td>1.8 (1.4)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>MPO (\mu\text{g}/\text{L})</td>
<td>3.5 (4.1)</td>
<td>1.6 (3.7)</td>
<td>0.3</td>
<td>6.6 (14.7)</td>
<td>0.004</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>Total MMP-9 (\text{ng/ml})</td>
<td>1.2 (1.3)</td>
<td>0.6 (0.7)</td>
<td>0.2</td>
<td>3.0 (3.7)</td>
<td>0.012</td>
<td>0.008</td>
<td>0.01</td>
</tr>
<tr>
<td>Pro-MMP-9 (\text{ng/ml})</td>
<td>0.2 (0.4)</td>
<td>0.08 (0.05)</td>
<td>0.2</td>
<td>0.4 (0.1)</td>
<td>0.03</td>
<td>0.008</td>
<td>0.009</td>
</tr>
<tr>
<td>Active MMP-9 (\text{ng/ml})</td>
<td>1.01 (1.063)</td>
<td>0.620 (0.595)</td>
<td>0.0093</td>
<td>2.713 (3.746)</td>
<td>0.057</td>
<td>0.012</td>
<td>0.002</td>
</tr>
<tr>
<td>TIMP-1 (\text{ng/ml})</td>
<td>7.0 (3.3)</td>
<td>9.5 (4.0)</td>
<td>0.048</td>
<td>14.3 (21.1)</td>
<td>0.02</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Pro-MMP-9/TIMP-1</td>
<td>0.009 (0.01)</td>
<td>0.002 (0.001)</td>
<td>0.07</td>
<td>0.099 (0.01)</td>
<td>0.3</td>
<td>0.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Active MMP-9/TIMP-1</td>
<td>0.055 (0.055)</td>
<td>0.009 (0.018)</td>
<td>0.0013</td>
<td>0.064 (0.102)</td>
<td>0.808</td>
<td>0.0070</td>
<td>0.004</td>
</tr>
</tbody>
</table>

SSc, systemic sclerosis; ILD, interstitial lung disease; IQR, interquartile range; ECP, eosinophil cationic protein; MPO, myeloperoxidase; MMP-9, matrix metalloproteinase 9; TIMP, tissue inhibitor of metalloproteinase.
and controls with bronchoscopy is that the obtainable numbers. An inherent difficulty in exploring patient groups is the tendency (ILD in SSc).

Correlations between active MMP-9 and TLC in percent of predicted in 16 systemic sclerosis patients. Solid circles represent patients with interstitial lung disease.

The corresponding correlations between active MMP-9 and reduction in TLC were \( r_s = -0.677; P = 0.009 \) (Fig. 1) and between active MMP-9/TIMP-1 and TLC \( r_s = -0.62; P = 0.016 \).

**Discussion**

In the present study we have demonstrated that BAL fluid levels of MMP-9 are enhanced in SSc patients with signs of ILD on HRCT, compared to SSc patients without ILD. Furthermore, the increased MMP-9 concentrations were associated with a reduction in TLC, an important consequence of ILD.

The study was performed in SSc patients treated in our clinic after having given informed consent to participate in a bronchoscopy investigation. Ideally, a very large cohort of individuals would have been included, but availability of patients, time for performing the study and resources set limits. The study material is relatively similar in size to other investigations of the field and has strength by the limits. The study material is relatively similar in size to other investigations of the field and has strength by the limits. The study material is relatively similar in size to other investigations of the field and has strength by the limits.

The present data imply MMP-9 to be produced locally in the lungs and predominantly by neutrophils. Using levels of MPO and ECP as markers, the study indicates that neutrophils and, to a lesser extent, eosinophils are activated in SSc patients with signs of ILD on HRCT. Besides pointing at the neutrophil, correlation analyses did not exclude the eosinophil as a potential additional source of MMP-9. Upon IL-8 stimulation, activated neutrophils release considerable amounts of unbound MMP-9, while eosinophils and mast cells release partly complexed MMP-9 and TIMP-1 after pro-inflammatory cytokine stimulation. The present result is in agreement with the previous observations of increased BAL fluid concentrations of IL-8, IL-6 and TNF alpha in ILD in SSc.

In this study, TIMP-1 levels were elevated in both groups of SSc patients compared to the control subjects and showed a tendency \( (P = 0.051) \) to correlate with BAL fluid mast cell numbers. An inherent difficulty in exploring patient groups and controls with bronchoscopy is that the obtainable number of subjects is commonly low, due to the invasive sampling procedure. It can therefore be debated whether correction for comparison between multiple groups needs to be applied or not. The data indicate, but do not definitely prove that mast cells may contribute to the elevated levels of TIMP-1 in SSc and other cellular sources cannot be excluded. It has been speculated that an excess of unbound TIMP-1 may stimulate fibroblast proliferation and potentially enhance development of fibrosis. The present study does not support this assumption as there was no indication of excessive TIMP-1, in relationship with either pro-MMP-9 or active MMP-9, in the SSc patients with ILD. In contrast, patients with SSc without ILD, had more TIMP-1 in relationship to the MMP-9 forms.

In the patient group without ILD, which comprised six patients with ISSc, DLCO was slightly reduced, whereas TLC was normal. Of these, one has developed severe isolated pulmonary hypertension and two have increased pulmonary arterial pressure measured by echocardiography. Isolated DLCO reduction with a normal TLC is a common abnormality in SSc due to thickening of the wall in small- and medium-sized pulmonary arteries and may antedate the development of isolated pulmonary hypertension, which is especially common in ISSc patients.

ILD may lead to remodelling of the lung resulting in a restrictive lung function pattern and lung fibrosis. In the present study of patients with SSc with and without ILD, the MMP-9 levels, regardless if expressed as total-, pro- or active MMP-9 were all inversely associated with TLC, consistent with a role for alveolar MMP-9 in remodelling and lung volume reduction. Furthermore, in an inflammatory environment the local degradation of the inhibitor TIMP-1 may be accentuated due to increases in neutrophil elastase and mast cell proteases. The more abundant pro-MMP-9 in SSc patients with ILD may also be locally activated by elevated levels of inducible enzymes such as MMP-3 (stromelysin), plasmin and inflammatory cell derived proteases.

It is suggested that the neutrophil may play an important role, also in the regulation of the inflammatory process in ILD in SSc which may lead to lung fibrosis, through MMP-9 production. MMP-9 may be able to potentiate the effects of IL-8 and establish a positive feedback loop for neutrophil attraction, activation and MMP-9 release. MMP-9 may also activate IL-1 beta, an auto-inducible cytokine central to the inflammatory reaction. Furthermore, MMP-9 has been suggested to create neo-epitopes, which trigger T cell activation in auto-immune diseases, a phenomenon designated "remnant epitopes that generate auto-immunity". Considering this, treatment with MMP-9 inhibitors may, in addition to reducing ECM degradation, inhibit auto-immune self-perpetuating reactions in ILD in SSc. Such an option that may be available in the future as a broad-spectrum MMP inhibitor has been found to reduce experimental lung fibrosis.

In summary, the results point to an extended role for activated neutrophils in ILD development in SSc due to increased release of MMP-9. Bronchoalveolar levels of MMP-9 were further inversely associated with TLC, suggesting a role of MMP-9 in the remodelling in ILD and lung fibrosis. The metalloprotease burden in the lung in SSc with ILD may represent a new therapeutic target in this condition.
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