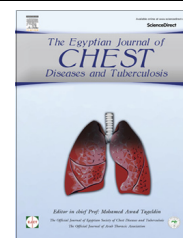




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## ORIGINAL ARTICLE

# Study the level of sputum matrix metalloproteinase-9 and tissue inhibitor metalloproteinase-1 in patients with interstitial lung diseases



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## KEYWORDS

Interstitial lung diseases;  
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 Sputum neutrophils

**Abstract** *Background:* Pulmonary fibrosis, the final result of a large variety of interstitial lung diseases, is characterized by an aberrant remodeling of extracellular matrix (ECM) with a profound disturbance of the normal lung architecture. This remodeling includes the exaggerated accumulation of ECM components in the interstitial and alveolar spaces and the disruption of the basement membranes. It has long been accepted that MMPs play an important role in the pathogenesis of pulmonary fibrosis, but the exact mechanisms are not well characterized. There are several interrelated processes—such as ECM remodeling, basement-membrane disruption, epithelial-cell apoptosis, cell migration, and angiogenesis—in which MMPs may play a central role, either by ECM direct cleavage or by generating bioactive mediators. TIMPs can modulate cellular processes such as cell growth, apoptosis and migration, and can be both anti- and pro-tumorigenic. This study aimed to examine the changes in induced sputum as regards MMP-9, TIMP-1 and levels of inflammatory cells in ILD patients compared with sputum of healthy non smokers.

*Subjects and methods:* Thirty subjects were included in this study and were classified into the following two groups: Group I included twenty patients diagnosed clinically, radiologically and physiologically as interstitial lung diseases. Group II included ten healthy non smoker subjects. Sputum induction was done and processed to assess matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and cytological examination with cellular count.

*Results:* In this study, we have demonstrated that levels of sputum MMP-9 and TIMP-1 were significantly increased in patients with interstitial lung diseases than normal persons with highly significant statistical differences ( $p = 0.001$ ). MMP-9 was positively correlated with number of neutrophils in the airway with highly significant statistical difference ( $p = 0.001$ ).

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## Background

The interstitial lung diseases are a clinically challenging and diverse group of over 150 disorders characterized by varying degrees of fibrosis and inflammation of the lung parenchyma or interstitium. The interstitium of the lung spans the region between alveolar epithelium and pulmonary vascular endothelium. This region includes a variety of cell types (fibroblasts, myofibroblasts, and macrophages) and matrix components (collagens, elastin, and proteoglycans) [1]. Pulmonary fibrosis, the final result of a large variety of interstitial lung diseases, is characterized by an aberrant remodeling of extracellular matrix (ECM) with a profound disturbance of the normal lung architecture. This remodeling includes the exaggerated accumulation of ECM components in the interstitial and alveolar spaces and the disruption of the basement membranes [2]. The pathologic findings in pulmonary fibrosis (excessive accumulation of ECM and remodeling of the lung architecture) are a consequence of disturbances in two physiologically balanced processes: proliferation and apoptosis of fibroblasts and accumulation and breakdown of ECM. When the normal balance between ECM deposition and turnover is shifted toward deposition or away from breakdown, excessive ECM accumulates [3]. Several possible origins of ECM producing mesenchymal cells have been described, and have included accumulation of resident lung fibroblasts, homing and fibroblastic differentiation of bone marrow-derived cells such as circulating fibrocytes or monocytes [4–7], or epithelial-mesenchymal transition (EMT) [8]. In addition to altered mesenchymal cells, abnormalities of the alveolar epithelium in patients with pulmonary fibrosis have been noted from the earliest descriptions of the disease process [9,10].

Matrix metalloproteinases (MMPs) are a family of zinc containing endopeptidases, which is a subset of the metzincin superfamily of metalloproteinases. These regulatory proteases are the extracellular matrix (ECM) remodelers characterized by their substrate specificity to degrade ECM proteins [11,12]. MMPs can be separated into 6 main classes according to their substrate specificity, cellular location and primary structure: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others [13–15]. Recently, a new classification system has been proposed based on MMP structure rather than on their substrate specificity: archetypal MMPs, matrilysins, gelatinases, and furin activable MMPs [16,17]. In general, MMPs levels are usually low in normal adult resting tissues, and with some exceptions, their production and activity are maintained at virtually undetectable levels. By contrast, their expression becomes elevated when there is a challenge to the system, such as wound healing, repair or remodeling processes, in diseased tissues and even in several cell types grown in culture [18].

It has long been accepted that MMPs play an important role in the pathogenesis of pulmonary fibrosis, but the exact mechanisms are not well characterized. There are several inter-related processes—such as ECM remodeling, basement-membrane disruption, epithelial-cell apoptosis, cell migration, and angiogenesis—in which MMPs may play a central role, either by ECM direct cleavage or by generating bioactive mediators [2]. The participation of MMPs in lung fibrosis has been analyzed in several interstitial lung diseases in humans and in experimental models such as those provoked

by bleomycin, paraquat plus hyperoxia and silica [19]. MMP-9, also called gelatinase B, contains additionally a type V collagen like domain that is highly glycosylated, which has been suggested to have an effect on substrate specificity [20].

MMP-9 gene expression and protein have also been shown to be elevated in lungs and BAL fluids from patients with IPF [21–25]. The majority of MMPs are not expressed in normal healthy tissues but are expressed in diseased tissues that are inflamed or undergoing repair and remodeling [26,27]. Pulmonary epithelial cells may also be a significant source of MMPs as they express MMP-1, -2, -7 and -9. Intracellularly, MMP secretion is primarily regulated by the prostaglandin (PG) and mitogen activated protein kinase (MAPK) signal transduction pathways.

Tissue inhibitors of metalloproteinases (TIMP) are a family of secretory proteins that are able to inhibit matrix metalloproteinase activities through non-covalent binding of pre- or active forms of MMPs at molar equivalence. By inhibiting MMPs, TIMPs may also influence MMP-mediated processes such as processing of cytokines, degradation of growth factor binding proteins, and the release of ECM-bound growth factors [11,28–31]. TIMPs can modulate cellular processes such as cell growth, apoptosis and migration, and can be both anti- and pro-tumorigenic [11,13,32,33]. TIMP-1 works as a natural inhibitor of MMP-9 and is found in most tissues and body fluids. By inhibiting MMPs activities, TIMPs are involved in tissue remodeling and regulation of ECM metabolism. The TIMP family consists of four members sharing important structural features as well as the ability of MMP inhibition [11,13].

Induced sputum (IS) collection is a non-invasive method for assessment of airway inflammation in the airways [34,35]. This study aimed to examine the changes in induced sputum as regards MMP-9, TIMP-1 and levels of inflammatory cells in ILD patients compared with sputum of healthy non smokers.

## Subjects and methods

### Subjects

Thirty subjects were included in this study and were classified into the following two groups: Group I included twenty patients diagnosed clinically, radiologically and physiologically as interstitial lung diseases. Group II included ten healthy subjects. Patients with history of COPD, bronchial asthma or recent respiratory tract infection were excluded from this study.

### Methods

A written informed consent was obtained from all subjects. The two groups underwent full history taking including history of smoking (current, ex, and non smoking), history of chest symptoms (cough, expectoration, wheezes and dyspnea) and history of any other co morbidities. Also full clinical examination (general, full local respiratory system examination including inspection, palpation, percussion and auscultation, with special regards to manifestations of right sided heart failure as lower limbs edema, congested neck veins, tenderness over right hypochondrium, and dullness on right parasternal area)

and chest X ray. Physiological assessment was also done using spirometry. Flow/volume loop was performed in all cases. Computerized tomography CT was done to group one to confirm diagnosis of interstitial pulmonary diseases. Sputum induction was done and was processed to assess matrix metalloproteinase-9 (MMP-9) by ELISA (MMP-9 BIOTRAK ELISA), tissue inhibitor of metalloproteinase-1 (TIMP-1) by ELISA (TIMP-1 BIOTRAK ELISA), cytological examination and cellular count. The total and the differential cell counts were expressed as corrected percentage. Routine laboratory investigations (CBC, Liver enzymes and serum creatinine) were also done. Sputum Induction and Processing were done according to Esa et al. [11].

*The assay of matrix metalloproteinases-9 in sputum is done by ELISA*

#### *Principles of assay*

The RayBio<sup>®</sup> human MMP-9 ELISA (enzyme-linked immunosorbent assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human MMP-9. This assay employs an antibody specific for human MMP-9 coated on a 96-well plate. Standards and samples are pipetted into the wells and MMP-9 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human MMP-9 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of MMP-9 bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm [36].

#### *Assay procedure*

All reagents and samples were brought to room temperature (18–25 °C) before use. 100 µl of each standard (see Reagent Preparation step 2) and sample was added into appropriate wells, covered well and incubated for 2.5 h at room temperature. The wells were washed 4 times with 1x wash solution (200 µl each). 100 µl of 1x prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 µl each). 100 µl of prepared Streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded and washed 5 times with 1x wash solution (200 µl each). 100 µl of TMB One-Step Substrate Reagent was added to each well and incubated for 30 min at room temperature in the dark. 50 µl of Stop Solution was added to each well then the absorbance was read at 450 nm immediately. The concentration of MMP-9 was calculated from the standard curve.

*The assay of tissue inhibitor metalloproteinases-1 in sputum is done by ELISA*

#### *Principles of assay*

The RayBio<sup>®</sup> human TIMP-1 ELISA (enzyme-linked immunosorbent assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TIMP-1. This assay employs an antibody specific for

human TIMP-1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TIMP-1 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TIMP-1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated Streptavidin is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TIMP-1 bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm [37].

#### *Assay procedure*

All reagents and samples were brought to room temperature (18–25 °C) before use. 100 µl of each standard (see Reagent Preparation step 2) and sample was added into appropriate wells, covered and incubated for 2.5 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 µl each). 100 µl of 1x prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 µl each). 100 µl of prepared Streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded and washed 5 times with 1x wash solution (200 µl each). 100 µl of TMB One-Step Substrate Reagent was added to each well and incubated for 30 min at room temperature in the dark. 50 µl of Stop Solution was added to each well then the absorbance was read at 450 nm immediately. The concentration of TIMP-1 was calculated from the standard curve.

#### *Statistical analysis*

Quantitative data were presented as mean and standard deviation (SD) values. *Student's t-test* used for comparisons between mean of two groups. Inter-group comparison of categorical data was performed by using fisher exact test (FET). Qualitative data were presented as frequencies and percentages. *Pearson's correlation coefficient* was used to determine significant correlations between the different variables. The significance level was set at  $p \leq 0.05$ . Statistical analysis was performed with SPSS version 19.0 (statistical package for scientific studies) for windows.

## **Results**

This study was done in the chest department of university hospital in the period between August 2011 and August 2014. Thirty subjects were included in this study and were classified into the following two groups: Group I included twenty patients diagnosed clinically, radiologically and physiologically as interstitial lung diseases. Group II included ten healthy non smoker subjects. Patients' demographic data are shown in Table 1. Group I included 14 (70%) male patients and 6 (30%) female patients with a mean age of  $49.15 \pm 3.12$ . The control included 10 (100%) male patients with a mean age of  $48.3 \pm 3.4$ . There was no significant statistical difference of mean ages between both groups. Patients' laboratory data are shown in Table 1. Total leukocytic count in sputum was significantly higher in group I ( $23.36 \pm 4.53$ ) than in the control group ( $17.38 \pm 1.95$ ). Neutrophils, eosinophils and lymphocytes

**Table 1** Patients' demographic data, laboratory results and PFT of both groups.

| Variable          | Group (I) ILD (20 cases)<br>Mean $\pm$ SD | Group (II) control group<br>Mean $\pm$ SD | <i>p</i> -Value |
|-------------------|---|---|-----------------|
| Age               | 49.15 $\pm$ 3.12                          | 48.3 $\pm$ 3.4                            | 0.5             |
| Sex, <i>n</i> (%) |   |   |                 |
| Male              | 14 (70.0)                                 | 10 (100)                                  | 0.146           |
| Female            | 6 (30.0)                                  | 0 (0.0)                                   |                 |
| MMP9              | 242.05 $\pm$ 16.7                         | 29.5 $\pm$ 10.05                          | 0.001**         |
| TIMP1             | 1493.0 $\pm$ 135.4                        | 91.2 $\pm$ 36.36                          | 0.001**         |
| MMP9/TIMP1        | 0.163 $\pm$ 0.015                         | 0.315 $\pm$ 0.036                         | 0.001**         |
| TLC               | 23.36 $\pm$ 4.53                          | 17.38 $\pm$ 1.95                          | 0.001**         |
| Neutrophils       | 61.44 $\pm$ 2.98                          | 21.93 $\pm$ 3.74                          | 0.001**         |
| Eosinophiles      | 1.12 $\pm$ 0.41                           | 0.50 $\pm$ 0.29                           | 0.001**         |
| Macrocytes        | 34.6 $\pm$ 2.91                           | 75.53 $\pm$ 4.18                          | 0.001**         |
| Lymphocytes       | 2.77 $\pm$ 0.96                           | 1.97 $\pm$ 0.92                           | 0.001**         |
| FEV1              | 1.2 $\pm$ 0.58                            | 3.15 $\pm$ 0.32                           | 0.037*          |
| FEV1%             | 34.05 $\pm$ 16.93                         | 89.8 $\pm$ 4.61                           | 0.001**         |
| FVC               | 1.34 $\pm$ 0.59                           | 4.06 $\pm$ 0.51                           | 0.001**         |
| FVC%              | 31.25 $\pm$ 15.62                         | 87.5 $\pm$ 5.13                           | 0.001**         |
| FEV1/FVC          | 85.7 $\pm$ 11.22                          | 75.4 $\pm$ 2.12                           | 0.008**         |

\* Significant.  
\*\* Highly significant.

**Table 2** Correlation between sputum MMP-9, TIMP-1 and ratio of MMP-9/TIMP1 with different variables in group (I).

| Variables    | MMP-9 (ng/ml) |          | TIMP-1 (ng/ml) |          | MMP-9/TIMP-1 |          |
|--------------|---------------|----------|----------------|----------|--------------|----------|
|              | ( <i>r</i> )  | <i>p</i> | ( <i>r</i> )   | <i>p</i> | ( <i>r</i> ) | <i>p</i> |
| FVC%         | 0.405         | 0.077    | -0.194         | 0.413    | 0.486        | 0.03*    |
| FEV1/FVC%    | -0.034        | 0.885    | -0.244         | 0.30     | 0.230        | 0.33     |
| Neutrophils  | 0.993         | 0.001**  | 0.324          | 0.163    | 0.417        | 0.067    |
| Eosinophiles | -0.121        | 0.61     | -0.359         | 0.120    | 0.267        | 0.254    |
| Macrocytes   | -0.959        | 0.001**  | -0.303         | 0.195    | -0.413       | 0.071    |
| Lymphocytes  | -0.242        | 0.303    | -0.069         | 0.774    | -0.118       | 0.621    |

\* Significant = less than 0.05.

\*\* Highly significant = less than 0.01.

were statistically highly significant more in group 1 than in the control group, while alveolar macrophages were statistically highly significant lower in group I than that in the control group. Sputum MMP-9 was statistically highly significant higher in group 1 with a mean value of 242.05  $\pm$  16.7 than in the control group with a mean value of 29.5  $\pm$  10.05. Also sputum TIMP-1 was statistically highly significant higher in group I with a mean value of 1493.0  $\pm$  135.4 than in the control group with a mean value of 91.2  $\pm$  36.36. Pulmonary function tests are shown in Table 1. Forced vital capacity (FVC) and forced expiratory volume in first seconds (FEV1) were significantly lower than that in the control group while the ratio of FEV1/FVC was significantly higher in group 1 than in the control group.

The correlation between inflammatory markers and different variables in group 1 is shown in Table 2 and Fig. 1. There was a positive correlation with highly significant statistical difference between neutrophils and MMP-9. Alveolar macrophages showed negative correlation with highly significant difference with MMP-9. As regards TIMP-1 and ratio of MMP-9/TIMP-1, neutrophils showed positive correlation with no significant difference while alveolar macrophages showed negative correlation with no significant difference. FVC% showed positive correlation with MMP-9 and MMP9-9/

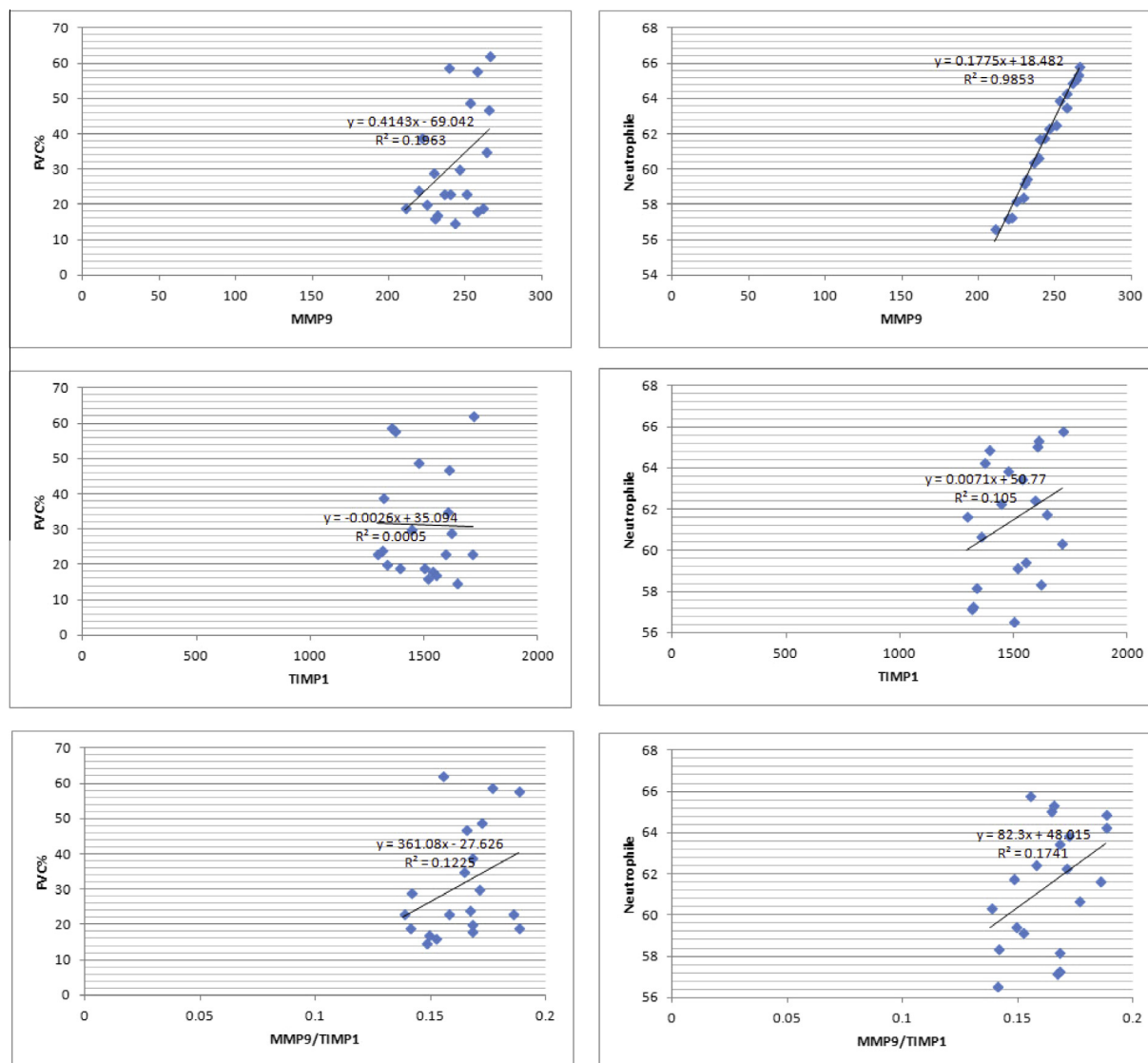
TIMP-1 ratio with highly significant statistical difference with MMP-9/TIMP-1. The CT findings and final diagnosis of group (I) are shown in Table 3. Fifteen cases were diagnosed with IPF, three cases NSIP (nonspecific interstitial pneumonia), one case sarcoidosis and one case rheumatoid arthritis. The diagnosis was confirmed in some cases by biopsy but in other cases by associated laboratory and radiological findings.

## Discussion

Pulmonary fibrosis is a chronic lung disease characterized pathologically by excessive accumulation of extracellular matrix (ECM) and remodeling of the lung architecture, and additionally characterized by recognizable clinical, physiologic, and radiographic findings [3]. Dysregulated pulmonary matrix remodeling resulting from failure to adequately repair damage caused by chronic inflammation is felt to underlie the development of pulmonary fibrosis. Excessive degradation can trigger the unregulated production of the matrix component that characterizes fibrosis [38].

MMPs are capable of degrading various components of the connective tissue matrix, and TIMPs are believed to play a significant role in remodeling after parenchymal damage, which





**Figure 1** Correlation between sputum inflammatory markers (MMP-9, TIMP-1, MMP-/TIMP1) with FVC% and neutrophils in group (I).

results in tissue destruction or in the induction of repair processes in pulmonary disease [22,39,40]. Among the MMPs, MMP-9 possesses the capacity to preferentially degrade collagen type IV, entactin, collagen type VII, as well as collagen type V in the interaction with interstitial fibrillar collagens [41,42]. The level of MMP-9 in the air spaces may relate to the degree of severity of inflammation of lung disease [43,44].

In this study, we have demonstrated that levels of sputum MMP-9 and TIMP-1 were significantly increased in patients with interstitial lung diseases than normal persons with highly significant statistical differences. These results were in agreement with the results obtained from previous studies [38,45]. In this study MMP-9 levels were positively correlated with sputum neutrophils. It is tempting to speculate that neutrophils are the major source of MMP-9 in the airways of those patients. This result was matched with another study that investigated MMP-9 in COPD and IPF [46]. MMP-9 may provoke the disruption of basement membranes, which seems to

be an important event in IPF pathogenesis that enhances the fibroblast invasion into the alveolar spaces. In addition, the disruption of the basement membrane may also contribute to the failure of an orderly repair of the damaged alveolar type I epithelial cells, affecting normal reepithelialization and, moreover, it may have an additional deleterious role by inducing epithelial apoptosis [2]. In fact, the integrity of the basement membrane is required to suppress programmed cell death as has been demonstrated in mammary epithelium and other tissues [2]. MMP-9 gene expression and protein have also been shown to be elevated in lungs and BAL fluids from patients with IPF [21,23]. The enzyme has been localized in epithelial cells, neutrophils, and macrophages with some staining in subepithelial fibroblasts.

The source of airway TIMP-1 in our patients remains unclear, since there was positive correlation between TIMP-1 and neutrophils but with no significant statistical difference. Possible sources of TIMP-1 could be cellular components of

**Table 3** CT findings and final diagnosis of group (I) ILD.

| Number of cases | CT finding   | Final diagnosis                                 |
|-----------------|--|---|
| 15 cases        | Peripheral, subpleural and, Basal distribution of:<br>Reticular opacities (fibrosis),<br>Honeycombing, Traction<br>bronchiectasis, Architectural<br>distortion, Minimal focal ground<br>glass appearance                                 | Idiopathic<br>pulmonary fibrosis<br>(IPF)       |
| 3 cases         | Peripheral, subpleural, basal,<br>symmetric diffuse ground glass<br>opacities, fine reticulations and<br>minimal honeycombing  | NSIP (nonspecific<br>interstitial<br>pneumonia) |
| 1 case          | Upper and mid lung distribution<br>of:<br>Micronodular opacities along<br>septae, peribronchovascular<br>bundles and pleura Irregular<br>linear opacities, Ground-glass<br>opacities, Honeycombing, Hilar<br>and mediastinal, adenopathy | Sarcoidosis                                     |
| 1 case          | Subpleural basal reticular pattern<br>Pleural thickening, Ground-glass<br>opacities, Honeycombing  | Rheumatoid<br>arthritis                         |

the extracellular matrix, like fibroblasts or myofibroblasts [47], or epithelial cells. Sputum alveolar macrophages were negatively correlated with all markers (MMP-9, TIMP-1 and molar their ratio). These findings explore that alveolar macrophages were decreased in all cases of pulmonary fibrosis. These findings matched with previous studies [38,46]. The molar ratio of MMP-9/TIMP-1 was significantly lower in ILD group than in the control group. It means that the imbalance between MMP-9 and its inhibitor TIMP-1 may play a role in tissue remodeling and lung fibrosis.

The molar ratio of MMP-9/TIMP-1 correlated with the number of neutrophils ( $r = 0.417$  and  $p = 0.067$ ). This is a marginally acceptable correlation, most likely due to the small sample size. These data suggest that the number of neutrophils may be more closely related to levels of MMP-9 than to levels of TIMP-1.

### Conclusion

MMP-9 and TIMP-1 were significantly increased in pulmonary fibrosis. MMP-9 was positively correlated with the number of neutrophils in the airway and is responsible for pulmonary injury while TIMP-1 is responsible for airway remodeling and failure to repair. MMP-9 and its cognitive inhibitor TIMP-1 may play a significant role in the process of lung fibrosis resulting from different etiologies.

### Limitations

Small sample size and variable causes of pulmonary fibrosis.

### Conflict of interest

No conflict of interest.

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