

## CATHEPSIN-K IS A SENSITIVE IMMUNOHISTOCHEMICAL MARKER FOR DETECTION OF MICRO-GRANULOMAS IN HYPERSENSITIVITY PNEUMONITIS

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**ABSTRACT.** Hypersensitivity Pneumonitis (HP) is an interstitial lung disease that occurs upon exposure to a variety of inhaled organic antigens. The presence of small non-caseating granulomas and isolated giant cells is not specific, but is considered a relevant histological feature for HP. The detection of granulomas is widely considered as easy on standard histological stains, but microgranuloma detection can be difficult and/or time consuming, especially in chronic HP cases. Cathepsin K (Cath-K) is a potent cysteine protease expressed at high levels in activated macrophages (osteoclasts, and epithelioid cells in granulomas), but is not expressed in resident macrophages thus representing a promising marker to rapidly detect and quantitatively evaluate microgranulomas in interstitial lung diseases. We analyzed the expression of Cath-K by immunohistochemistry in 22 subacute and chronic HP cases, using semi-quantitative scores. Control samples included normal lung tissue, and a variety of interstitial lung diseases: 3 Wegener's granulomatosis, 3 sarcoidosis, 3 tuberculosis, 1 berylliosis, 20 idiopathic pulmonary fibrosis (IPF), 2 Langerhans' cell histiocytosis, 5 nonspecific-interstitial pneumonia (NSIP), 5 cryptogenic organising-pneumonia (COP), 2 Airway-Centered Interstitial Fibrosis (ACIF), 5 desquamative interstitial pneumonia (DIP), 3 respiratory bronchiolitis interstitial lung disease (RB-ILD). Intense expression of Cath-K was demonstrated in epithelioid and giant cells in all cases containing granulomas (HP, sarcoidosis, Wegener's granulomatosis, berylliosis, tuberculosis). Among HP cases 19/22 (86,3%) contained granulomas that could be semiquantitatively evaluated. In all HP and control cases alveolar macrophages did not express Cath-K, including cases characterised by large collections of alveolar macrophages such as DIP and RB-ILD. *Conclusions:* Cath-K represents a sensitive and specific marker to detect and quantitate granulomatous reactions in interstitial lung diseases, and is particularly useful in chronic HP cases. (*Sarcoidosis Vasc Diffuse Lung Dis* 2010; 27: 57-63)

**KEY WORDS:** hypersensitivity pneumonitis, Cathepsin-K, granulomas, interstitial lung diseases

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### INTRODUCTION

Hypersensitivity Pneumonitis (HP), also referred as Extrinsic Allergic Alveolitis, is a diffuse interstitial granulomatous lung disease that occurs upon exposure to a large variety of inhaled organic antigens or, more rarely, to simple chemicals in different environmental settings (1, 2). HP is thought to involve a combination of immune complex, humoral, and T-cell-mediated immune reactions oc-

curing in the pulmonary microenvironment after sensitisation to the inhaled antigen (3-5) Although no gold-standard rules have been fully validated, the diagnosis of HP is mainly based on the contribution of various components including a careful anamnesis, physical examination, laboratory data, HRCT imaging, and histological examination (6-8).

HP can present as an acute, a subacute, or a chronic disease, although this distinction has been recently challenged (9). Usually, acute HP has an abrupt manifestation and the diagnosis is commonly based on clinical and anamnestic data. Instead, subacute and, more frequently, chronic HP have less typical symptoms, with variable clinical presentation, serology, and radiological images, and a lung biopsy is more often performed in these cases. A critical issue is the distinction of chronic HP and other interstitial pneumonias as non-specific interstitial pneumonia (NSIP) and idiopathic pulmonary fibrosis (IPF). In fact, when HP presents with severe parenchymal distortion and remodelling, its histology can mimic the usual interstitial pneumonia (UIP) pattern observed in IPF (7, 10-11). Although the prognosis of HP has been reported to be more severe when the UIP pattern is present, its precise distinction from a true IPF remains clinically relevant, since avoidance of antigen exposure and appropriate therapy may improve the progression of the disease (5, 11).

Histologically, HP is characterized by a variety of aspects, including the occurrence of interstitial non-caseating poorly formed epithelioid granulomas, bronchiolitis with various degrees of fibrosis and bronchiolar regeneration, peribronchiolar mononuclear cell infiltrates, foci of organizing pneumonia, and occasional clusters of endoalveolar foamy macrophages (1, 5-7, 10). Only rarely all these aspects are found together, and while a cellular chronic interstitial pneumonia with peribronchiolar accentuation should suggest the possibility of a HP, the finding of interstitial non-necrotizing granulomas is highly suggestive of the diagnosis (1, 6, 7, 11, 12). However, granulomas in HP are usually poorly formed and made of isolated giant cells or small aggregates of epithelioid cells (microgranulomas, less than 150  $\mu\text{m}$  in diameter), and their distinction from enlarged stromal cells or clusters of distorted alveolar macrophages is at times difficult, especially in small transbronchial biopsies, a type of sample that is increasingly utilised in the diagnostic flow chart of

interstitial lung diseases (13, 14). Immunohistochemistry can significantly help in recognising macrophages on tissue sections using CD68 or CD11c, but these markers can not be utilised to distinguish activated epithelioid cells from resident alveolar macrophages (15).

Cathepsin K (Cath-K) is a papain-like cysteine protease with high matrix (collagen, elastin)-degrading activity that plays an important role in osteoclast function and bone remodelling (16-18). The reactivity pattern of Cath-K in macrophages is unique among cathepsins, since cathepsin B and cathepsin L are expressed ubiquitously in CD68-positive tissue macrophages, epithelioid cells, and multinucleated giant cells, whereas Cath-K appears as specific for epithelioid and giant cells (18-21). In fact, Cath-K is not expressed by the large majority of non-stimulated resident macrophages in different human tissues (including alveolar macrophages), and has been recently described as a useful marker for microgranulomas in Crohn's disease (22).

In this study we have investigated the immunohistochemical expression of Cath-K in a series of chronic HP and other interstitial lung diseases in order to evaluate its potential diagnostic utility.

## METHODS

A series of 22 cases of HP were retrieved from the files of our Departments. The clinical, radiological and histological data of all cases were consistent with the diagnosis of HP, as defined by current criteria (8). Among the 22 retrieved cases 6 exhibited clinical, radiological and pathological criteria diagnostic of sub-acute HP, whereas 16 cases were diagnosed as chronic HP. Two cases with a clinical diagnosis of chronic HP were characterised by the complete set of histological features of the UIP pattern. All samples were surgical biopsies including at least two tissue fragments.

Control cases included 5 normal bone marrow biopsies obtained during staging protocol for lymphomas containing osteoclasts, and a variety of surgical biopsies: 3 normal lung tissue samples, 3 cases of pulmonary Wegener's granulomatosis, 3 sarcoidosis, 3 tuberculosis, 1 berylliosis, 20 cases of idiopathic pulmonary fibrosis (IPF/UIP), 1 alveolar haemorrhage, 2 Langerhans' cell histiocytosis, 5 nonspecific

interstitial pneumonia (NSIP), 5 organising pneumonia, 2 airway-centered interstitial fibrosis (ACIF), 5 desquamative interstitial pneumonia (DIP), and 3 respiratory bronchiolitis interstitial lung disease (RB-ILD).

#### *Immunohistochemical staining and antibodies*

Sections of HP and control cases were immunostained with two different mouse monoclonal antibodies recognizing human Cath-K (clone CK4 Novocastra, Newcastle - UK, and clone 3F9 Abcam, Cambridge, UK). Heat-induced antigen retrieval was performed using a microwave oven and 0.01 mol/L citrate buffer, pH 8.0, for 30 minutes. All samples were processed using a sensitive "Bond polymer Refine" detection system in an automated Bond immunostainer (Menarini Diagnostics, Florence, Italy). Sections incubated without the primary antibody served as a negative control.

#### *Immunohistochemical evaluation*

Epithelioid granulomas in HP cases were semi-quantitatively evaluated at scanning magnification

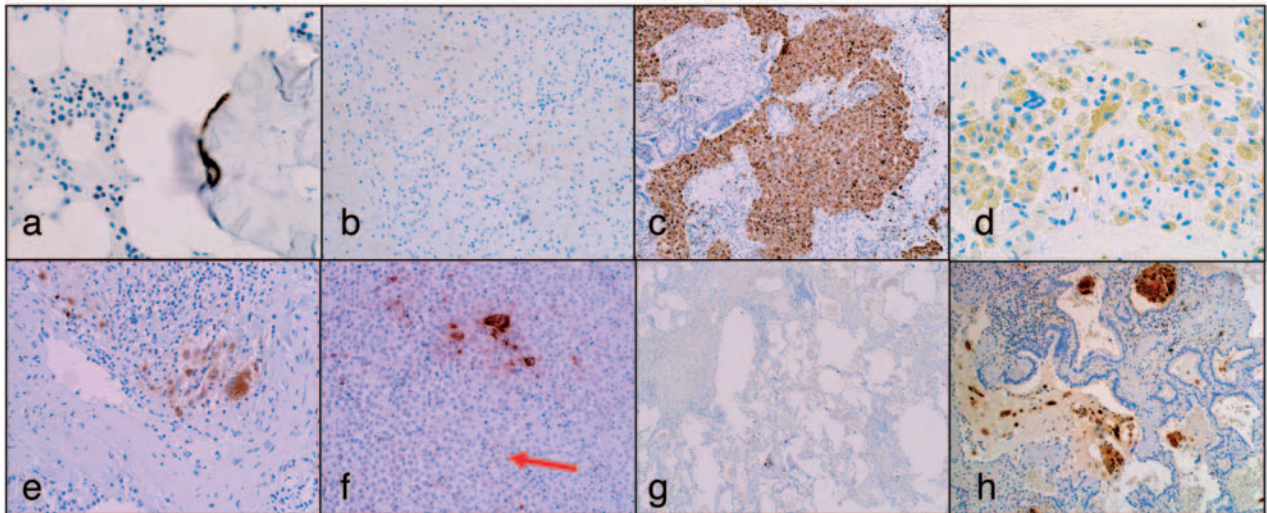
on Cath-K immunohistochemical preparations using the following scoring system: 0: no immunoreactivity; 1+: scattered small granulomas; 2+: numerous small granulomas; 3+: large granulomas.

#### RESULTS

The immunoreactivity in control and HP tissue samples was identical using both antibodies specific for Cath-K (clone CK4, Novocastra, Newcastle, UK and clone 3F9, Abcam, Cambridge, UK).

**Controls:** strong cytoplasmic Cath-K expression was evidenced in osteoclasts scattered along bone trabecules in all bone marrow biopsies (figure 1a). All interstitial bone marrow macrophages were completely negative for Cath-K, as were all types of cells within the haemopoietic microenvironment.

In normal lung tissue no immunoreactivity was observed with the exception of faint granular reactivity in rare alveolar macrophages. Cath-K immunoreactivity was also absent in alveolar collections of macrophages observed in smoking-related diseases (RB-ILD, and DIP) (figure 1b and c), as well as in iron-containing pigmented macrophages oc-



**Fig. 1.** Control samples of interstitial diseases.

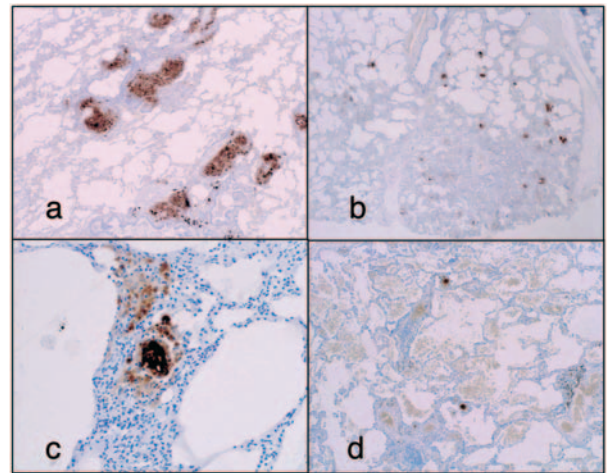
Intense Cathepsin-K (Cath-K) expression is evidenced in an osteoclast on a bone marrow trabecule (a 250x). Cath-K (b 100x) is not expressed in CD68 positive (c 100x) macrophages filling alveolar spaces in a case of desquamative interstitial pneumonia (DIP). Lack of Cath-K immunoreactivity in iron-containing pigmented macrophages occurring in alveolar haemorrhage (d 250x). Strong expression of Cath-K in epithelioid and giant cells occurring in Wegener's granulomatosis (e 100x). In Langerhans' cell histiocytosis Langerhans' cells are negative (arrow), whereas scattered epithelioid and giant cells are Cath-K positive (f 100x). Absence of Cath-K expression epithelioid reaction in a case of airway centered interstitial fibrosis (ACIF) (g 40x). Intraluminal Cath-K expressing muciphages and occasional giant cells in a case of UIP (h 100x).

curing in alveolar haemorrhage (figure 1d). In all investigated pulmonary samples from granulomatous diseases including sarcoidosis, berylliosis, tuberculosis, and Wegener's granulomatosis (figure 1e), strong Cath-K expression was demonstrated in epithelioid cells and giant cells in all recognisable granulomas. In all sarcoidosis samples granulomas were large and a 3+ score could be assigned. Langerhans' cells in pulmonary Langerhans' cell histiocytosis were Cath-K negative (figure 1f).

No epithelioid granulomas were detected either by morphology or Cath-K staining in all other pulmonary diseases including alveolar haemorrhage, NSIP, OP and the 2 cases of air-centered interstitial fibrosis (ACIF, figures 1d, 1g). In all these samples demonstration of the absence of granulomatous reaction could be reliably obtained on H&E preparations only after a careful and time consuming scrutiny, whereas the same goal could be easily obtained in a few seconds on Cath-K immunostained sections at low magnification (figure 1).

In 14 of 20 UIP samples clinically confirmed as IPF a variable number of Cath-K expressing macrophages and giant cells were observed within distorted lumens containing mucus within enlarged bronchioles and honeycomb lesions (figure 1h), but no granulomas could be observed in interstitial location. Low levels of granular Cath-K immunoreactivity was observed in myofibroblast clusters in both UIP (fibroblast foci, figure 3f) and organising pneumonia (endoalveolar Masson's bodies) samples as previously described (23), but their distinction from granulomas was not a problem in any sample.

In 19/22 HP cases (86,3%) variable numbers of small epithelioid non caseating granulomas could be easily recognised and quantitatively evaluated on Cath-K preparations. According to the scoring system described in the Methods, score 3+ was observed in 3/22 cases (13,6%), score 2+ in 3/22 cases (13,6%), and score 1+ in 13/22 cases (59%) (figure 2). In only 3/22 HP cases (13,6,3%) granulomas were absent as demonstrated by complete Cath-K negativity. In 2 cases of chronic HP confirmed by anamnestic and serologic data an UIP-like pattern was present. At morphological scrutiny the presence of epithelioid granulomas was not reported in these cases, whereas in both cases scattered microgranulomas could be demonstrated by Cath-K immunohistochemistry (figure 3).

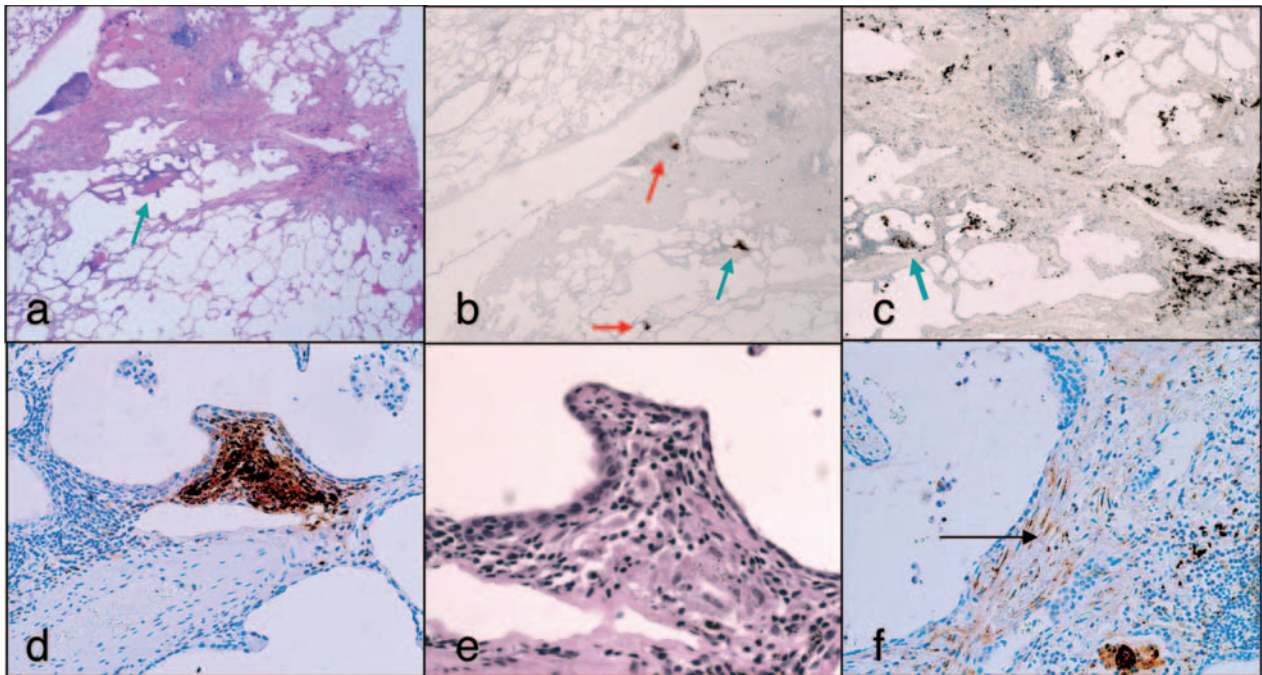


**Fig. 2.** Hypersensitivity pneumonitis: semiquantitative evaluation of granulomatous involvement in HP using Cath-K immunoreactivity.

The 3+ score as defined by the presence of numerous Cath-K non-necrotising epithelioid granulomas in a case of subacute HP (a 100x). The 2+ score was assigned to cases with numerous small Cath-K expressing granulomas (b 40x), and (c 250x) at higher magnification. The 1+ score was assigned to cases with rare small granulomas (d 40x). In all cases Cath-K+ macrophages were localised within the interstitial spaces.

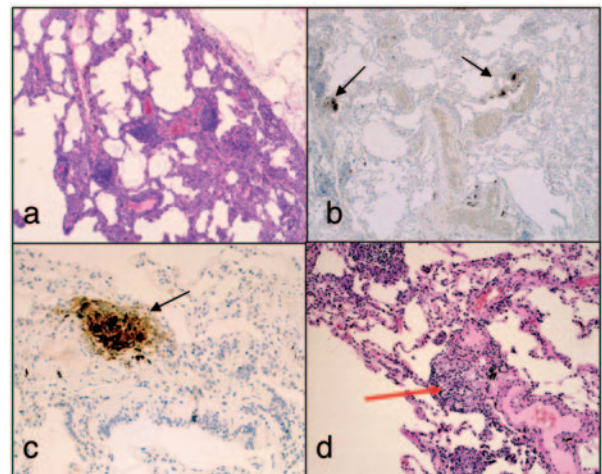
## DISCUSSION

In this paper we describe the immunohistochemical reactivity pattern of Cath-K in a variety of interstitial lung diseases, and suggest that this marker can significantly improve the sensitivity of detection of small granulomas in diseases where this finding is diagnostically relevant, as in chronic HP. In fact, in most cases of chronic HP granulomas are small, poorly formed and/or rare, and can be easily missed at morphological analysis on H&E preparations, especially at low magnification. In other granulomatous diseases, as in sarcoidosis, the granulomas are usually large and well formed, and can be easily evidenced at morphology. The specificity is also improved, since Cath-K is expressed at high levels in all granulomas, whereas alveolar macrophages are mostly Cath-K negative, also when accumulate within alveolar spaces following a variety of stimulation such as cigarette smoke (RB-ILD and DIP), or accumulation of substances such as iron (alveolar haemorrhage), or lipids (foamy macrophages). This heterogeneity is likely related to different molecular pathways triggered upon activation of macrophages



**Fig. 3.** A case of chronic hypersensitivity pneumonitis showing features of the UIP (usual interstitial pneumonia) pattern (a, 40x H&E stain). Scattered small granulomas (score 2+) are specifically evidenced by Cath-K immunostaining (b, 40x arrows), whereas CD68 on a serial section (c 40x) stains all the macrophages, so that the recognition of small granulomas using this marker is difficult (the same small granuloma is evidenced by the green arrows in b and c). Morphologically, the same granuloma shown at higher magnification in d (250x, Cath-K immunostaining), appears as a loose collection of epithelioid cells that can be easily neglected at routine analysis (e, H&E, 250x). Discrete Cath-K expression is demonstrated in a fibroblast focus (f, arrow, 100x)

(24). In diagnostic practice the high specificity of Cath-K can be of value in distinguishing small collections of true epithelioid cells from aggregates of alveolar macrophages, especially when only small or crushed samples are available (e.g. in transbronchial biopsies). Although well formed granulomas can be easily evidenced at morphology, the recognition of microgranulomas can be at time difficult or time consuming, necessitating careful scrutiny at high magnification. The reliable and sensitive demonstration of granulomatous reaction can particularly help in cases of chronic HP that represent the cases where most lung biopsies in HP are performed (5, 6, 8, 9, 11). In fact, chronic HP can be difficult to diagnose, and problems are related to the slow and progressive evolution of the illness, and to the lack of typical clinical and laboratory data. When the history, the clinical and radiological aspects are suggestive of HP, but the antigen exposition is difficult to suspect and/or demonstrate, the diagnosis of HP can be strengthened only after a lung biopsy (1, 2, 8, 11). Moreover, early diagnosis of a chronic phase of HP



**Fig. 4.** A case of chronic hypersensitivity pneumonitis characterised by heterogenous morphology including areas exhibiting a cellular NSIP pattern (a, H&E, 40x). A few small granulomas (score 1+) could be easily demonstrated in this case at low magnification in 3 out of 4 different tissue portions using Cath-K immunostaining (b 40x, c 250x, arrows). Microgranulomas on serial H&E sections could be found only after a careful and time-consuming examination at high magnification due to the confounding presence of abundant diffuse and nodular lymphoid infiltrate (d, H&E, 250x).

is critical, since irreversible or progressive disease can occur (25, 26).

Among the widely recognised histological features of HP, the occurrence of non-caseating, poorly formed granulomas, although not fully specific, is considered as particularly relevant, but the occurrence of granulomas can be morphologically demonstrated only in two thirds of HP cases (7, 11, 27). In our series granulomas could be detected with the aid of Cath-K immunohistochemistry in 19/22 (86,3%) HP cases. The specificity of other histological features of HP, including centrilobular accentuation of the inflammatory changes and occurrence of organising pneumonia is low, and only rarely all these diagnostic aspects are found together in subacute and chronic HP. In addition, a variety of other presentations have been described in HP including the NSIP and UIP patterns (10, 11, 25-27).

According to our data, when granulomas in HP cases were small or distorted could be better appreciated by immunohistochemistry than using morphology on H&E preparations (figure 3 and 4). Interestingly, the vast majority of HP cases were evaluated as score 1+, that is to say that they contained "scattered, small granulomas", confirming that granulomas in HP are frequently poorly small and difficult to be found by morphology. In some differential diagnosis the use of Cath-K can be particularly relevant, as in the distinction between chronic HP and the spectrum of lung diseases variably named as airway-centered interstitial fibrosis (ACIF), or idiopathic bronchiolocentric interstitial pneumonia (IBIP)(28,29), that share with HP different features such as the centrilobular fibrosis and bronchiolar metaplasia. The boundaries between ACIF/IBIP and HP have not been completely defined, and the precise evaluation of granulomatous reaction can represent an important element for their characterisation, and also for a better understanding of these newly described lung diseases (30).

Some HP histological presentations can closely mimic IPF, especially in advanced chronic cases (5, 10, 11, 31). In our series, both cases of HP with UIP-like pattern contained a few small interstitial granulomas that could be clearly evidenced by Cath-K immunostaining (figure 2). Interestingly, in a large proportion of IPF cases (14/20) some Cath-K expressing macrophages and isolated giant cells were present within enlarged bronchiolar lumens with

features of microscopic honeycombing (figure 1h). The localisation of these activated macrophages within structures containing inspissated mucus suggests that in IPF activation of macrophages occurs, but it is mainly related to their specialised function as muciphages, as also observed in other tissues (22, 32).

According to our study, Cath-K is not expressed in normal lung, but is over-produced in granulomatous diseases where this enzyme could have pathogenic relevance. In fact, Cath-K is a papain-like cysteine protease with high matrix (collagen, elastin) degrading activity, and is the most active human elastase so far described (16, 18). Cath-K is involved in various aspects of extracellular matrix turnover and collagen degradation, and plays an important role in several physiologic and pathologic conditions including osteoclast function and bone remodelling, cartilage destruction, scar formation, and also lung fibrosis and emphysema (16, 18, 19, 20, 29, 32-37). Further studies are needed to reveal the possible pathogenic role of this potent matrix-degrading enzymes in parenchymal abnormal remodelling occurring in pulmonary granulomatous diseases including HP.

In conclusion, Cath-K can represent a useful adjunct in histological practice to rapidly detect and quantitate minimal granulomatous reaction in interstitial lung diseases, and is particularly useful to either demonstrate or exclude the presence of microgranulomas in suspected cases of chronic HP cases. Further studies are needed to evaluate its pathogenic role in granulomatous diseases and to assess if it can be a target for pharmacologic manipulation.

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