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Elevated MMP-12 protein levels in induced sputum from COPD patients.

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Abstract

Background: Several Matrix Metalloproteinases (MMP's) are involved in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD). In mice, MMP-12 plays a crucial role in the development of cigarette smoke induced emphysema. The main purpose of this study was to investigate the role of MMP-12 in the development of COPD in human smokers.

Methods: Induced sputum samples were collected from stable COPD patients (n = 28), healthy smokers (n = 14), never smokers (n = 20) and former smokers (n = 14). MMP-12 protein levels in induced sputum were determined by ELISA and compared between the four study groups. MMP-12 enzymatic activity in induced sputum was evaluated by casein zymography and by cleaving of a fluorescence-quenched substrate.

Results: Median (IQR) MMP-12 levels were significantly higher in COPD patients when compared to healthy smokers, never smokers and former smokers (17.5 (7.1-42.1) v 6.7 (3.9-10.4) v 4.2 (2.4-11.3) v 6.1 (4.5-7.6) ng/ml, p = 0.0002). MMP-12 enzymatic activity was significantly higher in COPD patients compared to controls (4.11 (1.4-8.0) v 0.14 (0.1-0.2) µg/µl respectively, p = 0.0002).

Conclusion: MMP-12 is markedly increased in induced sputum from stable COPD patients when compared to controls, suggesting a role for MMP-12 in the development of COPD in smokers.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major health problem and it is predicted to be the 3rd most common cause of death worldwide by 2020.[1] The disease is mainly caused by cigarette smoking, which leads to chronic inflammation of the airways and parenchymal destruction resulting in emphysema. The destruction of lung tissue is caused by an imbalance between protease and anti-protease activity, where the excess of proteolytic enzymes such as matrix metalloproteinases (MMP's), cysteine and serine proteinases is insufficiently counterbalanced by a rise of anti-proteolytic molecules.[2]

Matrix metalloproteinases are a family of metalloproteases that contain a zinc atom at their active site and are able to degrade matrix macromolecules, including collagen, laminin and elastin.[3] In addition to their ability to degrade extracellular matrix components, some MMP's also cleave cytokines[4] and anti-proteolytic molecules.[5][6][7]

The knowledge on the functional activity of MMP's and the fact that MMP's such as MMP-9 and MMP-12 were demonstrated in alveolar macrophages[8][9] led to the hypothesis that MMP's could play a role in the destruction of lung parenchyma induced by cigarette smoke. More specifically, there is convincing evidence from animal models that MMP-12 is crucial in the development of smoke-induced emphysema. MMP-12 KO mice were completely protected from development of emphysema despite long term smoke exposure, while wild-type mice developed alveolar space enlargement.[10] In addition, MMP-12 KO mice had impaired recruitment of monocytes into the lung, possibly due to the loss of MMP-12 mediated generation of elastin fragments (chemotactic for monocytes).

The contribution of MMP-12 to smoke induced emphysema is probably not limited to a direct effect (destruction of extracellular matrix), but is most certain enhanced by indirect effects such as inactivation of α1- antitrypsin by MMP-12[11] and MMP-12 mediated recruitment of neutrophils (which in their turn are able to release proteolytic enzymes) to the lung in response to cigarette smoke.[4]

Altogether, these data suggest a pivotal role for MMP-12 in the development of smoke-induced emphysema in mice. However, in human subjects, the role of MMP-12 in the development of COPD is still unclear. Very few studies investigated the involvement of MMP-12 in cigarette smoke induced lung damage in human subjects, partly because of the lack of appropriate diagnostic tools to detect the MMP-12 protein in sputum, bronchoalveolar lavage (BAL) or lung tissue. One group described an association of polymorphisms in the MMP-12 gene with the rate of decline of lung function in smokers, which suggests a role for MMP-12 in the pathogenesis of COPD.[12] Recently, Molet and colleagues described elevated MMP-12 levels in BAL fluid (demonstrated by Western blot analysis) and BAL cells, as well as in bronchial biopsies of COPD patients when compared to controls.[13]

To explore the contribution of MMP-12 to the development of COPD in smokers, we collected induced sputum samples from never smokers, healthy smokers (smokers without airway obstruction), former smokers (quit smoking for more than one year) and stable COPD patients and compared the MMP-12 levels in induced sputum between these different groups.

METHODS

Subjects

In total, 76 subjects participated in the study and were classified in 4 different groups: COPD stage I (mild)-II (moderate) patients according to the GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria (www.goldcopd.com), never-smokers, healthy smokers and former smokers (subjects who quit smoking for more than a year, with a history of at least ten pack year). Patients were recruited from our outpatient pulmonary clinic, while controls were recruited by advertising as well as from the outpatient clinic. All patients with COPD had stable disease; patients with symptoms or clinical signs of a COPD exacerbation in the preceding 2 months were excluded. For safety reasons, patients suffering from severe and very severe COPD (GOLD stage III-IV) were excluded, as well as subjects with asthma, bronchiectasis or a respiratory infection in the preceding 4 weeks. Written informed consent was obtained from all subjects according to protocols approved by the Medical Ethical Committee of the Ghent University Hospital.

Sputum induction and processing

Sputum induction was performed as described previously[14] by inhalation of sterile, pyrogen-free, hypertonic saline at increasing concentrations (NaCl 3, 4, and 5% respectively) during 5 minutes, after the inhalation of salbutamol (2 x 200 µg). The hypertonic saline was nebulized via an ultrasonic nebulizer (Ultra-neb; Devilbiss Health Care Inc, Somerset, PA, USA). Each time after the inhalation of a specific saline concentration, subjects were encouraged to cough and expectorate an adequate sample. Spirometric values were obtained before and after induction by means of a watersealed spirometer (Vmax 20C , Sensormedics, Yorba Linda, CA, USA). Processing of induced sputum was performed as previously described.[15] To reduce salivary contamination, sputum plugs were selected and transferred in a polystyrene tube. Freshly prepared 0.1% solution of dithiotreitol (DTT; 10% Sputalysin, Boehringer-Calbiochem Corp, San Diego, CA, USA) was added in an amount equal to 4 times the weight of the sputum plugs. The tube was vortex mixed during 30 sec. and the sputum was incubated on a tube rocker for 15 min. at room temperature. An amount of phosphate buffered saline (PBS) equal to the DTT volume was then added and the sample was incubated for 5 more min. Next, the sample was filtered through a 70 µm cell strainer (Becton Dickinson Labware, NJ, USA) and centrifuged at 390g for 10 min. Supernatant was aspirated, aliquoted and stored at –80°C. The cell pellet was resuspended in PBS supplemented with 2% human serum albumin (Behring Diagnostics, San Jose, CA, USA) ; cells were counted in a haemocytometer and the cell concentration was adjusted to 0.7 x 10^6 cells/ ml. Cytospins were prepared by adding 60 µl of cell suspension into Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA, USA) and spun for 5 min. at 300 rpm. Two slides were stained with May-Grünwald-Giemsa for an overall count of leukocytes, bronchial epithelial cells and squamous cells. Slides were counted blind by two investigators. For cell differentiation, 400 nucleated cells per slide were counted and expressed as percentage of intact round nucleated cells, excluding squamous epithelial cells.

Generation of antibodies against MMP-12

MMP-12 protein was detected in induced sputum supernatant using an enzyme-linked immunosorbent assay developed in cooperation with industrial partners (Immunotech, Marseille, France and Schering-Plough LIR, Dardilly, France). Antibodies were generated as follows: the full length MMP-12 cDNA was cloned from a cDNA library constructed from CD34-derived dendritic cells. The clone was entirely sequenced and shown to correspond to the published sequence.[9] The MMP-12 cDNA was cloned in PME18S vector that was

electroporated in COS7 cells. Enriched supernatant was used to immunize Balb/C mice. Hybridomas were obtained as previously described[16], and screened by immunostaining of COS7 transfected cells. 23 hybridomas were obtained and their specificity was confirmed by Western Blot and by immunoprecipitation of radiolabelled recombinant MMP-12 (fig 1).

Development of ELISA for MMP-12 detection

Selection for ELISA was performed by testing the 23 antibodies obtained. The most sensitive ELISA used mAb 701E4.03 for capture and mAb 706F9.01 for detection of MMP-12. Recombinant MMP-12 was immunopurified with a third mAb, 603.E6.

Recombinant human MMP-12 with known concentrations was used as standard. MMP-12 in sputum supernatant was quantified by converting the optical density values of the samples to nanograms from the standard curve obtained with recombinant MMP-12. Sensitivity of the ELISA was less than 50 pg/ml. Specificity testing showed that there was no detection of other related MMPs (fig 2), including MMP-1, MMP-3 and MMP-9 (R&D systems, Abingdon, UK), with the ELISA for MMP-12 (kindly provided by Immunotech). Mean intra-batch co-efficient of variation of the ELISA was less than 6%.

Since the use of dithiotreitol (DTT) in sputum processing could possibly interfere with the detection of MMP-12 in sputum samples, we compared MMP-12 levels in sputum samples treated with or without DTT. Sputum samples were each time divided in two aliquots: one aliquot was treated with DTT (concentration matching the concentration used in the standard procedure as described above), while PBS was added to the other aliquot instead of DTT. Next, both samples were centrifuged (50.000g, 4°C, 90 min) in an ultracentrifuge (Ultracentrifuge Floor Model L5-65, Beckman Coulter, Fullerton, CA, USA) and sputum supernatant was stored at –80°C. Levels of MMP-12 as measured by ELISA were compared between the two aliquots. There was no difference in MMP-12 levels between these two methods ($n = 13$, $p = 0.89$). Moreover, there was a strong positive correlation ($r_s = 0.99$; $p = 0{,}000001$) between MMP-12 levels in both aliquots from the same sample (fig 3), so we concluded that there is no influence of DTT on the detection of MMP-12 in sputum samples.

Zymography

We performed casein zymography based on a previously described technique with slight modifications[17]. Ten µl of sputum supernatant were mixed with the same amount of non-reducing sample buffer. Electrophoresis was carried out on a Sodium dodecyl sulfate (SDS)-10 % polyacrylamide gel containing α-casein (Sigma) at a concentration of 1 mg/ml. Gels were then incubated in 2.5% (v/v) Triton X-100 for 30 min and soaked 16 hours in a buffer containing 10 mM CaCl2 and 100 mM NaCl at 37°C. Afterwards, the gels were stained with Coomassie brillant blue G250. Caseinolytic activity was detected as white lysis zones against a blue background. Quantitative evaluation of the gelatinolytic activity was performed by scanning the gel using a Bio-Rad multiImager imaging densitometer. We used recombinant MMP-12 (R&D systems, Abingdon, UK) in order to confirm the validity of the procedure and used bands obtained with rMMP-12 as standards. Caseinolytic activity was determined by scanning the lysis band in the area corresponding to rMMP-12.

MMP-12 activity assessed by fluorescence-quenched substrate cleaving

In addition to casein zymography, MMP-12 specific enzymatic activity was analysed by using a technique based on EnzoLyte^{TM}490 Kit (AnaSpec, San Jose, CA) slightly modified. This kit is optimized to detect the activity of MMP-12 in biological samples using a fluorescence-quenched substrate (EDANS/DabcylPlusTM FRET peptide). Upon cleavage into two separate fragments by MMPs, the fluorescence of this peptide is recovered, and can be monitored at excitation/emission wavelengths= 340nm/490nm. Sputum samples were incubated with 1mM

APMA (4-aminophenylmercuric acetate) during 1 hour in order to activate MMP-12. 50 µl of sample were incubated in a 96 well plate. The plate was incubated at room temperature for 45 minutes and protected from light. Stop solution from Anaspec (50µl) was added in each well and the fluorescence intensity was measured at 490nm after excitation at 340nm. MMP-12 purified enzyme used as positive control was purchased from Sigma (Saint Louis, Missouri).

Analysis of data

Statistical analysis was performed with SPSS version 12.0 for Windows. Data are presented as median (IQR) and statistical analysis was performed using non-parametric testing with Kruskal-Wallis tests. Significant differences between groups were further investigated with Mann Whitney U testing. Spearman's rank correlation was calculated to assess the correlation between MMP-12 level, lung function parameters and cell differential counts in induced sputum of study subjects. Statistical significance was defined as $p<0.05$.

RESULTS

Subject Characteristics

Twenty-eight patients with stable COPD (no exacerbation in the 2 preceding months) were included in the study (table 1). Four of them were classified as COPD GOLD stage I, while twenty-four patients had a disease severity of GOLD stage II. Seventeen of the COPD patients took inhaled steroids, while eleven of them did not. Fourteen COPD patients were active smokers, whereas fourteen COPD patients had quit smoking for more than one year. Twenty never smokers, fourteen healthy smokers and fourteen former smokers were studied in the control groups. All healthy subjects had normal spirometric values. The characteristics of the four study groups are summarised in table1. FEV_1 (Forced Expiratory Volume in one second) and FEV_1/FVC ratio were significantly lower in patients with COPD than in control groups.

Table 1

Table 1 Subject Characteristics

	COPD	Never Smokers	Smokers	Former Smokers
n	28	20	14	14
Sex ratio (M/F)	24/4	4/16	7/7	7/7
Age (years)	64.0 (52-72)*†‡	52.5 (42-56)	51.0 (49-53)	52.5 (46-63)
Smoking history (pack year)	42.0 (26-55)‡§	0.0 (0-0)	35.0 (16-43)	30.0 (9-42)
FEV_1 (L)	2.0 (1.7-2.3)¶	3.0 (2.7-3.4)	3.0 (2.5-3.3)	3.4 (2.7-3.7)
FEV_1 (% pred)	70.0 (63-78)¶	105.5 (97-118)	96.0 (91-101)	108.0 (100-113)
FEV_1/FVC (%)	56.0 (49-65)¶	80.5 (77-83)	78.0 (73-82)	78.5 (75-80)
bronchodilators (Y/N)	20/8	0/20	0/14	0/14
ICS (Y/N)	17/11	0/20	0/14	0/14

Data are presented as median (IQR). *p<0.01 v never smokers, †p<0.001 v smokers, ‡p<0.05 v former smokers, §p<0.001 v never smokers, ¶p< 0.001 v never smokers, smokers and former smokers. ICS= inhaled corticosteroids.

Differential cell counts of induced sputum

Total and differential cell counts of induced sputum are shown in table 2. The total inflammatory cell number in induced sputum was significantly higher in COPD patients compared to smokers without airway obstruction. The percentage of macrophages was lower in sputum of COPD patients compared to never smokers and active smokers, while absolute numbers of macrophages in induced sputum were not significantly different between groups. Induced sputum from COPD patients contained significantly more neutrophils than induced sputum from never smokers and active smokers (table 2).

Table 2

Table 2 Total and differential cell counts in induced sputum

	COPD	Never Smokers	Smokers	Former Smokers
Total inflammatory cells (x 10^3/mg)	14.0 (8-21)*	6.5 (5-15)	5.6 (4-8)	10.8 (8-18)*
Macrophages (%)	34.8 (18-43)*†	50.4 (38-68)‡	52.3 (30-66)	36.6 (24-52)
Macrophages (x 10^3/mg)	4.0 (2.1-6.4)	3.9 (2.9-5.1)	2.3 (1.8-3.5)	4.2 (2.6-5.6)
Neutrophils (%)	61.5 (56-75)*†	48.0 (27-59)‡	46.1 (32-66)	61.1 (47-75)
Neutrophils (x 10^3/mg)	9.1 (4.5-15.0)§¶	3.3 (1.0-9.8)	2.3 (1.5-4.5)‡	6.3 (3.9-13.0)
Eosinophils (%)	1.5 (0.5-3.1)†	0.2 (0.0-0.7)	1.0 (0.6-1.6)†	0.5 (0.0-1.8)
Eosinophils (x 10^3/mg)	0.1 (0.0-0.6)†	0.0 (0.0-0.1)*	0.1 (0.0-0.1)	0.1 (0.0-0.1)
Lymphocytes (%)	0.5 (0.2-1.3)†	1.4 (0.9-2.9)‡	0.2 (0.2-0.9)†	0.6 (0.3-1.4)
Lymphocytes (x 10^3/mg)	0.1 (0.0-0.2)	0.1 (0.1-0.2)	0.0 (0.0-0.0)†	0.1 (0.0-0.1)*
n	28	20	14	14

Data are presented as median (IQR). *p<0.05 v smokers, †p<0.01 v never smokers, ‡p<0.05 v former smokers, §p<0.05 v never smokers, ¶p< 0.01 v smokers

MMP-12 protein in induced sputum

MMP-12 protein could be detected by ELISA in all induced sputum samples (fig 4). COPD patients had significantly higher levels of MMP-12 in induced sputum (median 17.5 ng/ml (7.1-42.1)) when compared to never-smokers (4.2 ng/ml (2.4-11.3)), "healthy" smokers (6.7 ng/ml (3.9-10.4)) and former smokers (6.1 ng/ml (4.5-7.6)). There was no significant difference in MMP-12 level between the three control groups. MMP-12 was not significantly different (p = 0.87) in induced sputum from COPD patients who were active smokers (16.1 ng/ml (5.8-47.2)) compared to COPD patients who had quit smoking (18.2 ng/ml (10.1-38.9)). There was no difference (p = 0.21) in the level of MMP-12 protein in sputum from COPD patients who were taking inhaled corticosteroids (14.8 ng/ml (6.8-28.2)) compared to COPD patients who were not on inhaled corticosteroids (25.8 ng/ml (15.4-72.8)).

Zymography

To evaluate MMP-12 enzymatic activity, a number of sputum supernatants samples were analysed by casein zymography (7 COPD patients, 5 healthy smokers). As shown in figure 5a, functionally active MMP-12 could be detected in most samples from COPD patients, while this was not the case for samples from healthy smokers. The relative degree of enzymatic activity was calculated as described in the Materials and Methods section (fig 5b). There was a strong positive correlation between the amount of MMP-12 detected by the ELISA and the degree of enzymatic activity as evaluated by casein zymography ($r_s = 0.9$; $p = 0.01$).

MMP-12 activity assessed by fluorescence-quenched substrate cleaving

In addition to casein zymography, MMP-12 specific enzymatic activity was analysed by cleavage of a fluorescence-quenched substrate. For this purpose, ten samples from COPD patients were analysed and compared to ten control samples (five smokers, three never smokers and two former smokers). MMP-12 specific activity was significantly higher in COPD patients compared to controls (4.11 (1.4-8.0) v 0.14 (0.1-0.2) µg/µl respectively, p = 0.0002). There was a strong positive correlation between the degree of MMP-12 enzymatic activity and the amount of MMP-12 detected by the ELISA ($r_s = 0.79$; $p = 0.00002$).

Correlations between sputum MMP-12 and clinical and inflammatory parameters

An inverse correlation was found between MMP-12 protein levels in induced sputum and the FEV_1/FVC ratio (r_s = -0.44, p = 0.00007) as well as the degree of airway obstruction (r_s = -0.38, p = 0.0008) as measured by FEV_1 % when analysed for all subjects (fig 6). However, when correlation analysis was performed in each group separately, no significant correlations were found between MMP-12 protein levels and spirometric measurements. This suggests that the observed correlation was a result from the differences between the groups, rather than representing a true association.

There was no correlation between MMP-12 levels and the absolute number of macrophages, lymphocytes or eosinophils, while there was a positive correlation between MMP-12 and the absolute neutrophil counts (r_s = 0.41, p = 0.0004) in induced sputum when analysed for all subjects (fig 7). However, when analysing each group separately, a significant correlation between MMP-12 protein level and absolute neutrophil number could no longer be found, except in the group of never smokers (r_s = 0.64, p = 0.002).

DISCUSSION

For the first time, MMP-12 could be detected at the protein level in induced sputum. This is in itself of major importance: while several data from animal models suggest a possible role for MMP-12 in the development of COPD, very few data are available on the role of MMP-12 in human lung diseases, mainly because of the lack of appropriate diagnostic tools.

The major finding of this study was that the level of MMP-12 in induced sputum was significantly higher in COPD patients than in control groups: never smokers, former smokers as well as "healthy" smokers have lower MMP-12 levels compared to mild to moderate COPD patients (GOLD stage I and II). These findings suggest that MMP-12 is involved (next to other proteolytic enzymes) in the development of COPD and confirms earlier findings from animal models.[10]

An interesting finding was the difference in MMP-12 level between COPD patients and "healthy" smokers. As mentioned before, MMP-12 was clearly elevated in COPD patients, while it was not in smokers without airway obstruction. In other words, it seems as if MMP-12 is not induced in all smokers but only in those with COPD.

Importantly, MMP-12 levels were not only increased in COPD patients who were active smokers, but also in COPD patients who had quit smoking. These data suggest that MMP-12 in sputum is not induced by cigarette smoking per se, but by the disease itself. However, while there was no statistically significant difference between COPD patients that have quit smoking versus active smoking COPD patients, the mean MMP-12 levels were higher in the latter group, suggesting an additional effect of cigarette smoking on MMP-12 levels in induced sputum from COPD patients. It is important to remember that only patients with mild to moderate COPD were included in this study, and patients with severe and very severe COPD (GOLD stage III and IV) were excluded for safety reasons. Thus, in those patients with early stages of COPD, MMP-12 could possibly be an important biomarker of the disease. However, there is some overlap between the lowest MMP-12 levels in COPD patients and the MMP-12 levels in controls. Therefore, measurement of MMP-12 in induced sputum as a screening test for COPD is probably not useful. However, future studies in larger groups of subjects are needed to address this question.

The mechanisms by which MMP-12 is induced in COPD patients remain unclear. Data from animal models suggest that a local deficiency in Transforming Growth Factor β1 (TGF-β1)[18], or a rise in interleukin-13 (IL-13)[19] or interferon-γ (IFN-γ)[20] leads to overproduction of macrophage MMP-12. Recently, Grumelli et al showed that in human

subjects, lung macrophages release MMP-12 in response to interferon-inducible protein 10 (IP-10) and monokine induced by interferon (MIG), two chemokines that are secreted by lung macrophages and lymphocytes from emphysema patients.[21]

Alveolar macrophages appear to be the principal source of MMP-12 in the lung.[9] [13] However, recent data from in vitro studies suggest that MMP-12 can also be released by human bronchial epithelial cells in response to cigarette smoke.[22][23] Moreover, in vitro studies show a strong upregulation of MMP-12 transcription in immature, monocyte derived dendritic cells (DC) during differentiation from monocytes into DC.[24] So, whereas macrophages are most probably the most important source of MMP-12, both bronchial epithelial cells and DC might contribute to the total amount of MMP-12 in human lung. In induced sputum, MMP-12 levels did not correlate with the absolute number of macrophages. However, as already suggested by other authors[4], it is probably macrophage activation rather than macrophage number that accounts for the increased total secretion of MMP-12.

Only very few data are available on the role of MMP-12 in COPD in human subjects. Montaño and colleagues studied MMP activity and expression in alveolar macrophages from patients with COPD. They found increased macrophage elastolytic activity in COPD patients and suggest that this enzymatic activity corresponds to MMP-12.[25] Recent work from Molet and colleagues[13] demonstrated enhanced MMP-12 expression in BAL and in bronchial biopsies from COPD patients compared to controls (a heterogeneous group of smokers and non-smokers). As mentioned earlier, Grumelli et al showed that IP-10 and MIG, released by lung lymphocytes, upregulated MMP-12 secretion by lung macrophages and that this is mediated by the CXCR3 chemokine receptor on macrophages. Moreover, they demonstrated by immunohistochemistry that lung macrophages from emphysema patients express MMP-12, while lung macrophages from healthy smokers (without emphysema) do not.[21]

Our results show for the first time increased MMP-12 protein levels and MMP-12 related enzymatic activity in induced sputum from COPD patients compared to smokers without obstructive airway disease, former smokers and never smokers. Altogether, these data provide increasing evidence that MMP-12 is involved in the development of COPD in human subjects, and thus confirm the earlier findings from animal models.

Moreover, it becomes clear that MMP-12 could be an interesting target for new pharmacological treatments for COPD. In mice, a potent synthetic inhibitor of both human and murine MMP-12 (RS-113456) prevented progression of emphysema in smoke exposed animals.[26] These promising preclinical results, however, need to be confirmed in well-designed clinical trials in human patients.

Figure Legends

Figure 1 Autoradiography of SDS gel electrophoresis without (lane 1) or with (lane 2) β-mercaptoethanol of MMP12-transfected COS7 cells supernatant radiolabeled with ^{35}S. Immunoprecipitation of COS7 supernatant with mAbs 701E4.03 (lane 3) and 706F9.01 (lane 4).

Figure 2 Specificity of the ELISA for MMP-12. Detection of a serial dilution of recombinant MMP-12 protein by the ELISA (■). There is no detection of recombinant MMP-1 (Δ), MMP-3 (∇), or MMP-9 (□) with the ELISA, even at high concentrations.

Figure 3 Effect of the use of dithiotreitol (DTT) on the detection of MMP-12 in sputum. Aliquots from the same sputum sample were processed with or without the use of DTT. There is a strong positive correlation (r_s = 0,99; p = 0,000001) between the MMP-12 levels in aliquots treated with DTT and the corresponding aliquots processed without DTT. In conclusion, there is no influence of DTT on the detection of MMP-12 in sputum samples.

Figure 4 Elevated MMP-12 protein levels in induced sputum from COPD patients (n = 28) compared to never smokers (n = 20), healthy smokers (n = 14) and former smokers (n = 14). Horizontal bars represent median values.

Figure 5 Detection of functionally active MMP-12 by casein zymography. Functionally active MMP-12 is present in sputum samples from 4 out of 5 COPD patients (Fig 5a, lane 1-5), while no functionally active MMP-12 was detected in sputum from healthy smokers (Fig 5a, lane 6-7). Relative enzymatic activity was highest in COPD patients (Fig 5b). Data shown are representative of 12 subjects (7 COPD patients, 5 healthy smokers). Black bars represent COPD patients, white bars represent healthy smokers.

Figure 6 Relation between MMP-12 protein levels in induced sputum and FEV_1/FVC ratio (A) and FEV_1 (% predicted) (B). A significant inverse correlation was found for both FEV_1/FVC ratio (r_s = -0.44, p = 0.00007) and FEV_1 % predicted (r_s = -0.38, p = 0.0008) for all subjects. ■ COPD patients (n = 28), ○ controls (healthy smokers, never smokers, former smokers; n = 48).

Figure 7 Relation between MMP-12 protein levels and absolute neutrophil number in induced sputum. A significant positive correlation (r_s = 0.41, p = 0.0004) was found between the absolute neutrophil number and MMP-12 in induced sputum for all subjects. ■ COPD patients (n = 28), ○ controls (healthy smokers, never smokers, former smokers; n = 48).

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Abbreviations

COPD: Chronic Obstructive Pulmonary Disease
BAL: Bronchoalveolar lavage
DTT: dithiotreitol
ECM: Extracellular Matrix
ELISA: Enzyme Linked Immunosorbent Assay
FEV_1: Forced Expiratory Volume in 1 second
FVC: Forced Vital Capacity
GOLD: Global Initiative for Chronic Obstructive Lung Disease
ICS: Inhaled Corticosteroids
IFN-γ: Interferon-γ
IL-13: Interleukin-13
KO: knock-out
MMP: Matrix Metalloproteinase
PBS: phosphate buffered saline
SD: Standard Deviation
TGF-β1: Transforming Growth Factor-β1
TNF-α: Tumor Necrosis Factor-α

Competing interest statement

No competing interest declared.

Copyright statement

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Figure 1

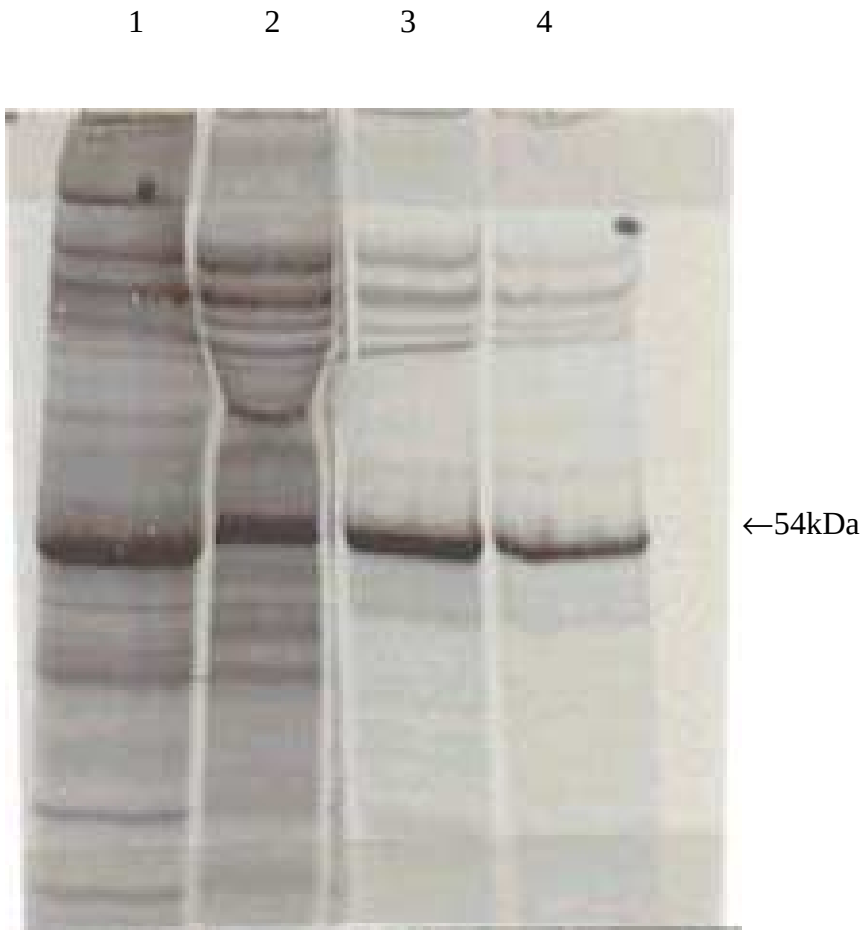


Figure 2

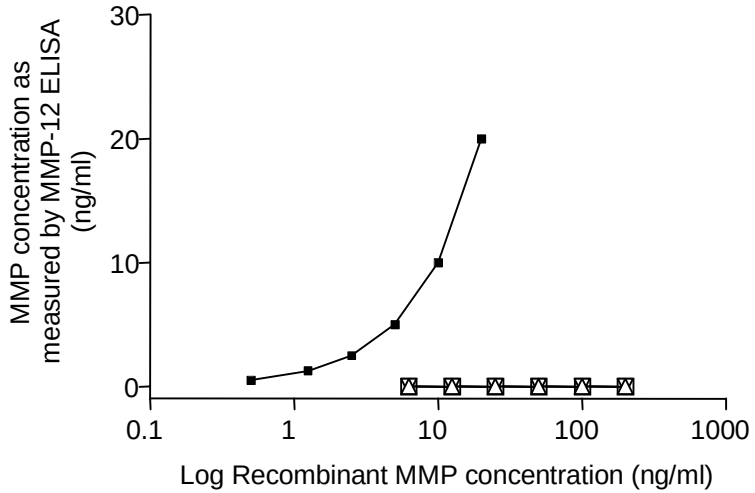
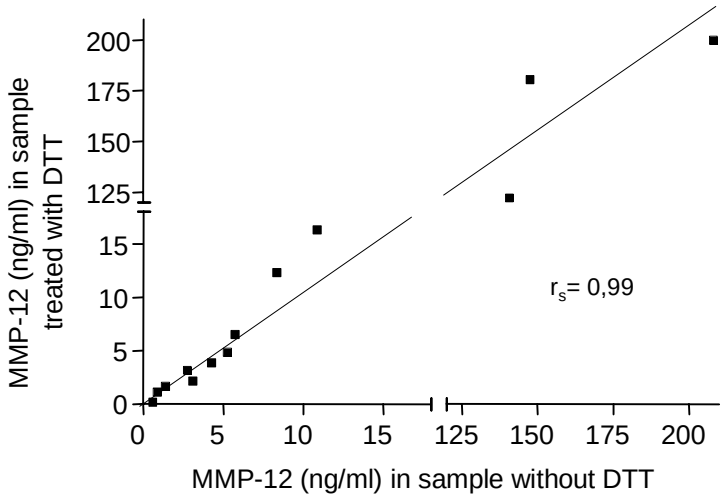


Figure 3



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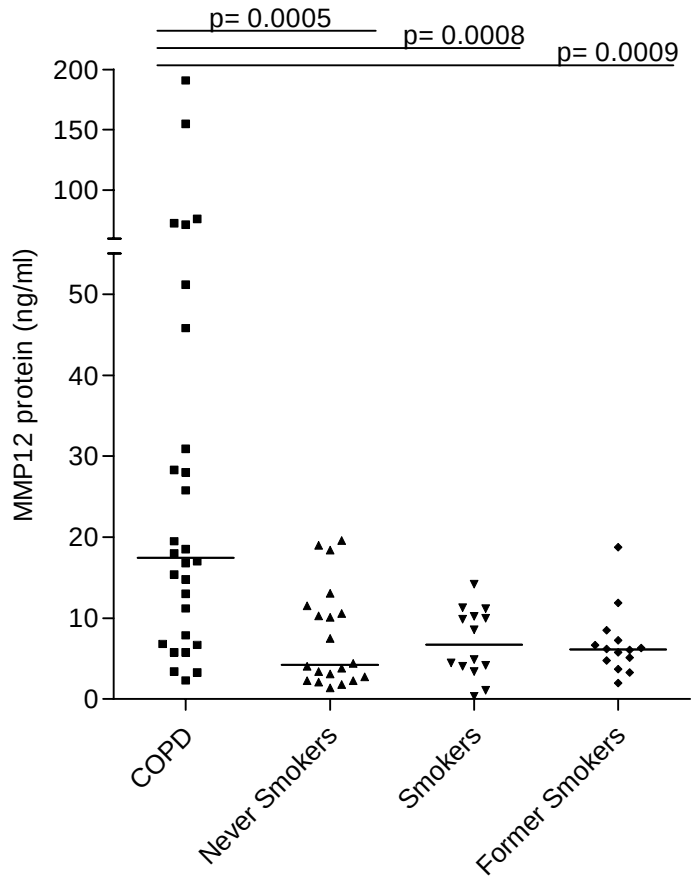


Figure 5

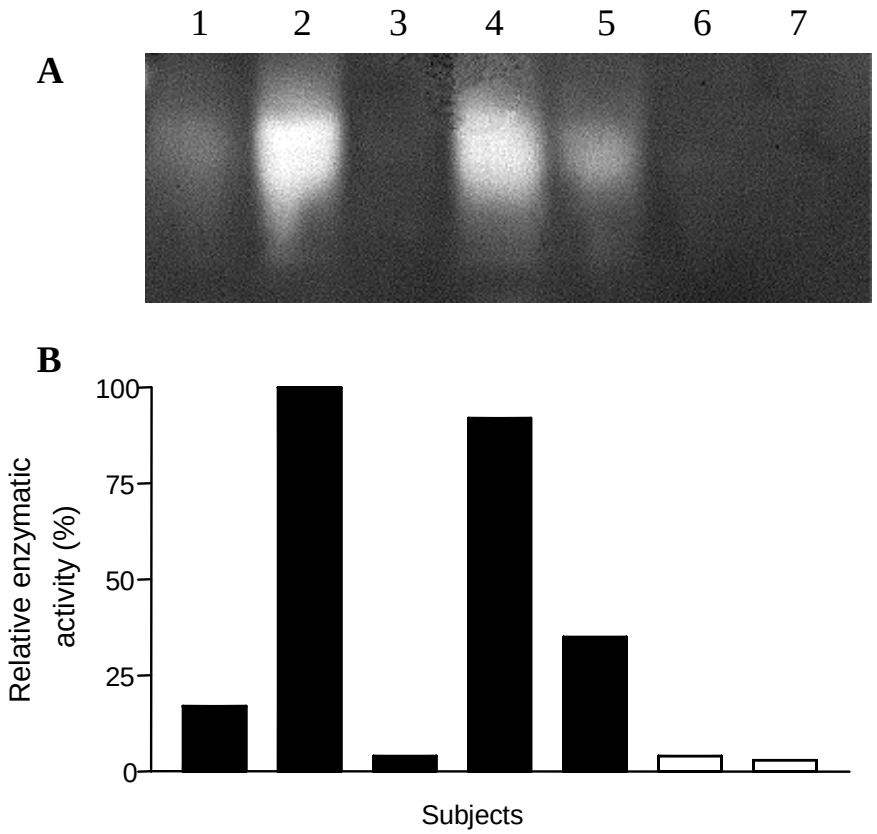
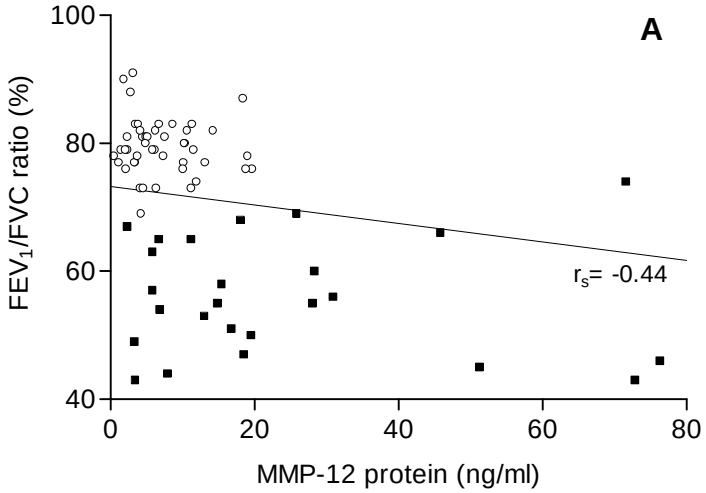


Figure 6



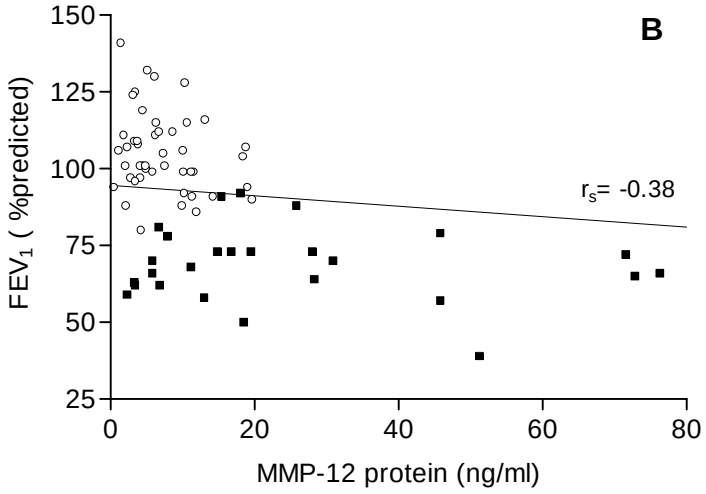


Figure 7