

# Matrix metalloproteinases -8, -9 and -12 in smokers and patients with Stage 0 COPD

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**Abstract:** COPD is underdiagnosed and its early assessment is problematic. It has been suggested that symptomatic smokers with normal FEV<sub>1</sub>/FVC (Stage 0 COPD, GOLD criteria) can develop COPD in the future. Potential early biomarkers in COPD include the matrix metalloproteinases (MMPs). It is not yet known, whether alterations in MMP expression are associated with smoking alone or with the risk of developing COPD. In this cross-sectional study MMP-8, MMP-9 and MMP-12 were determined from induced sputum and plasma by ELISA, immunocytochemistry, zymography, and/or Western blot in non-smokers (n = 32), smokers with symptoms (Stage 0, GOLD criteria) (n = 23) or without symptoms (n = 23). Only MMP-8 differentiated Stage 0 COPD from non-symptomatic smokers (p = 0.02). MMP-9 levels were significantly elevated in the induced sputum of non-symptomatic smokers and Stage 0 COPD (p = 0.01, p < 0.001) compared to non-smokers, but did not differ between the two subgroups of smokers. MMP-12 was higher only at Stage 0 compared to non-smokers (p = 0.04). MMP-8, MMP-9 and MMP-12 immunoreactivity was localized in macrophages and neutrophils, especially in smokers. MMP-8 levels correlated significantly with the small airway flow parameters (MEF50, MEF25) (p = 0.005 and p = 0.0004) and markers of neutrophil activation (myeloperoxidase, lactoferrin). In conclusion MMP-8 may differentiate Stage 0 from healthy smokers.

**Keywords:** cigarette smoking, GOLD, COPD, MMP, myeloperoxidase, oxidant, Stage 0

## Introduction

Chronic obstructive pulmonary disease (COPD) is leading cause of death worldwide, it also causes significant morbidity and disability (Calverley and Walker 2003). COPD is underdiagnosed and its early assessment is problematic. Even the early stages of the disease with normal lung function parameters (FEV<sub>1</sub>/FVC >0.7) possess inflammatory changes and structural abnormalities in the airways and lung parenchyma (Hogg et al 2004). The current international COPD classification, GOLD criteria (Pauwels et al 2001), takes these problems into consideration since there may be an apparent risk for COPD development in symptomatic smokers who have normal lung function parameters. The GOLD classification categorizes symptomatic subjects to have GOLD Stage 0 COPD. The usefulness of Stage 0 in predicting COPD development is still unclear (Vestbo and Lange 2002). However, recent studies do indicate that Stage 0 has importance, at least in predicting long-term mortality (Ekberg-Aronsson et al 2005; Mannino 2006; Stavem et al 2006). At present no biomarker has been found to differentiate these at-risk individuals from healthy cigarette smokers. Potential markers not only need to be evaluated in terms of disease development but they may also be implemented in smoking cessation protocols.

Biomarkers of tissue damage/remodeling in COPD include matrix metalloproteinases (MMPs) (Saetta et al 2001; Shapiro 2002; Barnes 2004). MMPs are activated by many different factors including cigarette smoke and oxidative stress (Rajagopalan et al 1996; Shapiro 2002; Nelson and Melendez 2004; Kinnula 2005; Kinnula et al 2005;

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Rahman and Adcock 2006). Markers of oxidative stress have been detected not only in COPD but also in cigarette smokers and subjects with Stage 0 COPD (Ryttila et al 2006).

Several MMPs including MMP-1, MMP-2, MMP-8, MMP-9 and MMP-12 are elevated both in experimental emphysema and human COPD, especially MMP-8, MMP-9 and MMP-12 have been associated with COPD (Hautamaki et al 1997; Beeh et al 2003; Vernooij et al 2004; Culpitt et al 2005; Demedts et al 2006; Elkington and Friedland 2006). The levels of these MMPs and TIMP-1 (the major endogenous inhibitor of MMP-8 and MMP-9) have not been earlier compared in non-smokers, non-symptomatic and symptomatic smokers (Stage 0 COPD). Knowing the effects of cigarette smoking on many signaling cascades, accumulation and activation of the inflammatory cells, and increased oxidative stress, it is likely that there is some increase/activation of MMPs in chronic smokers without airway flow limitation.

Induced sputum is a validated non-invasive method for the assessment of airway/tissue inflammation/injury in COPD (Djukanovic et al 2002). MMP-8, MMP-9, MMP-12 and TIMP-1 (a major endogenous inhibitor of MMP-8 and MMP-9) were investigated in induced sputum and plasma samples of healthy non-symptomatic smokers with normal lung function parameters by GOLD criteria and patients with GOLD Stage 0. MMP levels were also correlated with the levels of induced sputum neutrophils and macrophages, lactoferrin, myeloperoxidase (MPO) and nitrotyrosine to further characterize the association between these MMPs and the oxidative stress and inflammatory profile of the lung.

## Materials and methods

### Subjects

This cross-sectional study population included three groups: non-smoking healthy controls (NS) (n = 32), non-symptomatic smokers (S) (n = 23, mean 24 pack-years) and symptomatic smokers (chronic symptoms such as cough and phlegm, but no airway obstruction), ie, GOLD stage 0 (n = 23, mean 37 pack-years) (Table 1).

Of the non-smoking subjects, 20 were never-smokers and 12 ex-smokers (mean 15.7 pack-years) who had stopped smoking at least 20 years earlier. For comparison, MMPs and TIMP-1 were also analyzed from stable COPD patients (Stage I-III) (n = 10, mean 47 pack-years), (FEV<sub>1</sub> 62% of predicted, DLCO/VA 66% of predicted). The staging was based on clinical symptoms assessed with the St George's Respiratory Questionnaire, and airflow parameters on spirometry, according to the GOLD classification (American Thoracic Society (ATS) 1995; Pauwels et al 2001). Three

GOLD stage 0 patients had been prescribed  $\beta_2$ -agonists and inhaled corticosteroid therapy. The medications of the COPD Stage I-III patients were as follows: 4 patients received  $\beta_2$ -agonists, inhaled corticosteroid and anticholinergics; 4 patients received  $\beta_2$ -agonists plus inhaled corticosteroid; 1 received  $\beta_2$ -agonists and anticholinergics. None were receiving systemic corticosteroid therapy.

Exclusion criteria included allergies, asthma, a history of respiratory disease other than COPD, or respiratory infection less than 8 weeks before entering the study. The study was approved by the Ethics Committee of Helsinki University Hospital, Helsinki, Finland with written consent and registered (<http://www.hus.fi/clinicaltrials>).

### Pulmonary function tests

Flow-volume spirometry was performed with a pneumotachograph based spirometer connected to a computer (Medriko M 904, Kuopio, Finland) (Viljanen 1982). The pulmonary diffusing capacity for carbon monoxide (DLCO) and static lung volumes were measured with a single breath method according to the European Respiratory Society.

### Sputum induction

Sputum was induced by inhalation of hypertonic saline as recommended by the ERS Task Force and processed for differential counts of inflammatory cells as previously described (Ryttila et al 2000; Djukanovic et al 2002). Briefly, samples were treated by adding an equal volume, based on the weight of the sample, of dithioerythritol (DTE, Sigma, Germany) or phosphate-buffered-saline (PBS). The supernatant was frozen at -80 °C for biochemical and immunological analyses. Cell viability was assessed by Trypan blue in a Burkner chamber.

**Table 1** Patient characteristics

Variable	Non-smokers*	Smokers	Stage 0
Number	32	23	23
Age (yr)	56 (22-72)	51 (25-64)	53 (22-70)
Sex (F/M)	6/26	11/12	4/19
Pack years	15.7 (3-31)	24 (3-50)	37 (4-84)
Post bronchodilator:			
FVC % pred	101 (80-115)	97 (65-122)	87 (67-105)
FEV <sub>1</sub> % pred	104 (81-127)	97 (63-122)	85 (62-109)
FEV <sub>1</sub> /FVC	82.8 (75-90)	81 (70-92)	79 (70-89)
MEF50% PRED	99 (3-174)	88 (38-127)	71 (35-128)
MEF25% pred	120 (33-230)	109 (45-190)	64 (16-115)
Diffusion capacity % pred	104 (83-138)	96 (77-118)	93 (62-132)

\* This group includes 20 lifelong non-smokers and 12 ex-smokers more than 20 years after smoking cessation.

Cytochrome slides were prepared by Cytospin at 450 rpm for 6 min. One slide from each patient was stained with May-Grunwald-Giemsa-staining (Merck, Germany) for cell differential counts. A total of 400 cells were counted from each slide. The samples having less than 70% of squamous epithelial cells were accepted for further investigations. The slides were frozen at  $-20^{\circ}\text{C}$ .

### MMP-8, MMP-9, MMP-12, TIMP-1, and lactoferrin by ELISA

MMP-8, MMP-9, TIMP-1 and lactoferrin levels in sputum supernatant and plasma samples were determined by commercially available ELISA kits (Amersham Biosciences, Cardiff, UK) according to the manufacturers' instructions. MMP-12 was measured in induced sputum and plasma samples by a custom-made ELISA, as previously described (Demedts et al 2006). The detection limits were: 0.032 ng/ml for MMP-8, 0.6 ng/ml for MMP-9, 0.05 ng/ml for MMP-12, 1.25 ng/ml for TIMP-1 and 1.6 ng/ml for lactoferrin.

### Immunocytochemical staining of the MMPs, TIMP, MPO and nitrotyrosine

MMP-8, MMP-9, MMP-12, TIMP-1, MPO and nitrotyrosine were also assessed by immunocytochemistry. The cytospin samples were treated with Ortho Permeafix (Ortho Diagnostic Systems Inc., UK). The endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS and for immunostaining, Zymed ABC Histostain-Plus Kit was used according to the manufacturers' protocol. The samples were incubated with polyclonal rabbit anti-human MMP-8 (Hanemaaijer et al 1997), MMP-9 (NeoMarkers, Fremont, CA), TIMP-1 (Chemicon, Temecula, CA, USA), MPO (LabVision Corp., Fremont, US) or nitrotyrosine (Upstate Lake Placid, NY, USA) or monoclonal MMP-12 (R&D Systems Inc., Minneapolis, US) antibody, and negative control samples with Zymed Rabbit or Mouse (MMP-12) isotype Control and PBS overnight at  $4^{\circ}\text{C}$ , and stained with AEC (Zymed Laboratories Inc., South San Francisco, CA) and thereafter with Mayer's hematoxylin. MMP-8-, MMP-9-, MMP-12-, TIMP-1-, MPO- and nitrotyrosine positive and negative cells were counted (400 cells/cytospin).

### MMP-9 by zymography and Western blot analysis

Sputum supernatant (60  $\mu\text{g}$  protein) was incubated with Laemmli's sample running buffer. After electrophoresis on 8% sodium dodecyl sulphate-polyacrylamide gels (SDS-

PAGE) containing 1 mg/ml gelatine (Sigma, St. Louis, MO, USA), as a substrate, the gels were processed and stained with 0.1% Coomassie Brilliant Blue R250 as described (Sorsa et al 1997) for the detection of 92 kD MMP-9 (gelatinase B).

For the Western blot analysis, sputum supernatants were lyophilized (total protein content 40  $\mu\text{g}$  per well in 15  $\mu\text{L}$  volume). After 12% SDS-PAGE, the loaded samples were electrotransferred to nitrocellulose membranes (Schleicher & Shuell, Dassel, Germany). The membranes were first incubated with polyclonal rabbit anti-human MMP-9 (Calbiochem, Darmstadt, Germany) and after washing with 0.1% TTBS, treated with the secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The membranes were developed and quantified for MMP-9 by the Bio-Rad Model GS-700 Imaging Densitometer and Analyst<sup>TM</sup> program (Sorsa et al 1997).

### Statistical analysis

All statistical analyses were performed with the SPSS 10.0 software program (SPSS Inc., Chicago, IL, USA). As the data were not normally distributed, non-parametric tests were used for all comparisons. Data for individual variables from the several groups were first analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U-test. Correlations between variables were determined with the Spearman rank correlation coefficient. A p-value of  $<0.05$  was considered significant.

## Results

### Lung function characteristics of non-smokers, healthy smokers and subjects with Stage 0 COPD

Lung function characteristics of the subjects are shown in Table 1. All the subjects had normal airway function according to GOLD criteria (post bronchodilator  $\text{FEV}_1/\text{FVC} >0.7$ ). The cell profile of the induced sputum specimens in Figure 1 reveals the greater percentage of neutrophils and the lower percentage of macrophages in symptomatic smokers (Stage 0) than in non-smokers. Asymptomatic smokers and Stage 0 exhibited a higher total number of neutrophils ( $p = 0.005$  and  $p = 0.001$ ) than non-smokers.

### The levels of MMPs and TIMP-1 in the induced sputum and plasma samples by ELISA

Subjects with Stage 0 COPD had higher sputum MMP-8 levels than the non-symptomatic smokers and non-smokers ( $p = 0.02$  and  $p < 0.0001$ , Figure 2A). MMP-9 was higher in

Stage 0 and non-symptomatic smokers than in non-smokers ( $p < 0.0001$  and  $p = 0.01$ ), but no significant difference existed between non-symptomatic smokers and those at Stage 0 ( $p = 0.062$ , Figure 2B). Stage 0 patients had higher MMP-12 than non-smokers ( $p = 0.04$ ), but the difference between non-symptomatic smokers and Stage 0 patients was not significant ( $p = 0.14$ , Figure 2C) There were no differences in TIMP-1 levels between the subgroups (Figure 2D).

To confirm earlier findings on the elevation of MMP-8 and MMP-9 in COPD, their levels were also determined by ELISA in the induced sputum of Stage I–III COPD patients ( $n = 10$ ). MMP-8 and -9 were significantly elevated in COPD compared to healthy smokers (MMP-8:  $p < 0.0001$ ; MMP-9:  $p = 0.01$ ). We also recently found this to be the case with MMP-12 (Demedts et al 2006).

Plasma MMP-8 levels did not differ between the subgroups ( $p = 0.225$ ). Plasma MMP-9 concentrations of smokers were higher than those of non-smokers ( $p = 0.04$ ), but the difference between smokers without or with symptoms (Stage 0) was not significant ( $p = 0.62$ ). Plasma MMP-12 levels were significantly higher in Stage 0 patients when

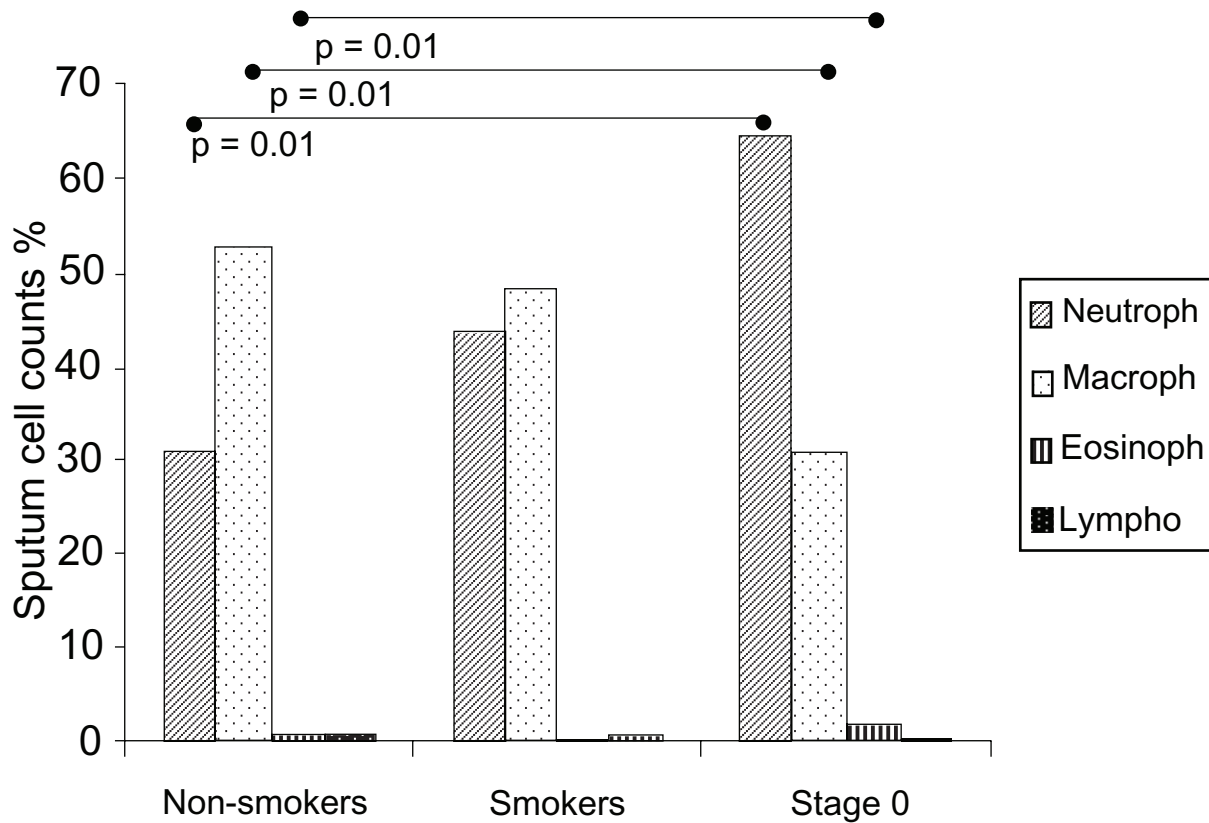
compared to the non-symptomatic smokers and non-smokers ( $p = 0.008$ ). Plasma TIMP-1 levels between the subgroups did not differ.

### Localisation of MMPs and TIMP-I in the alveolar macrophages and neutrophils

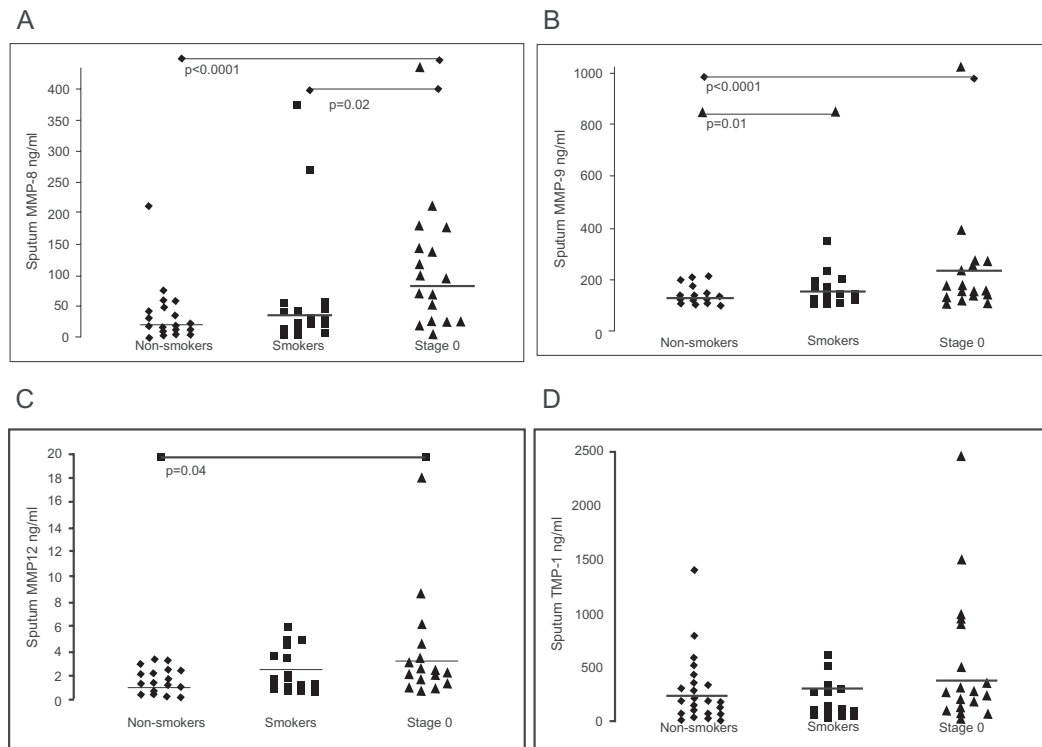
MMP-8, MMP-9 and TIMP-1 were localized in the neutrophils and macrophages. MMP-12 was mainly detected in the macrophages but could also be found in some neutrophils (Figure 3). Some ciliated epithelial cells exhibited weak MMP-8 and TIMP-1 immunoreactivity. Generally, the reactivity was similar in healthy smokers and those at Stage 0, whereas the expression in non-smokers was very weak (data not shown).

### Detection of active MMP-9 by zymography and Western blotting

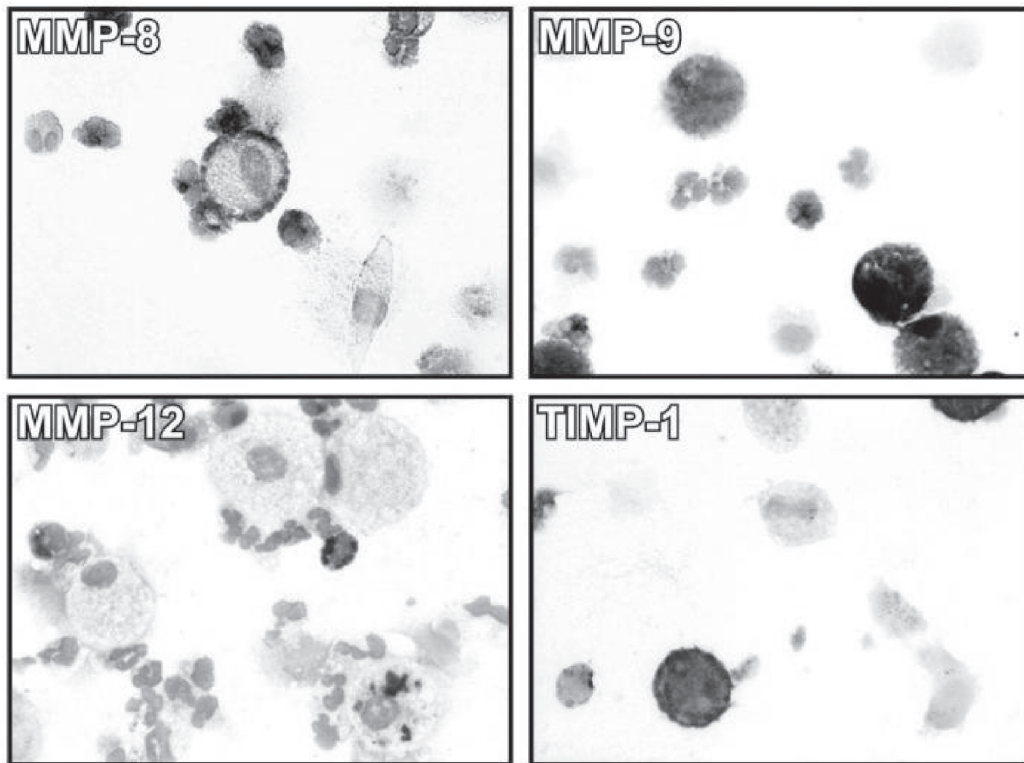
ELISA detected similar MMP-9 levels in healthy smokers and Stage 0 with individual variation. Therefore MMP-9 was also evaluated by zymography and Western blotting. However, even these methods exhibited extensive variability.



**Figure 1** Cell differential counts in induced sputum from non-smokers, smokers and Stage 0. Stage 0 patients had significantly higher percentage of neutrophils and eosinophils, and lower percentage of macrophages than non-smokers. There were no differences between asymptomatic smokers and Stage 0.



**Figure 2** MMP-8 (A), MMP-9 (B), MMP-12 (C) and TIMP-1 (D) levels by ELISA in the induced sputum from non-smokers, asymptomatic smokers and GOLD Stage 0. Mean values are shown with horizontal bars. The levels of MMP-8 were increased in Stage 0 when compared with asymptomatic smokers ( $p = 0.02$ ) and non-smokers ( $p < 0.0001$ ). The levels of MMP-9 were higher in Stage 0 ( $p < 0.0001$ ) and asymptomatic smokers ( $p = 0.01$ ) than in non-smokers. The MMP-12 levels were higher in Stage 0 than in non-smokers ( $p = 0.04$ ) and there were no significant differences in TIMP-1 levels between the groups.



**Figure 3** Immunocytochemistry for MMP-8 (A), MMP-9 (B), MMP-12 (C) and TIMP-1 (D) on the cytopins of induced sputum cells from a GOLD Stage 0 smokers. Clear immunoreactivity for each MMP was detectable in macrophages and neutrophils from all patient groups, especially from smokers and those at GOLD Stage 0.

Zymography revealed MMP-9 gelatinolytic activity (92 kD pro-MMP-9 and 77–82 kD active MMP-9) both in healthy smokers and those at Stage 0 with no major difference between the groups (Figure 4A). MMP-9 activation has been assessed also from the bands obtained from Western blot analysis (Sorsa et al 1997). The present results by densitometry were, however, variable (14% increase in the suggested MMP-9 active form in smokers compared to the non-smokers, n = 16) (n.s.) (Figure 4B).

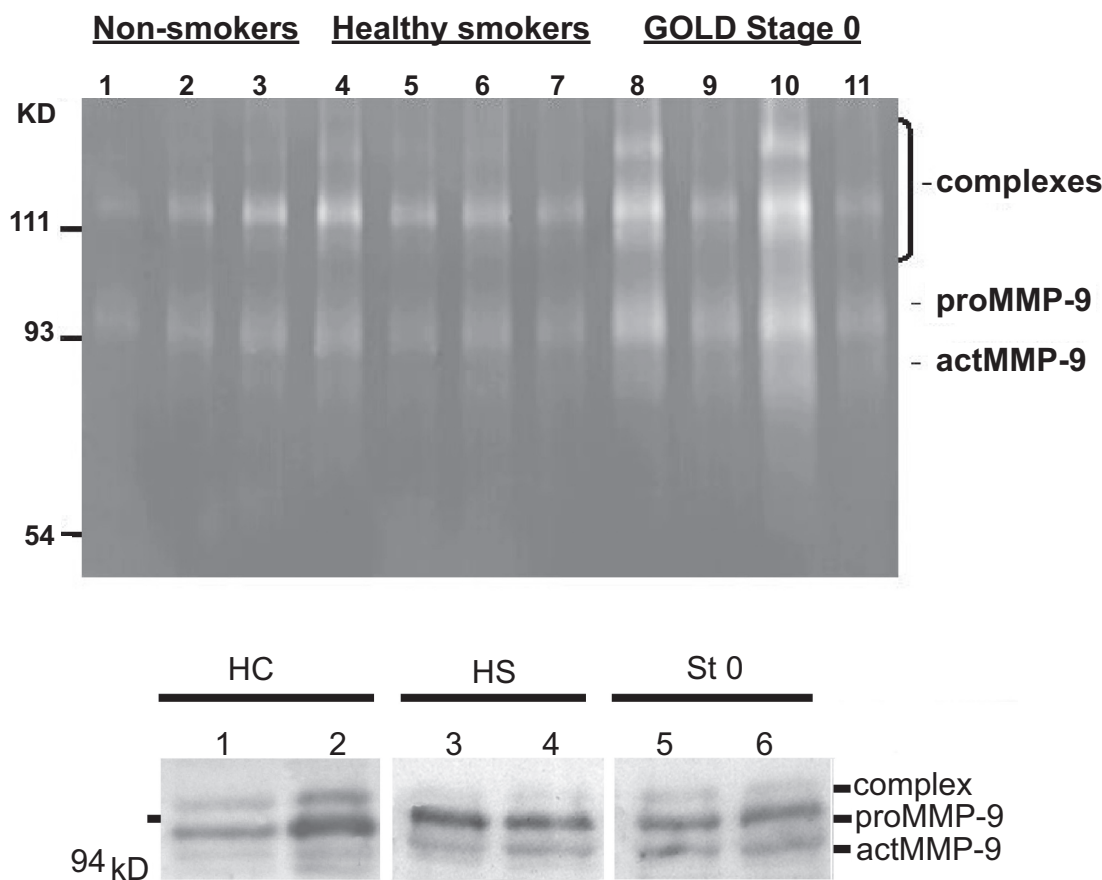
### MMP/TIMP ratios in the sputum and plasma

No difference was found in the MMP-8/TIMP-1, MMP-9/TIMP-1 or MMP-12/TIMP-1 ratios in the induced sputum of Stage 0, smokers and non-smokers. In contrast, the MMP-8/TIMP-1 and MMP-9/TIMP-1 ratio in plasma was substantially higher in healthy smokers when compared to non-smokers

(p = 0.009 and p = 0.02). Patients with Stage 0 had a higher MMP-12/TIMP-1 ratio in plasma than non-symptomatic smokers and non-smokers (p = 0.04 and p = 0.005).

### Correlation of MMPs and TIMP-1 with inflammatory cells and markers of oxidative stress

MMP-8, MMP-9, MMP-12 and TIMP-1 levels in the induced sputum, assessed by ELISA, generally correlated positively with the total number and percentage of neutrophils (MMP-8: p = 0.0001 and p = 0.0002; MMP-9: p < 0.0001 and p < 0.0001; MMP-12 p < 0.0001 and p = 0.129; TIMP-1: p = 0.002 and p = 0.004) (Figure 5). In addition, MMP-8 and MMP-9 levels negatively correlated with the percentage of macrophages (p = 0.02 and p = 0.0003). MMP-12 levels correlated positively with the total number of macrophages (p = 0.008).



**Figure 4** Zymography and Western blotting analysis of MMP-9 in the sputum specimens. Representative zymography of 11 specimens (A): Gelatinolytic activity at 92 kD (pro-MMP-9) and 77–82 kD (activated MMP-9) of non-smoking controls (lanes 1–3), cigarette smokers (lanes 4–7), and subjects at GOLD stage 0 (lanes 8–11). Individual variation occurred, but generally MMP-9 was activated in smokers and especially those at GOLD Stage 0. Representative Western blotting of 6 specimens (B): HC healthy non-smoking control (lanes 1–2), HS healthy smoker (lanes 3–4), St 0 Stage 0 (lanes 5–6). There was individual variation, when calculated from 16 specimens no significant differences between the groups could be seen. Molecular weight standards at the left.

Mean lactoferrin levels in induced sputum of GOLD Stage 0, smokers and non-smokers were  $59.7 \pm 18.0$ ,  $36.3 \pm 11.0$  and  $28.7 \pm 3.42$   $\mu\text{g/ml}$  ( $p = 0.071$ ) and in plasma  $772 \pm 121$ ,  $761 \pm 155$  and  $772 \pm 121$   $\text{ng/ml}$  ( $p = 0.616$ ), respectively. MMP-8, MMP-9 and MMP-12 correlated with sputum lactoferrin (Figure 6) and MMP-8 and MMP-9 correlated with the levels of MPO positive cells ( $r = 0.55$ ,  $p = 0.004$  and  $r = 0.49$ ,  $p = 0.01$ ) but not with nitrotyrosine.

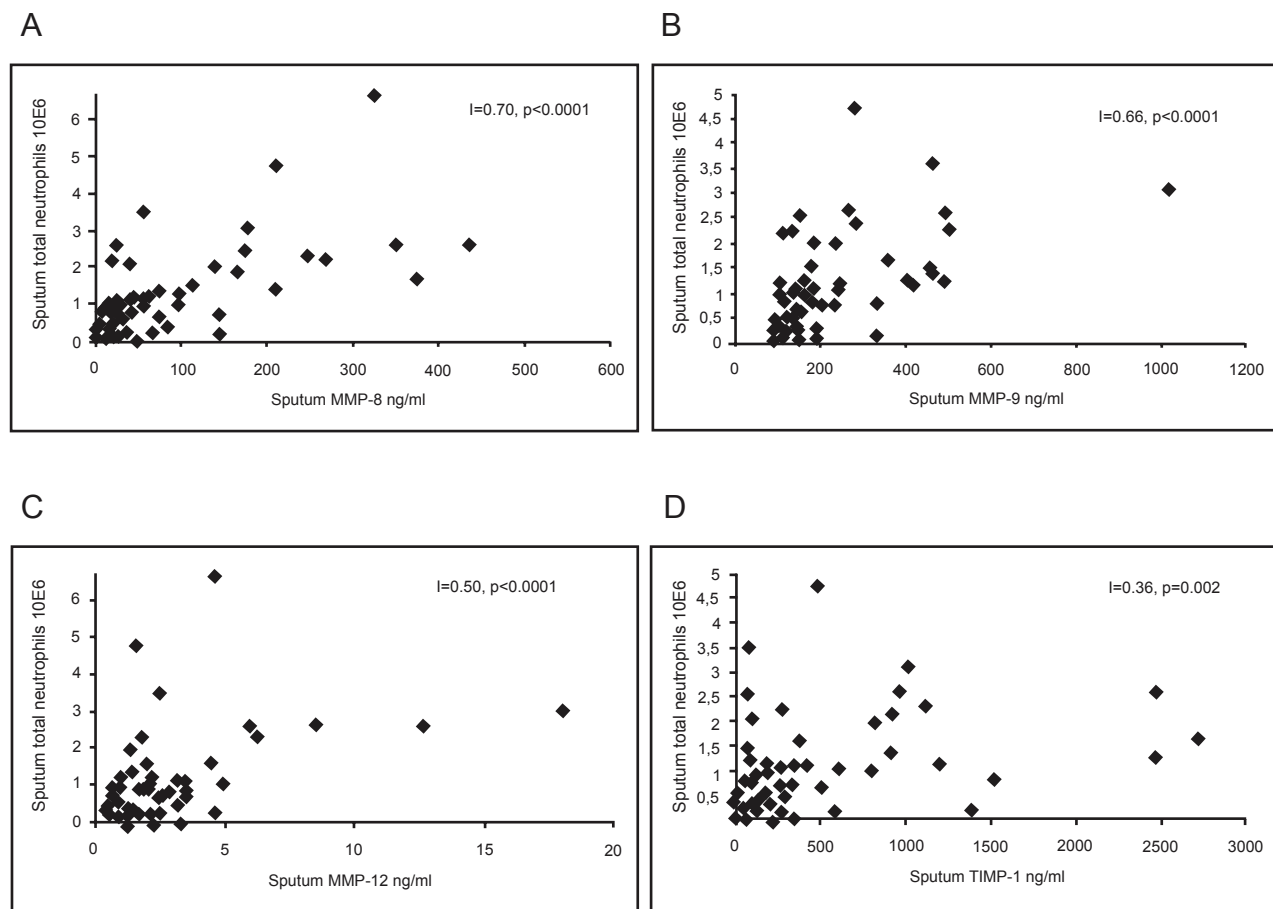
### Correlation between the levels of MMPs and lung function parameters

The purpose of this study was to evaluate the significance of MMPs at the early stages of COPD with normal lung function parameters based on GOLD criteria (FEV<sub>1</sub>/FVC). Special interest was with the airflow parameters in the small airways (MEF50, MEF25). When the various MMPs were correlated with the lung function parameters of all smokers with or

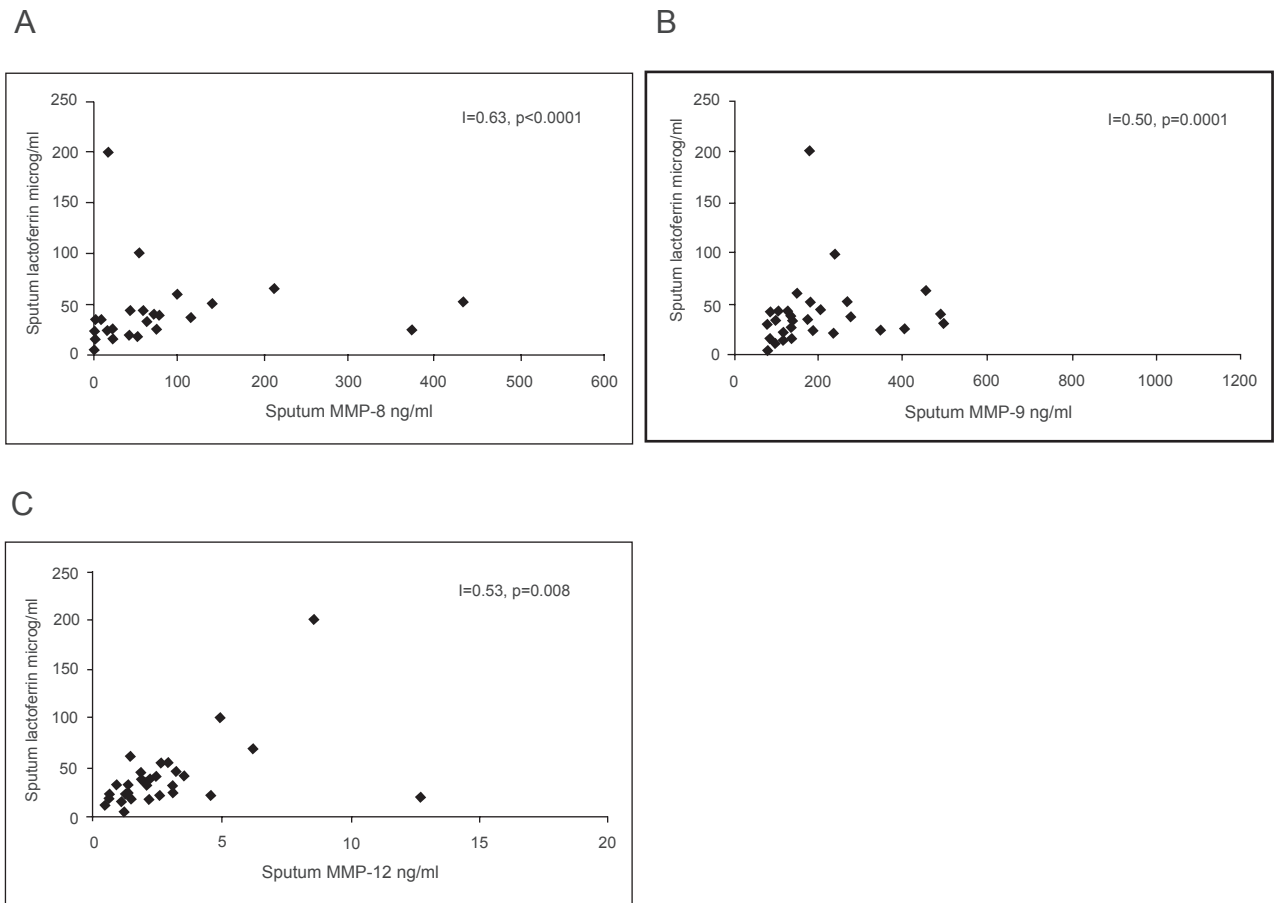
without COPD (GOL Stage 0) there was highly significant correlation with all lung function parameters (Table 2).

### Discussion

The present study suggests that MMP-8 can differentiate symptomatic smokers (Stage 0), ie, those who may be at risk for COPD development (Pauwels et al 2001; Willemse et al 2005; Mannino 2006; Stavem et al 2006) from non-symptomatic chronic smokers. MMP-8 also correlated with the lung function parameters, in particular the correlations with the small airway flow parameters were highly significant. Also MMP-9 and MMP-12 were already elevated in the induced sputum of Stage 0 COPD but neither MMP-9 nor MMP-12, with the exception of plasma MMP-12, appeared to differentiate healthy smokers from Stage 0 patients. MMPs can be activated by ROS, and this study also found a significant association especially between the levels of MMP-8



**Figure 5** Correlation between sputum MMP-8 (A) ( $n = 70$ ;  $r = 0.70$ ;  $p < 0.0001$ ), MMP-9 (B) ( $n = 69$ ;  $r = 0.66$ ;  $p < 0.0001$ ), MMP-12 (C) ( $n = 56$ ;  $r = 0.50$ ;  $p < 0.0001$ ), and TIMP-1 (D) ( $n = 68$ ;  $r = 0.36$ ;  $p = 0.002$ ) levels and total number of neutrophils.



**Figure 6** Correlation between MMP-8 (A) (n = 34; r = 0.63; p < 0.0001), MMP-9 (B) (n = 35; r = 0.50; p = 0.002) and MMP-12 (C) (n = 24; r = 0.53; p = 0.008) and lactoferrin levels in induced sputum.

and MMP-9 and neutrophils and markers of their activation (lactoferrin, MPO). However, as in biological samples in general, there was considerable individual variation in the levels of MMPs and TIMP-1 in both the induced sputum and the plasma specimens.

One problem in assessing MMPs in cigarette smokers has been the wide variation in the smoking history and time elapsed from smoking cessation of the control group (Cataldo et al 2000; Kang et al 2003; Culpitt et al 2005). We included a representative group of non-smokers and smokers, who were either symptom-free or exhibiting symptoms. Based on this study only MMP-8 could differentiate symptomatic smokers from healthy chronic smokers with normal FEV<sub>1</sub>/FVC ie, without major airway obstruction. The levels of MMP-8, MMP-9 and MMP-12 in the induced sputum of ex-smokers and lifelong non-smokers were similar suggesting that the effects of smoking on MMP-8, MMP-9 and MMP-12 levels may be reversible. Like in many biological measurements there was wide variation in MMP-values within the

groups with some outliers in each group. These values and the clinical characteristics of the subjects were carefully re-evaluated. To confirm the elevation of these MMPs in COPD as earlier published (Vernooy et al 2004; Culpitt et al 2005; Mercer et al 2005), additional measurements were conducted with the sputum specimens of Stage I–III COPD

**Table 2** Correlation between the levels of sputum MMPs and lung spirometric variables

Parameter	MMP-8	MMP-9	MMP-12	TIMP-1
FEV <sub>1</sub> % pred	r = -0.42 p = 0.0007	r = -0.39 p = 0.002	r = -0.32 p = 0.03	r = -0.17 p = 0.186
FVC% pred	r = -0.25 p = 0.05	r = -0.25 p = 0.05	r = -0.32 p = 0.03	r = -0.03 p = 0.82
FEV <sub>1</sub> /FVC	r = -0.36 p = 0.005	R = -0.36 p = 0.004	r = -0.13 p = 0.36	r = -0.31 p = 0.02
MEF50% pred	r = -0.35 p = 0.005	R = -0.35 p = 0.005	r = -0.27 p = 0.06	r = -0.21 p = 0.09
MEF25% pred	r = -0.44 p = 0.0004	R = -0.46 p = 0.0003	r = -0.3 p = 0.04	r = -0.16 p = 0.22



patients (Demedts et al 2006), detecting significantly higher levels of these MMPs in COPD subjects in comparison to healthy smokers.

Some previous studies have shown MMP-9 elevation in chronic cigarette smokers when compared to non-smokers (Lim et al 2000; Kang et al 2003), but no comparisons were made between asymptomatic and symptomatic smokers. Our recent study found higher MMP-12 levels in the induced sputum of COPD patients when compared to smokers and non-smokers (Demedts et al 2006), but again non-symptomatic and symptomatic smokers were not compared. Our present results with MMP-8 and Stage 0 are in line with previous suggestions about the special role of MMP-8 in the development of emphysema (Segura-Valdez et al 2000). Since MMP-9 levels as assessed by ELISA were very similar both in non-symptomatic smokers and individuals at Stage 0, its activation was further investigated by zymography and Western blot analysis which showed extensive inter-individual variability. MMP-12 levels were higher in patients with Stage 0 than in non-smokers but there was no difference between healthy smokers and individuals at Stage 0. Also MMP-12 showed high variability in the Western blotting analysis (not shown). This does not signify that MMP-12 has no relevance in COPD development (Russell et al 2002b; Demedts et al 2006), but the fact that MMP-12 is already increased in smokers without airway obstruction.

MMP-8, MMP-9 and MMP-12 are known to be expressed in alveolar macrophages (Russell et al 2002a; Vernooij et al 2004; Bracke et al 2005; Molet et al 2005) and MMP-8 and MMP-9 in neutrophils (Cataldo et al 2001; Barnes et al 2003; Beeh et al 2003; Takafuji et al 2003; Vernooij et al 2004; Culpitt et al 2005; Gueders et al 2005). These previous observations were confirmed here and the expressions were similar in healthy and symptomatic smokers. Although MMP-12 is produced by macrophages, its presence or absence in neutrophils has not been reported (Churg et al 2003; Bracke et al 2005; Molet et al 2005). In the present study some neutrophils clearly exhibited MMP-12 immunoreactivity. The relative functional role of alveolar macrophages and neutrophils as cellular sources of these MMPs in COPD is still unclear. The present findings support the importance of neutrophils as all MMPs correlated with neutrophils and markers of neutrophil activation.

MMPs were also elevated in the plasma of smokers, in agreement with previous observations (Nakamura et al 1998) and with the fact that COPD is a systemic disease, but also with the fact that MMPs are associated with other smoking-

related systemic diseases (Jones et al 2003; MacCallum 2005; Wouters 2005). This is the first study to report the elevated plasma MMP-12 levels in symptomatic smokers, but the relative role of all of these MMPs as systemic markers in COPD progression remains to be clarified.

TIMP-1 is a major endogenous inhibitor of MMP-8 and -9 and shown to be elevated in COPD (Beeh et al 2003; Owen et al 2003; Culpitt et al 2005; Higashimoto et al 2005). The sputum MMP-8/TIMP-1 and MMP-9/TIMP-1 ratio did not change significantly in smokers, pointing to a role for TIMP-1 as a natural defense mechanism in preventing tissue destruction.

The conformation of latent MMPs is maintained by thiol interactions between Cys-residues in the prodomain and the zinc atom present in the catalytic site of all MMPs. Disruption of this interaction is thought to represent the critical step in initiating the process of MMP autoactivation. ROS are known to reversibly react with -SH groups, such as those involved in preserving MMP latency (Rajagopalan et al 1996). Recent studies from our laboratory and others have indicated that cigarette smoke can elevate the oxidant burden also in healthy humans without airway obstruction (Ryttila et al 2006). The present study not only found increased levels of MMPs, especially MMP-8 in healthy smokers and those with symptoms (Stage 0), but also found a significant correlation between MMP-8, MMP-9 and the levels of MPO and lactoferrin, both of which are neutrophil-derived markers of oxidant generation. These findings further support the hypothesis that especially neutrophil derived oxidants, may significantly activate/induce the tissue destructive MMPs already in the lungs of healthy cigarette smokers. We did not find any association between MMPs and nitrotyrosine immunoreactivity showing also the complexity between smoking, oxidative stress and the expression of oxidized/nitrosylated proteins in sputum inflammatory cells.

In conclusion, MMPs, especially MMP-8, may function as sensitive biomarkers in COPD development. The relative role of various MMPs as markers of COPD and its progression remains to be clarified in future longitudinal investigations.

## Acknowledgments

Tiina Marjomaa, Merja Luukkonen and Ritva Keva are acknowledged for their excellent technical assistance, Antero Kokkonen and the Jaakko Pöyry Company for helping us in the recruitment of the non-smokers and smokers. We are very grateful to every subject who agreed to participate in this study.

This work was supported by the Sigrid Juselius Foundation, The Finnish Antituberculosis Association Foundation, Jansson Foundation, the Helsinki University Hospital (HUCH-EVO), the Academy of Finland and the ERS COPD Travel Grant for Best Posters 2005.

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