Sputum matrix metalloproteinase-12 in patients with chronic obstructive pulmonary disease and asthma: Relationship to disease severity

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Background: Matrix metalloproteinase (MMP)-12 has been implicated in the pathogenesis of both chronic obstructive pulmonary disease (COPD) and asthma. The influence of disease severity on sputum MMP-12 concentrations and activity is not known.
Objectives: We sought to examine the relationship between disease severity assessed by means of lung function and computed tomography (CT) and induced sputum MMP-12 concentrations and activity in patients with asthma and COPD.
Methods: In 208 subjects (109 asthmatic patients, smokers and never smokers, mild, moderate, and severe; 53 patients with COPD, smokers and exsmokers, mild, moderate, and severe; and 46 healthy control subjects, smokers and never smokers), we measured induced sputum MMP-12 concentrations (ELISA) and enzyme activity (fluorescence resonance energy transfer), sputum cell *MMP12* mRNA expression (quantitative PCR [qPCR]), diffusing capacity for carbon monoxide (DLCO), and CT assessment of emphysema (percentage of low-attenuation areas at less −950 Hounsfield units).
Results: Sputum MMP-12 concentrations are greater in patients with COPD and smokers with asthma than in healthy nonsmokers ($P = .003$ and $P = .035$, respectively) but similar to those seen in healthy smokers. In patients with COPD, disease severity, when measured by means of CT-assessed emphysema, but not by means of spirometry or DLCO values, is directly associated with sputum MMP-12 concentrations and activity. In the asthma groups there is no significant association between disease severity and sputum MMP-12 concentrations or activity.
Conclusions: Sputum MMP-12 concentrations and activity in patients with COPD are directly associated with the extent of emphysema measured by means of CT. This finding supports a role for MMP-12 in the pathogenesis of COPD and might suggest that blocking MMP-12 activity in patients with COPD could prevent the further development of emphysema.
(J Allergy Clin Immunol 2012;129:655-63.)

Key words: *Matrix metalloproteinase 12, tissue inhibitor of metalloproteinases 1 and 2,* MMP12 *expression, emphysema, chronic obstructive pulmonary disease, asthma, smoker*

Chronic obstructive pulmonary disease (COPD), the fourth most common cause of death in the Western world, and severe asthma, which occurs in up to 10% of patients with asthma, result in considerable morbidity and respond poorly to current therapies.[1,2] Smokers with asthma are an important phenotype of "difficult-to-control" asthma[3,4] because of poorly controlled disease[5] that is associated with an impaired therapeutic response to corticosteroids[4] and an accelerated decrease in lung function.[6] New drug targets are needed for patients with COPD and asthma, particularly those with severe disease or who are cigarette smokers.

Mechanisms of disease and factors leading to poor symptom control and remodeling of the lung in patients with chronic airway diseases are unclear. Matrix metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases that form a family of extracellular matrix proteolytic enzymes. They are primarily responsible for the degradation of extracellular matrix components during the remodeling processes essential for normal tissue growth and repair. In the lung inappropriate expression and excessive activity of several MMPs, including MMP-12 (macrophage elastase, EC

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Supported by an award (INF-GU-090) from the Translational Medicine Research Collaboration, a consortium made up of the Universities of Glasgow, Edinburgh, Aberdeen, and Dundee and the 4 associated NHS Health Boards (Greater Glasgow & Clyde, Lothian, Grampian, and Tayside), Scottish Enterprise, and Pfizer (formerly Wyeth) and supported financially by NHS Research Scotland (NRS) through the Scottish Primary Care Research Network.

Disclosure of potential conflict of interest: K. Nocka is employed by Pfizer. D. Crowther is a previous employee of Pfizer and is a visiting Fellow at the Cranfield University and an honorary lecturer at the University of Dundee. W. MacNee has received research support from Pfizer. D. K. Miller is a Pfizer/Wyeth employee. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 1, 2011; revised October 26, 2011; accepted for publication December 8, 2011.

Available online February 6, 2012.

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0091-6749/$36.00

doi:10.1016/j.jaci.2011.12.996

Abbreviations used
COPD: Chronic obstructive pulmonary disease
CT: Computed tomography
DLCO: Diffusing capacity for carbon monoxide corrected for hemoglobin and carboxyhemoglobin
FRET: Fluorescence resonance energy transfer
FVC: Forced vital capacity
%LAA−950: Low-attenuation areas at less than −950 Hounsfield units taken as an index of emphysema
MMP: Matrix metalloproteinase
qPCR: Quantitative PCR
TIMP: Tissue inhibitor of metalloproteinases

3.4.24.65), have been implicated in the tissue-destructive processes associated with chronic lung diseases, including COPD and asthma.[7-12] MMPs can also process and activate latent cytokines and chemokines, cleave cell-surface receptors, and facilitate leukocyte recruitment.[13,14] MMP synthesis can be stimulated and activity perpetuated by microbial infection[15] and have a major role during exacerbations of airway diseases. MMP activity is regulated by a family of specific protease inhibitors called tissue inhibitors of metalloproteinases (TIMPs).

The expression of MMP-12 is increased in alveolar macrophages from cigarette smokers,[16] and protein levels and enzymatic activity of MMP-12 are reported to be increased in the sputum of patients with COPD compared with those seen in smokers without airflow limitation, in whom MMP-12 levels were inversely correlated with lung function.[17] It might be the imbalance between MMP activity and the inhibitory action of TIMPs that is important in patients with COPD and asthma, and the maintenance of an adequate MMP/TIMP ratio might explain the absence of progressive airway obstruction in healthy smokers.[18]

Taken together, these findings suggest that excess MMP activity is an important therapeutic target for both COPD and asthma. However, there is no information about concentrations, activities, and expression of MMP-12, as well as the regulatory proteins TIMP-1 and TIMP-2, in well-characterized smokers and nonsmokers with asthma and COPD. Furthermore, it is unknown whether activation of MMP-12 occurs at an early stage of disease or accelerates disease progression. The aim of this study was to assess the influence differences in disease severity and smoking status have on these sputum mediators in patients with COPD and asthma.

METHODS

Subjects and study design

A cross-sectional study was performed in patients with asthma, patients with COPD, and healthy control subjects as part of the Glasgow COPD and Asthma Biomarker study. Clinical, physiologic, and immunologic plus imaging (asthma and COPD groups only) measurements were performed at baseline, and sputum measurements were repeated on 1 occasion in the asthma group and healthy control subjects and on 3 occasions in patients with COPD over a 6-week period. Participants recruited were patients with mild, moderate, and severe persistent asthma (Global Initiative for Asthma classification, both current smokers and never smokers)[19]; patients with mild, moderate, and severe COPD (Global Initiative for Chronic Obstructive Lung Disease stages I, II, and III; both current smokers and former smokers)[20]; and healthy smokers and nonsmokers (additional details are shown in the Methods section in this article's Online Repository at www.jacionline.org). The West Glasgow Research Ethics Committee approved the study, and all patients provided written informed consent.

Measurement

Additional details on measurement are shown in this article's Online Repository at www.jacionline.org.

Spirometry was performed to American Thoracic Society guidelines. In the asthma group airway hyperresponsiveness to methacholine was measured.[21]

Lung volumes and diffusing capacity for carbon monoxide (DLCO) were performed in the asthma and COPD groups by using the body box technique (Zan500 Body Plethysmography; nSpire Health Limited, Hertford, United Kingdom).

Sputum induction was performed by using 3%, 4%, and 5% hypertonic saline, as previously described,[22] and sputum was processed to collect cells and fluid by using the method of Pin et al.[23] Samples with cell viability of less than 40% or epithelial squamous cells of greater than 80% were discarded.

For volume computed tomographic (CT) scans of the chest, scans were performed at full inspiration by using 16-slice Brightspeed and 64 slice Lightspeed (GE CT scanner, Milwaukee, Wis) with the following parameters: 120 KV, 100 mAs, collimation of 1 mm, reconstruction slice thickness of 0.65 mm, reconstruction slice separation of 0.5 mm, and pitch of 1; the data were reconstructed with a CHST filter. All scans were evaluated centrally at the University of Edinburgh. Emphysema was quantified as low-attenuation areas at less than −950 Hounsfield units taken as an index of emphysema (%LAA−950) with the software Pulmonary Workstation 2.0 (VIDA Diagnostics, Iowa City, Iowa), as described previously.[24-26]

For MMP-12 assays, MMP-12 immunoreactivity was quantified by using ELISA,[27] and activity was quantified by using the fluorescence resonance energy transfer (FRET) assay.[27,28] These are described in Matsuoka et al[25] and in the Methods section in this article's Online Repository.

TIMPs were quantified by using fluorokine multiplex (LKT003; R&D Systems, Abingdon, United Kingdom).

For *MMP12* RNA qPCR, cDNA was generated (Qiagen Quantitect Reverse Transcription kit; Qiagen, Hilden, Germany), and qPCR was performed (Applied Biosystems 7900HT Fast Real-Time System; Applied Biosystems, Warrington, United Kingdom). An SyBr Green assay was used. Details of primers, cycling conditions, and bioinformatics are supplied in the Methods section in this article's Online Repository.

Statistical analysis

Additional details on statistical analysis are shown in the Methods section in this article's Online Repository. Mixed effects normal linear models were used to estimate the within- and between-subject variability for each marker while taking account of disease group and smoking status and testing for any group by smoking status interaction. Within disease groups, a similar model was used to assess the association of disease severity with biomarkers. Associations of MMP-12 with biomarkers were assessed by using the Spearman rank correlation coefficient with bootstrap 95% CIs. All analyses have been carried out in R version 2.11.0 software.[29]

RESULTS

Demographics and baseline characteristics

The number, age, and sex distribution of subjects within each of the clinical categories is listed in Table I along with other baseline characteristics. The asthma and healthy control groups were matched for age and smoking histories. The patients with COPD were older than the other groups ($P < .001$) and had a longer smoking history ($P < .001$ for pack years) and a lower DLCO percentage ($P < .001$). Sputum eosinophil percentages were increased in smokers and nonsmokers with asthma and in exsmokers with COPD compared with those seen in the healthy control groups. Percentage neutrophils in sputum were increased in exsmokers with COPD but not current smokers with COPD compared with those seen in healthy smokers. The percentage of macrophages in sputum proportions were increased in the COPD group compared with that seen in healthy nonsmokers

TABLE I. Demographics and clinical baseline characteristics

	Healthy subjects			Asthmatic patients			Patients with COPD				
	Nonsmoker	Smoker	*P* value	Nonsmoker	Smoker	*P* value	All	Exsmoker	Smoker	*P* value	*P* value*
No.	26	20		53	56		53	25	28		
Age (y)	49.9 (42.1-55.8)	53.3 (45.1-57.8)	.669	48.6 (38.0-54.2)	46.7 (40.7-52.9)	.793	65.0 (61.2-68.9)	66.2 (63.1-73.3)	62.0 (59.0-65.4)	**.004**	**<.001**
Sex, male/female	8/18	9/11	.369	22/31	27/29	.564	23/30	14/11	9/19	.101	.649
Disease duration (y)	–	–	–	21.0 (9.0-36.0)	18.5 (10.0-31.2)	.443	3.0 (1.0-7.0)	3.0 (1.0-7.0)	3.5 (1.8-7.0)	.989	**<.001**
FEV_1 prebronchodilator	2.91 (2.38-3.31)	2.63 (2.41-3.18)	.369	2.34 (1.88-2.78)	2.46 (1.75-2.90)	.820	1.40 (1.02-1.95)	1.32 (0.96-2.15)	1.43 (1.21-1.80)	.920	**<.001**
FEV_1 (% predicted) prebronchodilator	105.5 (95.2-115.0)	92.5 (87.5-100.2)	**.011**	79.0 (70.0-91.0)	78.0 (58.0-93.2)	.599	62.0 (45.0-77.0)	60.0 (39.0-76.0)	63.0 (48.8-78.0)	.648	**<.001**
FEV_1 (% predicted) postbronchodilator	–	–	–	91.0 (79.0-98.0)	84.0 (70.5-102.0)	.429	65.0 (48.0-80.0)	63.0 (46.0-77.0)	67.0 (50.8-80.2)	.692	**<.001**
FEV_1/FVC ratio postbronchodilator	82.0 (79.8-84.2)	78.0 (77.8-81.0)	.110	78.0 (69.0-83.0)	72.5 (66.8-79.2)	**.029**	64.0 (50.0-67.0)	57.0 (48.0-69.0)	65.0 (55.2-66.2)	.433	**<.001**
Years smoked	–	30.0 (28.0-37.2)	–	–	30.0 (22.8-40.0)	–	40.0 (35.0-50.0)	40.0 (30.0-48.0)	43.0 (39.8-50.0)	.100	**<.001**
Pack years smoked	–	31.0 (24.5-38.0)	–	–	32.5 (21.8-60.0)	–	50.0 (38.0-72.0)	40.0 (26.0-60.0)	63.0 (44.8-80.0)	**.005**	**<.001**
D_{LCO} (% predicted)	–	–	–	86.0 (80.0-92.8)	77.0 (65.5-85.5)	**.001**	60.0 (47.5-70.0)	58.5 (43.0-69.2)	60.0 (50.0-70.0)	.491	**<.001**
$FENO_{50}$ (ppb)	17.3 (9.4-21.8)	7.5 (5.1-16.8)	.056	26.4 (13.4-43.6)	9.0 (6.7-13.4)	**<.001**	11.1 (6.0-23.2)	15.0 (10.6-26.8)	6.4 (5.0-10.1)	**<.001**	.105
Total IgE (ng/mL)	2.0 (2.0-24.0)	2.0 (2.0-38.0)	.668	74.0 (2.0-388.0)	2.0 (2.0-669.0)	**.040**	2.0 (2.0-206.0)	2.0 (2.0-379.0)	2.0 (2.0-202.0)	.520	**.004**
Plasma cotinine (ng/mL)	0.9 (0.3-1.1)	29.2 (15.8-42.1)	**<.001**	0.4 (0.2-0.6)	32.5 (23.5-43.6)	**<.001**	27.2 (0.6-40.5)	0.6 (0.3-1.1)	40.5 (32.4-43.0)	**<.001**	.293
Inhaled steroid, no/yes	26/0	20/0	–	9/44	13/43	.479	23/30	7/18	16/12	.052	**<.001**
Beclometasone equivalent dose of inhaled steroid	–	–	–	800 (400-1000)	800 (700-1000)	.338	1250 (950-2000)	1000 (800-2000)	2000 (1000-2000)	.392	**<.001**
Total cell count (10^6/mL)	0.20 (0.09-0.54)	0.22 (0.18-1.24)	.268	0.34 (0.11-0.75)	0.48 (0.24-1.09)	.205	0.56 (0.22-1.05)	0.64 (0.20-1.68)	0.56 (0.25-0.99)	.694	.054
Neutrophils (%)	27.0 (16.0-59.2)	50.8 (32.6-69.8)	.101	44.5 (25.0-66.2)	59.8 (39.5-71.1)	.321	64.8 (40.6-76.9)	68.0 (57.9-75.6)	63.8 (32.5-81.5)	.359	**.007**
Eosinophils (%)	0.0 (0.0-0.5)	0.0 (0.0-0.4)	.789	1.0 (0.0-1.9)	0.5 (0.0-1.5)	.563	0.5 (0.0-1.0)	0.7 (0.0-1.5)	0.1 (0.0-1.0)	.273	**.001**
Macrophages (%)	40.0 (19.0-64.5)	29.2 (24.4-49.1)	.357	32.8 (17.9-53.6)	23.5 (14.9-39.5)	.174	21.2 (11.4-33.4)	19.8 (12.6-30.6)	21.8 (11.0-36.9)	.303	**.004**
%LAA−950	–	–	–	5.4 (2.8-8.8)	4.8 (2.7-7.1)	.258	8.1 (4.6-14.2)	13.3 (7.6-16.0)	5.4 (3.1-8.4)	**.001**	**<.001**

Data are depicted as medians (interquartile ranges) unless otherwise specified. Boldface *P* values indicate $P < .05$.
$Feno_{50}$, Exhaled nitric oxide measured at a flow rate of 50 ppb.
*Kruskal-Wallis tests comparing all clinical groups with data available.

($P = .03$) but were similar to the percentage seen in healthy smokers. The extent of emphysema (%LAA−950) was greater in exsmokers with COPD than in current smokers with COPD ($P = .001$) and in all patients with COPD compared with smokers or nonsmokers with asthma ($P = .002$) but not between smokers with asthma and smokers with COPD ($P = .645$). In subgroups based on severity of disease (see Table E1 in this article's Online Repository at www.jacionline.org),[19,20] there was an association between age, duration of disease, and increasing severity of emphysema, as assessed by %LAA−950 but not with pack years smoking history. Compared with the other COPD categories, there was a higher proportion within the severe category who were exsmokers (11/24) than current smokers (5/29; $P = .036$, Fisher exact test).

Sputum MMP-12 concentrations and activity

The protein concentrations and enzyme activities of MMP-12 in the sputum fluid of subjects in the different clinical categories are listed in Table II and in subjects with different severities of disease in Table E2 in this article's Online Repository at www.jacionline.org.

Patients with COPD. Sputum MMP-12 concentrations were increased in the COPD group ($P = .003$), including both current smokers ($P = .011$) and exsmokers ($P = .007$), compared with those seen in healthy nonsmokers but not when compared with those seen in healthy smokers ($P = .165$). Sputum MMP-12 concentrations were similar between current smokers and exsmokers with COPD. Sputum MMP-12 enzyme activity was not increased in the COPD group compared with that seen in healthy

TABLE II. MMP-12 concentration and activity and TIMP-1 and TIMP-2 concentrations in sputum fluid from different patient categories at baseline

	MMP-12 concentration (ng/mL)	MMP-12 activity (ng/mL)	TIMP-1 (mg/mL)	TIMP-2 (mg/mL)
Healthy control subjects				
All	158 (100-310)	294 (165-511)	60.6 (32.7-11.5)	10.2 (5.0-15.2)
Nonsmoker	118 (100-307)	237 (165-418)	35.2 (14.8-67.4)	8.0 (4.1-11.2)
Smoker	208 (139-306)	308 (220-606)	101.8 (60.8-149.2)	13.9 (10.4-18.5)
P value*	.131	.119	**.001**	**.003**
Patients with asthma				
All	173 (100-313)	326 (207-644)	77.1 (47.7-131.0)	12.8 (8.5-18.1)
Nonsmoker	100 (100-227)	477 (240-906)	60.5 (46.7-116.5)	10.6 (6.3-18.5)
Smoker	260 (141-365)	271 (179-489)	96.7 (55.0-158.0)	13.1 (9.2-16.8)
P value*	**.003**	**.020**	**.022**	.355
P value§	.657	**.004**	**.011**	**.033**
P value‖	.640	.314	.825	.592
Patients with COPD				
All	296 (175-438)	380 (165-1012)	135.6 (68.3-165.0)	14.9 (10.0-21.5)
Exsmoker	326 (166-695)	756 (272-1694)	113.4 (68.3-166.1)	19.8 (10.0-29.2)
Smoker	276 (175-352)	231 (165-522)	141.1 (78.7-158.6)	13.3 (10.0-17.7)
P value*	.353	**.006**	.624	.151
P value†	**.003**	.102	**<.001**	**<.001**
P value‡	.165	.942	.323	.622
P value§	**.007**	**.003**	**.001**	**.001**
P value‖	.270	.173	.258	.915

Boldface *P* values indicate $P < .05$.
*Smokers versus nonsmokers or exsmokers.
†All versus healthy nonsmokers.
‡All versus healthy smokers.
§Nonsmokers or exsmokers versus healthy nonsmokers.
‖Smokers versus healthy smokers.

nonsmokers ($P = .102$) or healthy smokers ($P = .942$). Exsmokers with COPD had higher concentrations of sputum MMP-12 enzyme activity than current smokers with COPD ($P = .006$). Sputum MMP-12 concentrations and activity did not differ significantly among the mild, moderate, and severe COPD groups (Fig 1 and see Table E2).

Patients with asthma. Sputum MMP-12 concentrations were increased in smokers with asthma compared with those seen in healthy nonsmokers ($P = .035$) but not when compared with those seen in healthy smokers ($P = .64$). Smokers with asthma had higher concentrations of sputum MMP-12 than nonsmokers with asthma ($P = .003$). Sputum MMP-12 enzyme activity was increased in nonsmokers with asthma compared with that seen in healthy nonsmokers ($P = .004$). Sputum MMP-12 concentrations and activity did not differ significantly among the mild, moderate, and severe asthma groups (Fig 1 and see Table E2).

Healthy control subjects. Among healthy control subjects MMP-12 concentrations and activity were similar in smokers compared with those seen in nonsmokers ($P = .131$ and $P = .119$, respectively).

Sputum TIMP-1 and TIMP-2 concentrations

The protein concentrations of TIMP-1 and TIMP-2 in the sputum fluid of subjects in the different clinical categories are listed in Tables II and E2.

Patients with COPD. Sputum TIMP-1 and TIMP-2 concentrations were increased in patients with COPD (TIMP-1, $P < .001$; TIMP-2, $P < .001$), including both current smokers ($P < .001$ and $P = .002$, respectively) and exsmokers with COPD (TIMP-1, $P = .001$; TIMP-2, $P = .001$), compared with those seen in healthy nonsmokers but not when compared with those seen in healthy smokers. Sputum TIMP-1 and TIMP-2 concentrations did not differ significantly among the mild, moderate, and severe COPD groups.

Patients with asthma. Sputum TIMP-1 and TIMP-2 concentrations were increased in smokers ($P < .001$ and $P = .004$, respectively) and nonsmokers with asthma ($P = .011$ and $P = .033$, respectively) compared with those seen in healthy nonsmokers but not when compared with those seen in healthy smokers.

Healthy control subjects. Sputum TIMP-1 and TIMP-2 concentrations were increased in healthy smokers compared with those seen in healthy nonsmokers ($P = .001$ and $P = .003$, respectively).

Sputum MMP-12 concentration or activity described as ratios with TIMP-1 or TIMP-2

Results are listed in Table E3 in this article's Online Repository at www.jacionline.org. For each of the 4 ratios of MMP-12 concentration or MMP-12 activity with either TIMP-1 or TIMP-2, there were no significant differences among all subjects in the COPD or asthma groups compared with all control subjects, categories of disease severity in the asthma or COPD groups, and smokers and nonsmokers in the healthy control subjects.

Within the COPD and asthma groups, there were significantly reduced ratios associated with smoking. Among patients with COPD, the smokers had lower ratios than the exsmokers for MMP-12 concentration/TIMP-1 ($P = .019$), MMP-12 activity/TIMP-1 ($P < .001$), and MMP-12 activity/TIMP-2 ($P = .004$). Among asthmatic patients, the smokers had lower ratios than nonsmokers for MMP-12 activity/TIMP-1 ($P = .002$) and MMP-12 activity/TIMP-2 ($P = .003$).

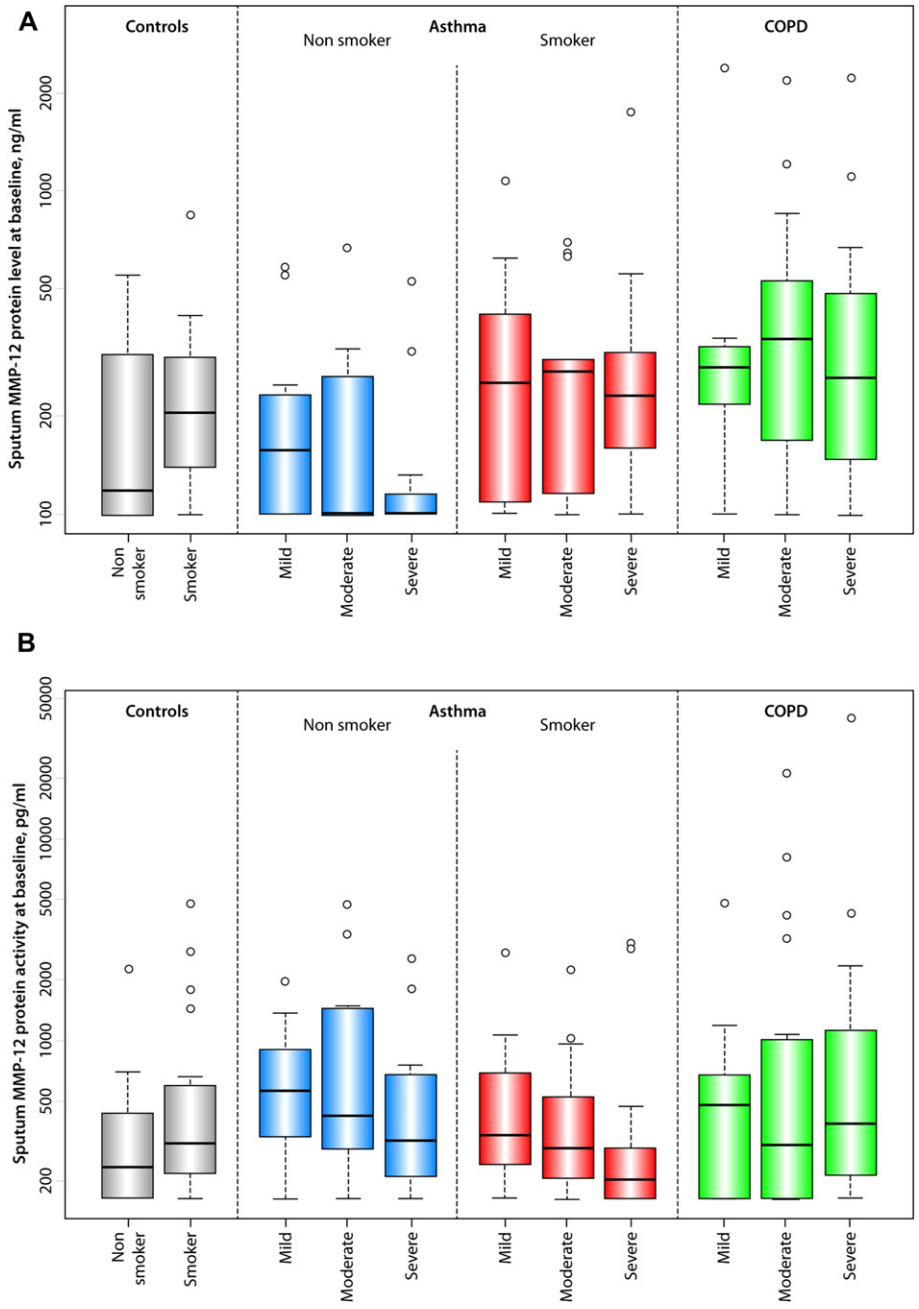

FIG 1. Sputum MMP-12 protein levels **(A)** and activity **(B)** in patients with different severity levels of COPD and asthma and in healthy control subjects. There were no significant differences ($P > .05$) among patients with mild, moderate, and severe disease in each category.

Effect of age and dose of inhaled corticosteroid on sputum MMP and TIMP measurements

Neither age nor dose of inhaled corticosteroid had a significant effect on MMP-12 concentrations and activity or TIMP-1 and TIMP-2 concentrations in the COPD group (adjusting for severity). In the asthma group TIMP-1 and TIMP-2 concentrations slightly increased with age ($P = .01$ and $P = .036$, respectively; data not shown).

Plasma MMP-12 protein and enzyme activity

There were no significant differences in the plasma MMP-12 concentrations between the clinical or smoking categories (Table III). The MMP-12 activity was just at or less than the lowest detection limit of the assay, suggesting plasma levels of less than 165 pg/mL. We were therefore unable to measure the distribution of MMP-12 activity in plasma because of a lack of assay sensitivity.

Sputum cell *MMP12* mRNA expression levels

RNA was isolated from cells processed from induced sputum, and the expression of *MMP12* mRNA was quantified by means of qPCR. Levels were normalized to the expression of β-actin and expressed as the $\log_{10}$ fold change in threshold cycle compared with the mean expression of the healthy nonsmoking control

TABLE III. Plasma MMP-12 concentration and TIMP ratios in healthy volunteers, patients with COPD, and asthmatic patients

	Healthy subjects		Asthmatic patients		Patients with COPD	
	Nonsmoker	Smoker	Nonsmoker	Smoker	Exsmoker	Smoker
No.	26	20	53	56	25	28
MMP-12 (ng/mL)	232 (113-355)	188 (130-384)	239 (133-451)	224 (137-335)	211 (149-298)	262 (187-348)
TIMP-1 (μg/mL)	88.6 (74.9-100.9)	86.4 (77.4-108.7)	84.6 (78.6-95.4)	87.4 (80.5-104.0)	100.0 (92.4-134.2)	86.1 (78.2-95.3)
TIMP-2 (μg/mL)	142 (119-175)	117 (100-157)	144 (116-172)	123 (96-167)	168 (126-193)	119 (92-155)
MMP-12/TIMP-1	2.1 (1.6-3.0)	2.1 (1.4-3.2)	2.4 (1.5-5.4)	2.3 (1.6-3.8)	1.7 (1.4-2.7)	3.2 (1.9-4.6)
MMP-12/TIMP-2	1.4 (1.0-2.6)	1.9 (0.9-3.1)	1.8 (0.8-3.1)	1.6 (1.0-3.1)	1.4 (0.9-1.9)	2.0 (1.5-3.9)

Plasma MMP-12 activity was undetectable in most subjects, and therefore no data are presented. Between-group comparisons were performed by using the Kruskal-Wallis test: $P > .05$ for all groups.

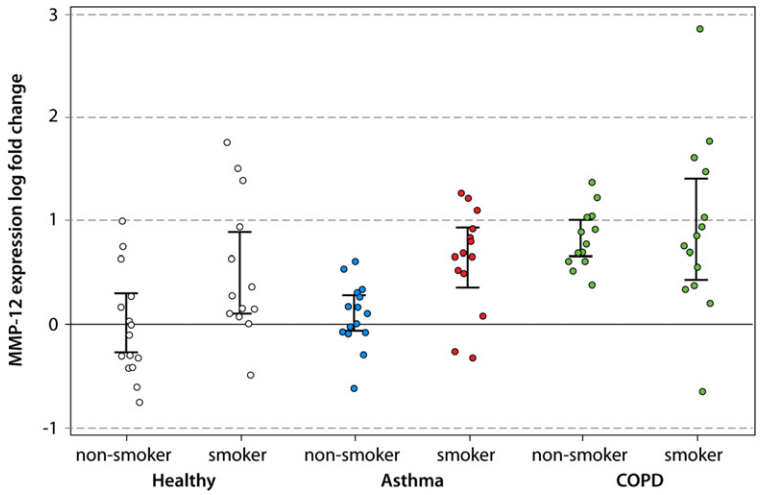

FIG 2. Sputum leukocyte *MMP12* mRNA expression measured by using qPCR and expressed as a $\log_{10}$ fold change in the asthma and COPD smoking categories relative to the mean expression value of the healthy nonsmoking control group normalized for β-actin. A scatter plot including the 95% CI for the mean is shown.

group (Fig 2). Expression levels were unrelated to sputum total leukocyte count or any component of the differential cell count (correlation coefficients): macrophages, −0.050 (95% CI, −0.26 to 0.164; $P = .649$); neutrophils, 0.012 (95% CI, −0.201 to 0.224; $P = .911$); eosinophils, 0.304 (95% CI, 0.099 to 0.486; $P = .013$); lymphocytes, 0.045 (95% CI, −0.169 to 0.255; $P = .686$); and epithelial cells, 0.068 (95% CI, −0.147 to 0.277; $P = .541$). ANOVA demonstrated that the *MMP12* expression was affected by smoking ($P = .00001$) and subject category (healthy subjects, asthmatic patients, and patients with COPD: $P = .0006$). Smokers had higher levels than nonsmokers among the healthy subjects ($P = .032$) and the asthmatic patients ($P = .003$), but in patients with COPD, there was no difference between smokers and exsmokers ($P = .961$). In the clinical categories patients with COPD had higher *MMP12* expression levels than both asthmatic patients ($P = .001$) and healthy subjects ($P < .001$); there was no significant difference between asthmatic patients and healthy subjects ($P = .24$). There was a nonsignificant tendency for *MMP12* expression levels to increase with disease severity in asthmatic patients ($P = .075$) but not in patients with COPD ($P = .526$). In the whole study group the sputum cell *MMP12* mRNA expression correlated with sputum MMP-12 protein levels ($r = 0.49$; 95% CI, 0.31 to 0.63; $P < .001$) but not with activity ($r = 0.03$; 95% CI, −0.18 to 0.24; $P = .815$).

Correlation of sputum MMP-12 protein levels and activity with lung function, CT-quantified emphysema, and sputum cell counts

Lung function. There was no significant association between sputum MMP-12 protein concentrations or activity with postbronchodilator percent predicted FEV_1 or FEV_1/forced vital capacity (FVC) ratio in any of the clinical or smoking categories, except for a modest negative correlation between sputum MMP-12 concentration and postbronchodilator FEV_1 and FEV_1/FVC ratio in smokers with asthma (correlation coefficient: $r = -0.256$ [95% CI, −0.464 to −0.01] and −0.315 (95% CI, −0.534 to −0.057], respectively). There were no significant associations between sputum MMP-12 protein levels or activity and DLCO percent predicted in any of the groups (eg, for the COPD groups, $r = -0.093$ [95% CI, −0.372 to 0.173] and 0.014 (95% CI, −0.239 to 0.276], respectively).

CT measure of emphysema. Sputum MMP-12 protein concentration ($r = 0.377$; 95% CI, 0.126 to 0.591) and MMP-12 activity ($r = 0.442$; 95% CI, 0.198 to 0.639; Fig 3) were each associated with the extent of emphysema (%LAA−950) in the COPD group.

Sputum cell counts. Sputum MMP-12 protein concentration correlated positively with sputum neutrophil counts in all groups: smokers with asthma ($r = 0.324$; 95% CI, 0.058 to 0.559), nonsmokers with asthma ($r = 0.452$; 95% CI, 0.164 to 0.676),

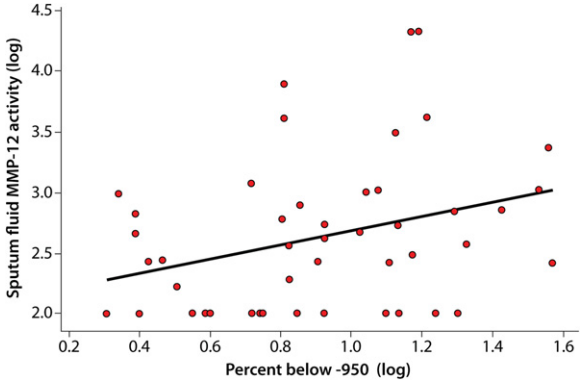

FIG 3. Increasing sputum MMP-12 enzyme activity is associated with emphysema score measured by means of CT assessment of emphysema (%LAA−950) in patients with COPD ($r = 0.442$; 95% CI, 0.198-0.639).

patients with COPD ($r = 0.305$; 95% CI, 0.054 to 0.539), and healthy control subjects ($r = 0.334$; 95% CI, 0.009 to 0.592). Similarly, MMP-12 activity correlated positively with neutrophil count in smokers with asthma ($r = 0.352$; 95% CI, 0.088 to 0.606), nonsmokers with asthma ($r = 0.708$; 95% CI, 0.454 to 0.867), patients with COPD ($r = 0.566$; 95% CI, 0.356 to 0.711), and healthy control subjects ($r = 0.630$; 95% CI, 0.384 to 0.789).

DISCUSSION

This study demonstrated, for the first time, an association between airway fluid MMP-12 protein and enzyme activity and disease severity in patients with COPD when assessed based on the extent of emphysema measured by means of CT scans but not based on spirometric results or DLCO values. In the asthma groups there was no association between disease severity and sputum MMP-12 concentrations or activity.

This study assessed, for the first time, the influence of smoking status on sputum MMP-12 concentrations, enzyme activity and gene expression, and TIMP concentrations in patients with stable COPD and asthma compared with those seen in healthy smoking and nonsmoking subjects. We found that smoking status has an important influence on airway MMP-12 and TIMP concentrations. In patients with COPD, median sputum MMP-12 activity and expression and TIMP concentrations are higher compared with those seen in healthy nonsmokers. In asthmatic patients median sputum MMP-12 concentrations and expression are increased in smokers with asthma compared with those seen in nonsmokers with asthma.

MMPs compromise a structurally and functionally related group of proteolytic enzymes, which play a key role in the tissue remodeling and repair associated with inflammation.[30] The elastin-degrading function of MMP-12[31] has potential importance for lung remodeling in patients with COPD, and its importance was confirmed in MMP knockout mice that were protected against smoke-induced emphysema.[32] In human subjects confirmation of specific involvement has been hampered by the adequacy of immunoreactive and functional MMP-12 measurements. Using extensively validated assays, we have confirmed and extended the observations of Demedts et al,[17] who reported that sputum MMP-12 concentrations in patients with mild-to-moderate COPD were higher than those in healthy smokers and that levels were similar between smokers and exsmokers with COPD. In the present study we found that median sputum MMP-12 concentrations in patients with COPD (mild, moderate, and severe; smokers and exsmokers) were higher than those in healthy nonsmokers. However, in contrast to the study by Demedts et al, we observed increased MMP-12 concentrations in the sputum of healthy smokers compared with those seen in nonsmokers. This is in keeping with the observation of equivalently increase sputum MMP-12 levels in smokers with either mild disease (stage 0, Global Initiative for Chronic Obstructive Lung Disease criteria) or no symptoms compared with nonsmokers,[33] and these observations might be valuable in understanding whether MMP-12 could contribute to the onset and progression of disease.

Increased MMP-12 concentrations have been localized by means of immunocytochemistry to macrophages in bronchoalveolar lavage fluid and biopsy specimens in patients with COPD,[33,34] and by using expression data, *MMP12* is one of the most highly transcribed genes in alveolar macrophages from healthy smokers.[16] We observed increased *MMP12* expression in sputum cells, suggesting that sputum MMP-12 is derived from airway leukocytes. We observed that *MMP12* expression was markedly higher in patients with COPD who were smokers and exsmokers compared with healthy smokers. This suggests that factors in addition to smoking are driving *MMP12* expression in patients with COPD. For example, in patients with COPD, we have identified increased cytokine concentrations in sputum fluid, primarily the chemokines CCL4 and CXCL9, which are secreted by IFN-γ–activated macrophages that might recruit mononuclear cells into the airways to amplify the macrophage population, and IFN-γ and CXCL9, which strongly induce MMP-12 production by macrophages.[9] The concentration of these mediators in sputum correlated strongly with MMP-12 levels and suggests that MMP-12 turnover is much more dynamic in smokers and in patients with COPD. This is supported by the observation that the increased *MMP12* expression in patients with COPD is not always reflected by increased MMP-12 concentrations and activity compared with those seen in healthy smokers, suggesting some post-transcriptional regulation; for example, MMP-12 is secreted as an inactive proenzyme that requires activation, and thereafter the active form of MMP-12 is able to autodigest, thereby limiting its activity. MMP-12 in plasma is further regulated by binding to and inactivation by α_2-macroglobulin and α_1-antitrypsin,[35] which might partly explain our difficulties with its detection in plasma. Thus the dynamics of MMP expression, protein, and activity is likely to be more informative of its specific role in patients with COPD.

In the present study MMP-12 activity was higher in exsmokers with COPD compared with that seen in smokers with COPD, and this suggests that the smoking effect, increasing MMP-12 activity, was not reversible and that more severe disease can be associated with dysregulated MMP-12 function.

In patients with COPD, disease severity, when assessed by the extent of emphysema on CT scanning but not by using spirometric values, was significantly associated with sputum MMP-12 concentrations and activity. In addition, we found no association between sputum MMP-12 levels and DLCO values, and although the degree of emphysema measured by means of CT often correlates with DLCO in patients with COPD, this association is not always found.[25] The association of an index of emphysema with MMP-12 activity supports a role for MMP-12 in the pathogenesis of emphysema and might suggest that blocking MMP-12 activity

in patients with COPD might prevent the further development of emphysema. This hypothesis must be tested in clinical trials of MMP-12 inhibitors in patients with COPD. Alternatively, the increased sputum MMP-12 levels in patients with COPD might be a marker of severity in those who already have emphysema.

Sputum MMP-12 levels were similar in the healthy smokers and patients with COPD, and in future studies, it would be interesting to perform CT scans in healthy smoking volunteers to examine whether higher levels were associated with early CT changes of emphysema. Given that smoking does not cause COPD in all patients yet smoking does increase MMP-12 concentrations, it does suggest that tissue susceptibility to the effect of MMP-12 and other inflammatory mediators results in the development of COPD rather than only MMP-12 itself.

There is limited information in the literature about MMP-12 concentrations, activity, and mRNA expression in asthmatic patients. Increased MMP-12 can be visualized by using immunohistochemistry within the extracellular matrix associated with airway smooth muscle in the large airways of patients who died from asthma compared with control subjects.[36] We found that MMP-12 concentrations were higher in smokers compared with those seen in nonsmokers with asthma and were inversely associated with postbronchodilator FEV_1 and FEV_1/FVC ratio in smokers with asthma. It has been suggested that an increase in MMP-12 concentration[9] might be one of the factors associated with the accelerated loss of lung function seen in smokers with asthma.[37] We found that nonsmokers with asthma had higher median levels of MMP-12 activity and TIMP-1 and TIMP-2 concentrations compared with those seen in healthy nonsmokers. None of these median concentrations were higher than those seen in healthy smokers, illustrating the strong effect that smoking has on these mediators. In allergen-induced experimental lung injury MMP-12 expression was upregulated,[38] whereas a genetic deficiency in MMP-12 resulted in a marked overall reduction in airway inflammation.[39] Airway smooth muscle cells express *MMP12* mRNA in tissue from healthy and asthmatic subjects,[40] and using explanted airway smooth muscle cells, exogenous TGF-β had no effect, but IL-1β and TNF-α strongly induced *MMP12* gene expression and protein activity, which was suppressed by dexamethasone. MMP-12 can also be produced by epithelial cells in smokers.[12] Our MMP-12 data showed increased expression levels in sputum cells from smokers with asthma compared with those seen in healthy nonsmoking subjects, but these levels were not increased compared with those seen in healthy subjects who smoked.

MMP-12 might be more involved in exacerbations than in stable disease, especially because it is increased in viral infections. The participants' disease was stable at the time of study, and therefore an important follow-up to this study would be to monitor sequential sputum MMP-12 concentration and activity over time and during exacerbations in either asthmatic patients or patients with COPD. Finally, other than the primary end point of sputum MMP-12 levels, this is an exploratory (hypothesis generating) study with the opportunity to study differences between different patient populations. The usual convention is not to adjust for multiple comparisons for this type of study, and the interpretation of such results should be treated with caution.

In conclusion, sputum MMP-12 concentrations, enzyme activity, and mRNA expression are influenced by smoking status in both patients with COPD and asthmatic patients. Smokers with asthma have higher concentrations of sputum MMP-12 than never smokers with asthma, but neither sputum MMP-12 concentrations nor activity differ among the mild, moderate, and severe asthma groups, suggesting that MMP-12 is not directly linked to disease progression in asthma among nonsmokers. Disease severity in patients with COPD, when assessed based on the extent of emphysema measured by using CT but not by using spirometry or diffusing capacity is directly associated with sputum MMP-12 concentrations and activity. Interventions directed at inhibiting MMP-12 activity should be investigated for their potential to prevent the development or deterioration in patients with COPD.

We thank Annick DeVries, Anna Tommasi, Agnieszka Kieloch, and Trupti Dhanani for analysis of TIMP samples.

Key messages

- **Sputum MMP-12 concentrations and activity in patients with COPD, but not in asthmatic patients, are directly associated with the extent of emphysema measured by means of CT.**
- **Blocking MMP-12 might prevent the progression of emphysema.**

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METHODS

Subjects

All subjects were taking stable medication for 4 weeks and had no exacerbation of disease for 4 weeks. Smokers were defined as smoking at least 10 pack years and currently smoking 5 or more cigarettes per day.

Asthma. Age range was 18 to 75 years, and duration of asthma was 6 months or longer. Symptoms of episodic wheezing, chest tightness, and/or dyspnea were present. Objective confirmation based on methacholine airway hyperresponsiveness (airway hyperactivity determined by a ≥20% decrease in FEV_1 at a methacholine dose of ≤8 mg/mL) or where this was not safe (when FEV_1 <60% of predicted value) by evidence of airflow variability with greater than or equal to 12% and 200-mL increase in FEV_1 after 2.5 mg of nebulized albuterol.

COPD. Patients with COPD were recruited from hospital clinics and the community based on the following criteria. Age range was 40 to 80 years, and the duration of COPD was 6 months or greater. Symptoms required were cough and breathlessness, history of smoking, FEV_1/FVC ratio after bronchodilator of less than 70%, and FEV_1 of 30% to 80% of predicted value with reversibility to 2.5 mg of albuterol of less than 12% and 200 mL. Current smoking status did not influence recruitment to the study.

Healthy control subjects (smokers and nonsmokers). Age range was 18 to 80 years. No known respiratory disease or chronic respiratory symptoms were present, with normal spirometric results (FEV_1/FVC ratio, >80%; FEV_1, >80% of predicted value).

Measurements

Clinical and physiologic measurements. Exhaled nitric oxide was measured at 50 mL/s in concordance with standardized guidelines (Niox Flex; Aerocrine, Solna, Sweden).[E1] Exhaled carbon monoxide levels were measured with a handheld Smokerlyser (Bedfont Scientific Ltd, Bedford, United Kingdom).

Blood samples were collected into lithium heparin bottles for plasma. Standard sputum induction[E2] and processing[E3] for cytology and fluids were performed. Induced sputum cell RNA was isolated (Ambion mirVana RNA kit; Applied Biosystems, Warrington, United Kingdom) and stored at −80°C until use. RNA quality was assessed with Bioanalyser microfluidic chips (Agilent Technologies, Santa Clara, Calif), and RNA with an integrity number of less than 6.4 was discarded. Samples with cell viability of less than 40% or squamous epithelial cells of greater than 80% were discarded. All samples were individually coded to ensure blinding of laboratory personnel to all patients' details.

MMP-12 immunoreactivity was quantified by using ELISA, and MMP-12 activity was quantified by using the FRET assay,[E3] as detailed below. MMP-12 is a primary end point, and therefore these assays were developed to improve on existing methods (by screening for the best buffers, by using a capture FRET rather than a homogeneous assay, and by using an alkaline phosphatase enzyme conjugate amplification step to improve sensitivity) and extensively validated (by demonstrating good interassay and intra-assay precision and good accuracy using quality control validation samples and by careful demonstration that MMP-9 up to 4-log order excess did not interfere with the assay). These are described in Matsuoka et al.[E4]

Anti–MMP-12 mAb (catalog no. DDX 0284; Dendritics, Bioparc Laennec, Lyon, France) was coated for 1 hour at 37°C in pH 9.6 carbonate buffer. The plate was washed and blocked with Superblock (Thermo Fisher Scientific, Rockford, Ill) for 1 hour at 37°C and washed before addition of sample/standard. Sputum samples were diluted 1:4 in ESD100 buffer (Cell Technologies, Inc, Mountain View, Calif) supplemented with Immunoglobulin Inhibiting Reagent (0.05 mg/mL; Sera Laboratories International Ltd, Bolney Grange Business Park, West Sussex, United Kingdom). The samples/standards were incubated overnight at 4°C before washing with Tris-buffered saline containing 0.05% Tween-20. The detection antibody (catalog no. DDX0281, Dendritics) directly conjugated to alkaline phosphatase (in-house conjugation) was incubated in Tris buffer for 1 hour at room temperature. Detection used an alkaline phosphatase amplification system (catalog no. 19589-019; Invitrogen, Paisley, United Kingdom), as per the manufacturer's instructions. The assay's lower limit of detection was 25 pg/mL.

Sputum fluid MMP-12 enzyme activity was quantified as follows. Briefly, anti–MMP-12 mAb (catalog no. DDX0282, Dendritics) was coated in pH 9.6 carbonate buffer on StarWell plates (catalog no. 441653; Nunc, Fisher Scientific UK, Loughborough, United Kingdom) for 2 hours at room temperature. The plate was washed and blocked with Superblock (Thermo Fisher Scientific) for 2 hours at room temperature. The plate was washed again before addition of sample or standard and diluted in BlottoB (B553-0500; Rockland Immunochemicals, Inc, Gilbertsville, Pa), followed by an overnight incubation at 4°C. Sputum supernatant was diluted 1:2 for assay. The FRET substrate (MMP-12 FRET substrate III; Anaspec, Fremont, Calif) was incubated on the plate for 24 hours at room temperature before a single end point was read. The results are recorded in relative fluorescent units per minute and titrated against a standard curve in picograms per milliliter. The lower limit of detection for the MMP-12 FRET assay is 82.3 pg/mL.[E2]

Validation of MMP and TIMP measurements

The validations of MMP and TIMP measurements is detailed below and by LaPan et al.[E2] The intra-assay and interassay precision of the read-back values for 6 assay calibrators are shown in Table E4.

The precision of measurements of MMP-12 concentrations is shown in Table E5. Test samples were randomized on the assay plate layout and tested in duplicate. Between-plate variation was minimized by incorporating standard curves and internal quality control samples, and some samples were assayed on different plates for reproducibility.

Sputum samples were collected 4 times over 6 weeks from 24 patients with COPD. ANOVA demonstrated no effect of replicates on concentrations or activities.

A multiplex kit (catalog no. LKT003) supplied by R&D Systems was used to determine the concentrations of TIMP-1 and TIMP-2. This kit was used on a Bioplex analyzer (BioRad, Hercules, Calif), which uses a dual laser and a flow-based sorting and detection platform. The assays are based on the sandwich immunoassay principle with precoated analyte-specific antibodies bound to color-coded microparticles, and respective detection antibodies are biotinylated. Streptavidin phycoerythrin conjugate binds the biotinylated detection antibodies. One laser determines the analyte being detected (color-coded microparticles), and the other measures the phycoerythrin-derived signal, which is proportional to the concentration of analyte. Samples were diluted 1:50 for assay and the kit to which the protocol was adhered.

Protein levels were measured with the Pierce 660-nm Protein Assay Product no. 1861426 (working range, 62.5-2000 μg/mL; Pierce, Cheshire, United Kingdom).

In sputum supernatants the concentrations of human cytokines (IL-1β, IL-1Ra, IL-2, soluble IL-2 receptor, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40/70, IL-13, IL-15, IL-17, and TNF-α), interferons (IFN-α and IFN-γ), chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL9, and CXCL10), and growth factors (epidermal growth factor, fibroblast growth factor β, granulocyte colony-stimulating factor, GM-CSF, hepatocyte growth factor, and vascular endothelial growth factor) were quantified simultaneously by using a sensitive cytokine antibody bead kit (lot no. 497124) from Invitrogen. The validation and sensitivity details are available at http://products.invitrogen.com/ivgn/product/LHC6003. The assay was a standard Luminex fluoroimmunoassay, and activity was measured with a Bio-Plex System platform (Bio-Rad Laboratories Ltd, Hemel Hempstead, United Kingdom). Samples were tested in duplicates, and concentrations were calculated by means of interpolation into a standard curve of known values by using NIBSC calibration standards. Concentrations that were less than the lower level of detection rather than being described as zero were assigned a concentration that was half of the lower level of detection for the purposes of using the data in analyses that required log transformation.

Sputum cells were harvested in RNase-free Eppendorf tubes, and the RNA in the cell pellet was stabilized by adding 0.6 mL of lysis reagent (Ambion mirVana RNA isolation kit, Applied Biosystems) and stored at −80°C until use.

For sputum RNA, total RNA was isolated with the Ambion mirVana isolation kit, according to the manufacturer's instructions, with inclusion of on-column DNAse treatment. Typical yields from this technique are in the range of 5 μg of total RNA. Total RNA quality metrics (RINs) were assessed

by using Bioanalyser microfluidic chips (Agilent), and RNAs with RINs of less than 6.4 were discarded from further analysis.

For sputum cDNA generation, the Qiagen Quantitect Reverse Transcription kit was used according to the manufacturer's instructions by using 200 ng of total RNA as input. qPCR was performed with the Applied Biosystems 7900HT Fast Real-Time System. An SyBr Green assay (SyBr Green Master mix/Fast-optical 96 well plate from Applied Biosystems), which allows for specificity of primers to be checked, was used with the following primers and cycling conditions: *MMP12* forward, 5′-*AGGAATCGGGCCTAAA ATTG-3′*; *MMP12* reverse, 5′-*TGCTTTTCAGTGTTTTGGTGA-3′*; β-actin forward, 5′-*ACAGAGCCTCGCCTTTGCCG-3′*; and β-actin reverse, 5′-*TTGCACATGCCGGAGCCGTT-3′*.

Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 repeats of 95°C for 15 seconds/60°C for 1 minute, and a dissociation stage was added at the end point of the cycle to ensure a single product. This was performed by using representative batches of 15 subjects from each of the following categories: smokers and exsmokers with moderate-to-severe COPD, smokers and nonsmokers with severe asthma, healthy smokers, and nonsmoking healthy control subjects.

MMP12 RNA levels were determined relative to β-actin by using the ΔΔ cycle threshold (ΔΔCt) method and the mean of the healthy nonsmoker subjects as baseline for comparison to determine fold change.

Serum total IgE measurements were determined by using the enzyme immunoassay, according to the manufacturer's instructions (catalog no. E88-108; Bethyl Laboratories, Inc, Montgomery, Tex) and calibrated to an international standard. The lower limit of detection was 2 ng/mL.

Statistical analysis

At each visit, the mean, median, and range were estimated for each biomarker for asthma and COPD and for the smoking and severity disease subgroups. Mixed effects normal linear models were used to estimate the within- and between-subject variability for each marker while taking account of disease group and smoking status and testing for any group by smoking status interaction. For the within-disease group, a similar model was used to assess the association of disease severity with biomarkers. Associations of MMP-12 concentrations with levels of plasma and sputum cytokines and other biomarkers were assessed by using the Spearman rank correlation coefficient with bootstrap 95% CIs. Reproducibility of biomarker measurements across visits was assessed by using the Bland-Altman method to determine the upper and lower limits of agreement. Bootstrap 95% CIs for these limits of agreement were calculated. Where necessary, the biomarker values were subjected to a natural log transformation to ensure that the assumptions of the Bland-Altman approach were satisfied. The *t* test, Wilcoxon test, ANOVA, Kruskal-Wallis test, and exact Fisher test were used, as appropriate. All analyses have been carried out in R version 2.11.0 software.[E5] *P* values are not adjusted for multiple testing and have to be considered descriptive.

RESULTS

Intra-assay and interassay precision of MMP-12 measurement

The intra-assay and interassay precision of MMP-12 measurement in duplicate samples from 3 separate aliquots of each of the 3 validation samples (low, mid, and high) in 5 independent analytic runs was determined. For intra-assay precision, the percentage coefficient of variation for each validation sample was determined in each analytic run. The interassay precision (percentage coefficient of variation) was determined in 5 analytic runs. This generated 15 MMP-12 concentration values (3 aliquots in 5 runs) for intra-assay data and 5 for interassay data. Results showed good intravariation and intervariation (Table E4).

Sputum MMP-12 concentration or activity described as ratios with TIMP-1 or TIMP-2

Results are listed in Table E3. For each of the 4 ratios of MMP-12 concentration or MMP-12 activity with either TIMP-1 or TIMP-2, there were no significant differences between all subjects in the COPD or asthma groups compared with all control subjects, categories of disease severity in the asthma or COPD groups, and smokers and nonsmokers among the healthy control subjects.

Within the COPD and asthma groups, there were significantly reduced ratios associated with smoking. In the COPD group the smokers had lower ratios than the exsmokers for MMP-12 concentration/TIMP-1 ($P = .019$), MMP-12 activity/TIMP-1 ($P < .001$), and MMP-12 activity/TIMP-2 ($P = .004$). In the asthma group the smokers had lower ratios than the nonsmokers for MMP-12 activity/TIMP-1 ($P = .002$) and MMP-12 activity/TIMP-2 ($P = .003$). The healthy control nonsmokers had the highest ratios, and compared with these, the ratios were significantly lower in smokers with COPD for MMP-12 concentration/TIMP-1 ($P = .027$), MMP-12 activity/TIMP-1 ($P < .001$), and MMP-12 activity/TIMP-2 ($P = .011$). The nonsmoking asthmatic patients also had lower ratios than healthy nonsmokers of MMP-12 concentration/TIMP-1 ($P = .008$) and MMP-12 concentration/TIMP-2 ($P = .022$). There was a significant association between sputum MMP-12 concentration with MMP-12 activity and TIMP-1 and TIMP-2 concentrations in all subjects, irrespective of clinical category (data not shown).

Correlation of sputum MMP-12 protein and sputum mediators

The concentration of a pragmatic range of inflammatory and immunoregulatory cytokines, chemokines, and growth factors was measured in sputum fluid, and the average result obtained from 2 specimens per subject was used for analysis. The association between the mean concentrations and the sputum MMP-12 protein concentration and enzyme activity is shown in Table E6. There was a significant association between sputum MMP-12 with many individual mediators. In the study cohort most of the mediator concentrations themselves correlated, likely reflecting a spectrum of generalized inflammation. The most striking pattern was the association between MMP-12 activity and 18 of the 27 detectable mediators in the COPD category compared with 8 in the healthy category, reflecting increased inflammation in this patient group.

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TABLE E1. Demographics and clinical baseline characteristics of patients with different severities of asthma and COPD

	Asthmatic nonsmoker				Asthmatic smoker				Patient with COPD			
	Mild	Moderate	Severe	*P* value	Mild	Moderate	Severe	*P* value	Mild	Moderate	Severe	*P* value
Age (y)	44.4 (37.1-53.3)	47.4 (41.6-53.0)	51.2 (42.0-58.4)	.611*	44.9 (42.7-49.0)	42.0 (34.4-53.4)	51.5 (44.3-56.3)	.080*	64.7 (59.6, 67.8)	62.8 (60.4, 66.4)	66.4 (62.0, 73.6)	.229*
Sex												
Male	6	5	11	.213‡	11	8	8	.563‡	6	9	8	.833‡
Female	11	12	8		8	9	12		8	14	8	
Disease duration (y)	20.0 (9.0-26.0)	23.0 (8.0-44.0)	21.0 (15.0-42.5)	.181*	14.0 (6.0-39.5)	17.0 (12.0-30.0)	20.0 (13.5-32.0)	.841*	3.5 (1.0-5.8)	3.0 (2.0-9.5)	5.0 (2.8-7.8)	.380†
FEV_1 prebronchodilator	2.36 (1.96-3.17)	2.36 (2.01-2.78)	2.16 (1.50-2.58)	.194*	2.67 (2.04-2.81)	2.50 (2.11-3.35)	1.96 (1.22-2.62)	**.020***	1.98 (1.65-2.69)	1.45 (1.29-2.00)	0.96 (0.81-1.04)	**<.001***
FEV_1 (% predicted) prebronchodilator	87.0 (83.0-96.0)	81.0 (72.0-97.0)	72.0 (61.0-78.0)	**.005***	75.0 (65.0-95.0)	83.0 (76.0-100.0)	65.0 (52.8-83.0)	**.028***	83.0 (78.5-90.5)	62.0 (58.0-73.0)	37.0 (34.5-42.8)	**<.001***
FEV_1 postbronchodilator	2.7 (2.1-3.3)	2.6 (2.3-3.4)	2.4 (2.0-3.0)	.430*	2.9 (2.3-3.2)	2.6 (2.2-3.5)	2.1 (1.8-2.8)	**.050***	2.1 (1.8, 2.8)	1.5 (1.3, 2.0)	1.1 (0.9, 1.1)	**<.001***
FEV_1 (% predicted) postbronchodilator	95.0 (86.0-98.0)	95.0 (79.0-102.0)	80.0 (75.0-92.0)	**.014***	84.0 (71.0-102.0)	86.0 (79.0-106.0)	75.5 (65.0-94.0)	.096*	88.0 (82.2-93.2)	66.0 (57.5-75.0)	40.0 (35.8-47.2)	**<.001***
Years smoked	–	–	–	–	27.0 (21.0-33.0)	25.0 (17.0-40.0)	31.5 (29.5-40.8)	.102*	40.0 (31.2-42.2)	43.0 (40.0-50.0)	40.0 (28.8-50.0)	.330*
Pack years smoked	–	–	–	–	35.0 (21.5-61.5)	25.0 (21.0-40.0)	34.0 (22.8-60.0)	.416†	44.5 (32.5-68.8)	64.0 (45.0-80.0)	40.5 (29.0-60.5)	.118†
D_{LCO} (% predicted)	82.0 (80.0-92.0)	88.0 (82.2-93.5)	86.0 (79.0-95.0)	.670*	77.0 (68.5-81.0)	80.0 (74.0-91.0)	68.0 (58.0-80.5)	.102*	64.5 (56.2-71.0)	61.0 (50.0-70.0)	43.0 (35.0-59.5)	**.014†**
$F_{ENO_{50}}$ (ppb)	30.5 (19.8-42.6)	16.6 (13.4-39.4)	24.3 (10.5-43.7)	.688†	10.2 (7.8-13.1)	8.8 (6.9-12.9)	8.8 (5.3-13.6)	.732†	13.5 (6.6-25.1)	8.5 (6.1-21.3)	12.1 (6.0-18.5)	.884*
Serum cotinine (ng/mL)	0.4 (0.2-0.6)	0.3 (0.2-0.5)	0.5 (0.3-0.9)	.140*	37.1 (31.3-44.3)	25.5 (20.9-36.9)	31.3 (22.9-41.7)	.232*	33.5 (0.3-40.9)	27.7 (1.1-41.0)	1.1 (0.4-30.3)	.172*
On inhaled corticosteroids												
No	9 (52.9%)	0 (0.0%)	0 (0.0%)	**<.001‡**	13 (68.4%)	0 (0.0%)	0 (0.0%)	**<.001‡**	10 (71.4%)	12 (52.2%)	1 (6.2%)	**<.001‡**
Yes	8 (47.1%)	17 (100%)	19 (100%)		6 (31.6%)	17 (100%)	20 (100%)		4 (28.6%)	11 (47.8%)	15 (93.8%)	
Beclometasone equivalent dose of inhaled corticosteroids	400 (350-400)	500 (400-800)	1200 (800-2000)	**<.001†**	200 (200-425)	800 (800-800)	1000 (800-2000)	**<.001†**	1400 (1100-1700)	1500 (1000-2000)	1000 (900-2000)	–§

Data are depicted as medians (interquartile ranges). Boldface *P* values indicate statistical significance.

$Feno_{50}$, Exhaled nitric oxide measured at a flow rate of 50 ppb.

*ANOVA.

†Kruskal-Wallis test.

‡Fisher exact test.

§At least 1 group too small.

TABLE E2. MMP-12 concentrations and activities, TIMP-1 and TIMP-2 concentrations, and MMP/TIMP ratios in sputum from patients with different severities of asthma and COPD

	Asthmatic nonsmoker			Asthmatic smoker			Patient with COPD		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
No.	17	17	19	19	17	20	14	23	16
MMP-12 concentration (ng/mL)	158 (100-226)	100 (100-265)	100 (100-116)	257 (114-398)	275 (115-300)	233 (160-319)	284 (220-330)	346 (170-521)	263 (148-480)
MMP-12 activity (ng/mL)	566 (337-827)	424 (294-1463)	317 (213-633)	338 (245-678)	294 (208-525)	204 (165-292)	476 (165-649)	306 (165-1017)	390 (216-1114)
TIMP-1 (mg/mL)	63.8 (42.3-110.0)	54.4 (51.2-90.7)	69.3 (52.5-117.1)	96.7 (50.6-157.4)	110.6 (53.4-142.7)	85.8 (63.1-169.8)	114.7 (63.3-140.2)	145.0 (82.6-169.8)	153.1 (51.3-160.6)
TIMP-2 (mg/mL)	9.87 (5.96-16.26)	10.57 (7.41-16.03)	14.00 (8.91-19.91)	11.66 (8.29-16.38)	14.06 (9.00-24.95)	13.21 (9.47-15.64)	12.91 (10.32-21.28)	16.21 (9.96-20.35)	17.72 (9.95-24.60)
MMP-12 concentration/ TIMP-1	3.1 (2.1-4.5)	2.6 (1.9-4.5)	2.1 (1.1-3.2)	2.2 (1.5-3.0)	3.4 (1.8-4.0)	2.6 (1.9-4.2)	2.9 (1.8-3.9)	3.0 (1.6-5.6)	3.2 (2.3-4.0)
MMP-12 activity/TIMP-2	18.3 (14.5-25.7)	15.8 (13.1-18.8)	10.1 (8.3-19.7)	13.9 (11.3-33.3)	17.0 (12.2-24.2)	19.4 (14.4-28.8)	18.1 (10.1-32.9)	20.7 (12.9-27.3)	19.7 (14.3-29.0)
MMP-12 concentration/ TIMP-1	8.7 (6.8-12.4)	4.7 (3.0-29.9)	5.7 (2.6-12.1)	5.3 (2.1-7.8)	6.9 (1.5-7.6)	2.3 (1.8-6.6)	3.6 (2.5-6.8)	3.7 (1.8-9.9)	5.2 (4.1-11.8)
MMP-12 activity/TIMP-2	59.6 (41.5-91.4)	40.6 (23.0-165.4)	36.5 (22.3-59.0)	31.4 (18.1-58.1)	34.8 (12.4-53.4)	17.6 (14.4-30.0)	32.9 (17.9-44.5)	25.8 (14.2-56.1)	37.2 (22.2-72.6)

Data are depicted as medians (interquartile ranges).

TABLE E3. MMP-12/TIMP ratios (in nanograms per milligram) in sputum fluid from different patient categories at baseline

	MMP-12/TIMP-1	MMP-12/TIMP-2	MMP-12 activity/ TIMP-1	MMP-12 activity/ TIMP-2
Healthy control subjects				
All	3.29 (2.15-7.92)	22.74 (11.38-29.22)	5.20 (2.94-15.73)	37.29 (23.51-60.40)
Nonsmoker	5.50 (2.24-9.37)	24.11 (14.26-33.55)	8.91 (3.55-17.51)	40.13 (27.54-58.79)
Smoker	3.11 (1.95-3.86)	22.28 (10.51-26.11)	4.89 (2.71-11.64)	27.17 (23.38-56.89)
P value*	.115†	.269†	.185†	.193†
Asthmatic patients				
All	2.60 (1.67-4.15)	16.47 (10.93-24.28)	6.06 (2.32-10.71)	33.93 (17.13-64.19)
Nonsmoker	2.68 (1.73-4.41)	16.16 (9.82-22.29)	7.13 (3.34-15.70)	42.94 (27.63-95.84)
Smoker	2.51 (1.66-3.99)	17.36 (11.82-31.18)	3.86 (1.72-7.49)	27.32 (15.35-49.37)
P value*	.988†	.263†	**.002**†	**.003**†
P value‖	**.008**†	**.022**†	.872†	.535†
P value¶	.684†	.926†	.312†	.319†
Patients with COPD				
All	3.01 (2.04-4.07)	20.42 (12.15-29.11)	4.29 (1.92-9.26)	29.36 (16.43-53.49)
Exsmoker	3.34 (2.89-5.80)	24.14 (14.05-30.86)	8.21 (4.50-14.99)	47.39 (29.58-107.34)
Smoker	2.64 (1.60-3.25)	19.86 (11.53-27.06)	2.79 (1.77-4.63)	22.59 (15.23-36.22)
P value*	**.019**†	.530†	**<.001**†	**.004**†
P value‡	.107†	.517†	.051†	.288†
P value§	.746†	.575†	.726†	.990†
P value‖	.617†	.798†	.862†	.384†
P value¶	.479†	1.000†	**.048**†	.125†

Boldface *P* values indicate $P < .05$.

*Smokers versus nonsmokers or exsmokers.

†Wilcoxon test.

‡All versus healthy nonsmokers.

§All versus healthy smokers.

‖Nonsmokers or exsmokers versus healthy nonsmokers.

¶Smokers versus healthy smokers.

TABLE E4. Intra-assay and interassay precision (95% CI) of MMP-12 measurement

	Intravariation		Intervariation	
	CV (%)	Bias (%)	CV (%)	Bias (%)
MMP-12 ELISA	0.9 to 12.6	−10.0 to 2.4	0.0 to 13.4	−3.6 to 2.1
MMP-12 activity	3.6 to 16.0	−15.3 to 11.3	0.2 to 9.4	−16.7 to 11.8

CV, Coefficient of variation.

TABLE E5. Intra-assay and interassay precision (95% CI)

Quality control sputum samples	Intravariation CV (%)	Intervariation CV (%)
MMP-12 ELISA	3.1 to 6.3	6.6 to 12.1
MMP-12 Activity	7.9 to 10.7	8.8 to 13.2

TABLE E6. Spearman correlation coefficients (with bootstrap CIs) of sputum cytokine, chemokine, interferon, and growth factor concentrations (in picograms per milliliter) associated with sputum MMP-12 protein and enzymatic activity in different clinical categories

	Healthy control subjects		Patients with COPD		Smokers with asthma		Nonsmokers with asthma	
	Protein concentration	Enzyme activity	Protein concentration	Enzyme activity	Protein concentration	Enzyme activity	Protein concentration	Enzyme activity
IL-1β	0.168 (−0.169 to 0.444)	0.058 (−0.280 to 0.325)	0.242 (−0.035 to 0.497)	**0.429** (0.165 to 0.652)	0.204 (−0.093 to 0.456)	**0.528** (0.296 to 0.707)	0.283 (−0.060 to 0.538)	**0.326** (0.001 to 0.611)
IL-1Ra	**0.345** (0.067 to 0.605)	**0.470** (0.199 to 0.691)	0.161 (−0.112 to 0.432)	0.071 (−0.201 to 0.316)	0.286 (−0.009 to 0.533)	0.133 (−0.139 to 0.386)	**0.521** (0.260 to 0.699)	**0.586** (0.350 to 0.763)
IL-2	−0.057 (−0.346 to 0.266)	−0.171 (−0.437 to 0.103)	0.058 (−0.252 to 0.359)	**0.448** (0.216 to 0.650)	−0.033 (−0.314 to 0.246)	0.191 (−0.049 to 0.423)	0.186 (−0.125 to 0.465)	0.120 (−0.186 to 0.413)
sIL-2R	0.146 (−0.164 to 0.481)	0.214 (−0.108 to 0.527)	0.135 (−0.141 to 0.420)	**0.363** (0.053 to 0.641)	0.162 (−0.117 to 0.432)	0.249 (−0.005 to 0.489)	**0.373** (0.091 to 0.657)	**0.374** (0.062 to 0.610)
IL-5	**0.330** (0.035 to 0.608)	**0.395** (0.063 to 0.662)	−0.299 (−0.534 to −0.008)	−0.181 (−0.431 to 0.117)	0.098 (−0.210 to 0.401)	−0.133 (−0.410 to 0.161)	**0.339** (0.039 to 0.580)	**0.413** (0.138 to 0.670)
IL-6	**0.384** (0.078 to 0.659)	**0.590** (0.365 to 0.745)	**0.396** (0.129 to 0.629)	0.181 (−0.085 to 0.443)	**0.477** (0.253 to 0.673)	**0.348** (0.042 to 0.574)	**0.545** (0.272 to 0.716)	0.244 (−0.086 to 0.528)
IL-10	0.247 (−0.075 to 0.510)	0.281 (−0.041 to 0.528)	**0.284** (0.067 to 0.478)	**0.446** (0.174 to 0.638)	0.107 (−0.150 to 0.355)	0.201 (−0.066 to 0.464)	0.087 (−0.232 to 0.379)	0.112 (−0.204 to 0.419)
IL-12	**0.487** (0.194 to 0.717)	**0.580** (0.355 to 0.737)	**0.477** (0.216 to 0.667)	**0.368** (0.106 to 0.580)	**0.434** (0.151 to 0.652)	**0.377** (0.155 to 0.563)	**0.560** (0.286 to 0.738)	**0.481** (0.193 to 0.689)
IL-15	−0.121 (−0.446 to 0.164)	−0.126 (−0.394 to 0.183)	0.124 (−0.180 to 0.435)	**0.405** (0.162 to 0.637)	0.085 (−0.173 to 0.363)	0.150 (−0.079 to 0.409)	**0.386** (0.121 to 0.628)	0.294 (−0.025 to 0.565)
IL-17	0.091 (−0.221 to 0.384)	−0.019 (−0.282 to 0.264)	0.054 (−0.224 to 0.328)	0.282 (−0.006 to 0.538)	0.063 (−0.181 to 0.304)	0.165 (−0.139 to 0.417)	0.218 (−0.102 to 0.494)	0.016 (−0.299 to 0.358)
TNF-α	0.091 (−0.201 to 0.395)	0.103 (−0.166 to 0.377)	−0.269 (−0.549 to 0.018)	0.016 (−0.260 to 0.281)	0.127 (−0.161 to 0.426)	−0.011 (−0.273 to 0.258)	0.283 (−0.013 to 0.506)	0.263 (−0.027 to 0.518)
IFN-α	0.068 (−0.232 to 0.348)	0.092 (−0.201 to 0.386)	−0.110 (−0.376 to 0.200)	0.079 (−0.194 to 0.372)	0.126 (−0.148 to 0.363)	0.031 (−0.192 to 0.255)	0.246 (−0.075 to 0.513)	0.178 (−0.117 to 0.479)
IFN-γ	0.182 (−0.141 to 0.504)	−0.020 (−0.327 to 0.310)	−0.105 (−0.375 to 0.179)	**0.305** (0.039 to 0.527)	0.125 (−0.171 to 0.367)	0.240 (−0.012 to 0.478)	0.286 (−0.022 to 0.529)	0.100 (−0.231 to 0.363)
CCL2 (MCP-1)	**0.443** (0.174 to 0.704)	**0.625** (0.414 to 0.765)	**0.474** (0.205 to 0.700)	**0.387** (0.117 to 0.625)	**0.539** (0.290 to 0.719)	**0.340** (0.069 to 0.555)	**0.573** (0.328 to 0.752)	**0.393** (0.097 to 0.627)
CCL3 (MIP-1α)	0.081 (−0.239 to 0.383)	0.031 (−0.284 to 0.338)	**0.267** (0.002 to 0.522)	**0.511** (0.266 to 0.720)	**0.323** (0.037 to 0.549)	**0.432** (0.173 to 0.646)	**0.308** (0.033 to 0.546)	**0.287** (0.043 to 0.523)
CCL4 (MIP-1β)	0.246 (−0.046 to 0.476)	0.226 (−0.066 to 0.498)	**0.351** (0.079 to 0.573)	**0.673** (0.471 to 0.833)	**0.298** (0.014 to 0.579)	**0.505** (0.281 to 0.671)	**0.394** (0.139 to 0.648)	0.124 (−0.164 to 0.415)
CCL5 (RANTES)	0.056 (−0.300 to 0.396)	−0.105 (−0.408 to 0.228)	0.164 (−0.095 to 0.453)	**0.405** (0.110 to 0.662)	−0.131 (−0.385 to 0.151)	0.138 (−0.132 to 0.415)	0.087 (−0.221 to 0.421)	0.154 (−0.195 to 0.446)
CXCL8 (IL-8)	**0.431** (0.134 to 0.687)	**0.645** (0.458 to 0.767)	**0.588** (0.355 to 0.763)	**0.543** (0.292 to 0.716)	**0.506** (0.222 to 0.714)	**0.425** (0.171 to 0.631)	**0.570** (0.321 to 0.736)	**0.326** (0.025 to 0.566)
CXCL9 (MIG)	−0.059 (−0.352 to 0.271)	0.209 (−0.061 to 0.478)	**0.421** (0.183 to 0.606)	**0.562** (0.299 to 0.744)	**0.317** (0.065 to 0.567)	0.100 (−0.172 to 0.358)	**0.443** (0.166 to 0.669)	**0.350** (0.076 to 0.596)
CXCL10 (IP-10)	−0.099 (−0.380 to 0.194)	0.161 (−0.117 to 0.418)	**0.352** (0.069 to 0.561)	**0.317** (0.036 to 0.593)	**0.257** (0.008 to 0.486)	0.122 (−0.133 to 0.363)	0.315 (−0.001 to 0.575)	0.223 (−0.099 to 0.469)
CCL11 (eotaxin)	0.006 (−0.262 to 0.288)	0.182 (−0.115 to 0.462)	0.292 (−0.024 to 0.546)	0.183 (−0.095 to 0.512)	**0.428** (0.135 to 0.652)	−0.011 (−0.274 to 0.250)	0.237 (−0.090 to 0.510)	0.166 (−0.141 to 0.444)
EGF	**0.309** (0.025 to 0.553)	**0.474** (0.217 to 0.635)	0.198 (−0.089 to 0.446)	0.025 (−0.282 to 0.308)	0.211 (−0.064 to 0.438)	0.107 (−0.203 to 0.393)	**0.342** (0.036 to 0.625)	0.145 (−0.186 to 0.478)
FGF-β	0.099 (−0.180 to 0.378)	0.171 (−0.133 to 0.422)	0.203 (−0.058 to 0.449)	**0.589** (0.328 to 0.770)	0.102 (−0.184 to 0.365)	0.210 (−0.068 to 0.450)	0.296 (−0.018 to 0.578)	0.321 (−0.013 to 0.641)
G-CSF	**0.342** (0.074 to 0.581)	**0.432** (0.144 to 0.652)	**0.453** (0.210 to 0.652)	**0.568** (0.317 to 0.770)	**0.474** (0.237 to 0.663)	**0.442** (0.202 to 0.666)	**0.584** (0.349 to 0.745)	**0.420** (0.137 to 0.641)
GM-CSF	−0.004 (−0.332 to 0.313)	0.131 (−0.194 to 0.452)	**−0.383** (−0.609 to −0.133)	−0.108 (−0.387 to 0.160)	0.041 (−0.238 to 0.291)	−0.109 (−0.363 to 0.123)	0.163 (−0.105 to 0.438)	0.136 (−0.207 to 0.413)
HGF	0.311 (−0.012 to 0.551)	0.314 (−0.005 to 0.572)	0.303 (−0.002 to 0.547)	**0.549** (0.327 to 0.742)	0.308 (−0.001 to 0.567)	**0.362** (0.100 to 0.586)	**0.500** (0.251 to 0.688)	**0.395** (0.117 to 0.621)
VEGF	0.219 (−0.075 to 0.504)	0.185 (−0.161 to 0.480)	0.246 (−0.054 to 0.498)	**0.529** (0.295 to 0.717)	0.126 (−0.168 to 0.406)	0.280 (−0.012 to 0.545)	0.260 (−0.027 to 0.532)	0.013 (−0.273 to 0.290)

Cytokines: IL-1β, IL-1Ra, IL-2, sIL-2R, IL-3,* IL-4,* IL-5, IL-6, IL-7,* IL-10, IL-12, IL-13,* IL-15, IL-17, and TNF-α; interferons: IFN-α and IFN-γ; chemokines: CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL9, and CXCL10; and growth factors: EGF, FGF-β, G-CSF, GM-CSF, HGF, and VEGF. *Data are not shown for these. Concentrations are all less than detection limits.

EGF, Epidermal growth factor; *FGF-β*, fibroblast growth factor β; *G-CSF*, granulocyte colony-stimulating factor; *HGF*, hepatocyte growth factor; *IP-10*, interferon-inducible protein 10; *MCP-1*, monocyte chemoattractant protein 1; *MIG*, monocyte induced by gamma interferon; *MIP-1β*, macrophage inflammatory protein 1β; *sIL-2R*, soluble IL-2 receptor; *VEGF*, vascular endothelial growth factor. Boldface type indicates a significant correlation.