



# Low sputum MMP-9/TIMP ratio is associated with airway narrowing in smokers with asthma

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**ABSTRACT** Asthmatic smokers have poor symptom control and accelerated decline in lung function. A reduced ratio of matrix metalloproteinase (MMP)-9/tissue inhibitors of metalloproteinases (TIMPs) in nonsmokers with asthma has been implicated in airway remodelling. We tested the hypothesis that sputum MMP-9 activity/TIMPs ratios are reduced in smokers compared with never-smokers with asthma and are associated with reduced lung function and altered computed tomography (CT) measures of airway wall dimensions.

Lung function, airway dimensions by CT, and induced sputum concentrations (and activity) of MMP-9 and TIMP-1 and -2 were measured in 81 asthmatics and 43 healthy subjects (smokers and never-smokers). Respiratory epithelial *MMP9* and *TIMP* mRNA was quantified in 31 severe asthmatics and 32 healthy controls.

Sputum MMP-9 activity/TIMP-1 and TIMP-2 ratios, and nasal epithelial *MMP9/TIMP1* and *MMP9/TIMP2* expression ratios were reduced in smokers with asthma compared with never-smokers with asthma. Low sputum ratios in asthmatic smokers were associated with reduced post-bronchodilator forced expiratory volume in 1 s (FEV<sub>1</sub>), FEV<sub>1</sub>/forced vital capacity ratio and segmental airway lumen area.

The association of a low sputum MMP-9 activity/TIMP-1 ratio with persistent airflow obstruction and reduced CT airway lumen area in smokers with asthma may indicate that an imbalance of MMP-9 and TIMPs contributes to structural changes to the airways in this group.



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## Introduction

A considerable proportion of patients with asthma, particularly those with severe disease [1], or who are cigarette smokers [2] have poorly controlled asthma, which can be associated with corticosteroid resistance [3], altered patterns of airway inflammation [4], airway remodelling [4] and persistent airflow obstruction [5]. The mechanisms leading to corticosteroid insensitivity and remodelling in asthma are poorly understood [3, 4].

Matrix metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases that form a family of extracellular matrix (ECM) proteolytic enzymes. Activity of MMPs is regulated by specific protease inhibitors called tissue inhibitors of metalloproteinases (TIMPs). Inappropriate expression and activity of several MMPs, including MMP-9, and imbalances in the MMP-9/TIMP ratio have been implicated in the pathogenesis of asthma [6–12]. In nonsmokers with asthma a reduced sputum MMP-9/TIMP ratio is associated with more severe airway obstruction [13, 14] and with computed tomography (CT) measures of increased airway wall area and thickness [14] as well as with increased CT lung scan abnormalities [15]. Based on these findings it has been proposed that high concentrations of TIMP-1 in relation to MMP-9 can lead to increased ECM deposition and increased myofibroblast proliferation, through TIMP-1 induced cell growth, and that a low sputum MMP-9/TIMP ratio may be a biomarker of airway remodelling in asthma [10–12]. Studies of asthma have recruited predominantly never-smokers; much less is known about MMP-9 activity and TIMP in smokers with asthma. Acute exposure of bronchial epithelial cells from patients with asthma to cigarette smoke *in vitro* reduces the MMP-9/TIMP-1 ratio suggesting that an interaction between asthma and cigarette smoking may potentiate structural changes to the airways [16]. We tested the hypothesis that MMP-9 activity/TIMP ratios are reduced in sputum from smokers with asthma compared with never-smokers with asthma and are associated with reduced lung function and altered CT measures of airway wall dimensions.

## Materials and methods

### Subjects

Participants were recruited with mild, moderate and severe persistent asthma (Global Initiative for Asthma classification) [17]; participants with asthma included both current smokers and never-smokers, and healthy smokers and never-smokers. All subjects were on stable medication and had had no exacerbation of disease for 4 weeks. Smokers were defined as individuals with  $\geq 10$  pack-years who currently smoke five or more cigarettes per day. Asthma subjects had to demonstrate either reversibility of forced expiratory volume in 1 s (FEV<sub>1</sub>) or airway hyperresponsiveness. The West Glasgow Research Ethics Committee approved the study and all patients gave written informed consent.

### Study design

The study was designed to examine the relationship between sputum MMP-9 activity/TIMP-1 and TIMP-2 ratios and the expression of MMP-9/TIMP-1 and MMP-9/TIMP-2 with sputum cytology, lung function and CT measures of airway dimensions in smokers and never-smokers with asthma. It was a cross-sectional study, performed in subjects with asthma and healthy controls, as part of a chronic obstructive pulmonary disease and asthma biomarker study [18].

### Measurements

Additional details of the measurements can be found in the online supplementary material.

Spirometry was performed according to American Thoracic Society guidelines [19] and airway hyperresponsiveness to methacholine was measured. Reversibility was defined as  $>12\%$  and 200 mL improvement in FEV<sub>1</sub> following salbutamol.

Exhaled nitric oxide fraction was measured (Niox Flex; Aerocrine, Solna, Sweden) at  $50 \text{ mL}\cdot\text{s}^{-1}$  (FeNO<sub>50</sub>) in concordance with standardised guidelines [20].

Lung volumes and diffusing capacity of the lung for carbon monoxide (DLCO) were performed using the body box technique (Zan 500 Body Plethysmography; nSpire Health Limited, Hertford, UK).

Sputum induction was performed as previously described [18].

CT scans of the chest were performed at full inspiration using 16-slice Brightspeed and 64-slice Lightspeed (GE Healthcare, Milwaukee, WI, USA) with the following parameters: 120 KV, 100 mA·s, collimation 1 mm, reconstruction slice thickness 0.65 mm, reconstruction slice separation 0.5 mm and pitch of one. The data were reconstructed with a CHEST filter. All scans were evaluated centrally at the University of Edinburgh (Edinburgh, UK). Airway dimensions were measured using the software Pulmonary Workstation 2.0 (VIDA Diagnostics, Iowa City, IA, USA) to plot an airway path from which airway

profiles were generated on cross-sections orthogonal to the airway path. Airway dimensions were measured in the right lower lobe posterior basal segmental bronchus (designated RB10). The following CT airway values were obtained: RB10 % wall area; RB10 wall thickness (mm); RB10 lumen area (mm<sup>2</sup>).

MMP-9 assays were carried out using the MMP-9 enzyme immunoassay and MMP-9 activity kits (catalogue numbers DMP900 and F9M00, respectively; R&D Systems Europe Ltd, Abingdon, UK). TIMPs were quantified by using fluorokine multiplex assay (LKT003; R&D Systems Europe Ltd).

MMP-9 (Affymetrix number 203936\_s\_at), TIMP-1 (201666\_at) and TIMP-2 (231579\_s\_at) mRNA expression levels were analysed using Affymetrix U133+2 chips (Affymetrix, Santa Clara, CA, USA) and quantified by global normalisation to sputum cell and nasal respiratory epithelial cell samples obtained from healthy never-smokers (n=15), healthy smokers (n=13), smokers with asthma (n=15) and never-smokers with asthma (n=15). Samples were selected by asthma severity and quality of RNA. Details of RNA isolation and quality are included in the online supplementary material.

### Analysis

Continuous variables were summarised as median (interquartile range). Their comparison between different patient groups was by Wilcoxon test or Kruskal–Wallis test. Categorical variables were summarised as frequencies and percentages per category and were compared using Fisher's exact test. Linear regression models were used to predict each marker from disease group, smoking status and additional covariates, testing for any group by smoking status interaction. Associations of log-transformed MMP-9/TIMP ratios with sputum neutrophil percentage and CT RB10 airway dimension measurements were assessed using the Pearson correlation coefficient with bootstrap 95% confidence intervals. The analyses were exploratory for the purpose of hypothesis generation, and statistical significance was accepted if  $p < 0.05$ . All analyses were carried out in R version 2.15.0 (The R Project for Statistical Computing. [www.r-project.org](http://www.r-project.org)).

## Results

### Demographics and baseline characteristics

81 asthmatics (35 smokers and 46 never-smokers) and 43 healthy subjects (19 smokers and 24 never-smokers) took part in the study. The asthma and healthy control groups were matched for age and smoking histories (table 1). None of the patients were on oral corticosteroids or anti-interleukin-5 therapy. The never-smokers with asthma were similar to the smokers with asthma in terms of age, duration of asthma, body surface area, atopic status, spirometry, dose of inhaled corticosteroid, treatment with montelukast, induced sputum total cell counts, eosinophil and neutrophil proportion, and CT measurements of airway wall thickness, but had a higher DLCO (86% versus 77% predicted,  $p = 0.002$ ) and  $F_{eNO_{50}}$  (23 ppb versus 9 ppb,  $p < 0.001$ ).

### Sputum MMP-9/TIMP activity and concentration ratios

The ratio of sputum MMP-9 activity to sputum TIMP-1 or sputum TIMP-2 was lower in smokers with asthma compared with never-smokers with asthma ( $p = 0.002$  and  $p = 0.001$  respectively), and in healthy smokers compared with healthy never-smokers ( $p = 0.018$  and  $p = 0.024$ , respectively) (fig. 1 and table 2). The difference in these ratios was due to elevated sputum TIMP concentrations in smokers compared to never-smokers (in both asthma and healthy controls) (table 2). There was no significant difference between groups according to asthma severity (table 3).

The ratios of sputum MMP-9 concentration to sputum TIMP-1 or sputum TIMP-2 were similar between smokers with asthma and never-smokers with asthma and between both asthma groups and healthy never-smokers and smokers, respectively (fig. 1 and table 2). There was no significant difference between groups according to asthma severity (table 3).

Linear regression analyses of interaction demonstrated that neither age nor dose of inhaled corticosteroid had a significant effect on MMP-9/TIMP ratios and that smoking had a significant effect on MMP-9 activity/TIMP-1 ( $r = -0.738$  (95% CI  $-1.276$ – $-0.200$ ),  $p = 0.008$ ) and on MMP-9 activity/TIMP-2 ratios ( $r = -0.518$  (95% CI  $-0.960$ – $-0.077$ ),  $p = 0.022$ ).

### Nasal respiratory epithelial cell and sputum cell MMP-9/TIMP expression ratios

Median (interquartile range) nasal respiratory epithelial cell MMP9/TIMP1 and MMP9/TIMP2 expression ratios were lower in smokers with severe asthma compared with never-smokers with severe asthma (0.01 (0.00–0.03) versus 0.05 (0.04–0.09)  $\times 10^{-3}$  relative fluorescence units (RFU),  $p < 0.001$  and 0.02 (0.01–0.06) versus 0.10 (0.05–0.14)  $\times 10^{-3}$  RFU,  $p < 0.001$ , respectively). Ratios were also lower in healthy smokers compared with healthy never-smokers ( $p < 0.001$  for both ratios) (online supplementary table E1). Sputum cell MMP9/TIMP1 and TIMP2 expression ratios were higher in smokers with severe asthma compared with

TABLE 1 Demographics and clinical baseline characteristics

	Asthma			Healthy control		
	Never-smoker	Smoker	p-value <sup>#</sup>	Never-smoker	Smoker	p-value <sup>†</sup>
Subjects n	35	46		24	19	
Age years	49.2 (42.0–53.7)	48.7 (42.6–54.8)	0.980	49.2 (41.9–55.0)	52.9 (44.9–58.8)	0.594
Male sex	15 (42.9)	21 (45.7)	0.825	7 (29.2)	9 (47.4)	0.341
Disease duration years	20.0 (9.5–30.0)	18.5 (10.0–33.8)	0.791			
Body surface area m <sup>2</sup>	1.90 (1.74–2.18)	1.84 (1.69–2.02)	0.132	1.76 (1.69–1.93)	1.86 (1.68–2.00)	0.283
Pre-bronchodilator FEV <sub>1</sub> % pred	81.0 (71.5–94.0)	77.5 (58.0–92.8)	0.330	108.0 (99.8–116.2)	92.0 (87.0–99.0)	<b>0.002</b>
Post-bronchodilator FEV <sub>1</sub> % pred	91.0 (80.5–98.0)	83.5 (69.5–101.2)	0.262			
Smoking pack-years		33.5 (23.0–60.0)			30.0 (24.0–38.0)	
DLco % pred COHb	86.0 (81.0–92.0)	77.0 (65.0–84.0)	<b>0.002</b>			
FeNO <sub>50</sub> ppb	23.2 (11.6–45.5)	9.3 (6.9–11.0)	<b>&lt;0.001</b>	15.5 (9.2–18.3)	9.1 (6.1–22.1)	0.333
Beclometasone-equivalent dose of inhaled steroid µg	800 (400–1050)	800 (750–1000)	0.506			
Sputum total cell count × 10 <sup>6</sup> ·mL <sup>-1</sup>	6.56 (5.17–8.59)	5.81 (4.97–7.55)	0.213	6.5 (5.63–7.75)	5.42 (5.18–6.14)	0.118
Sputum neutrophils %	49.8 (27.9–69.5)	58.0 (36.0–70.2)	0.759	26.2 (15.2–57.0)	50.8 (32.6–69.8)	0.075
Sputum eosinophils %	1.00 (0.00–1.88)	0.50 (0.00–1.50)	0.631	0.00 (0.00–0.49)	0.00 (0.00–0.42)	0.876
Sputum macrophages %	32.8 (16.6–52.9)	24.0 (15.5–40.5)	0.261	42.0 (20.6–64.5)	29.2 (24.4–49.1)	0.287
Computed tomography						
RB10 % wall area mm <sup>2</sup>	61.6 (58.7–65.5)	65.7 (61.1–68.1)	0.062			
RB10 wall thickness mm	1.77 (1.55–2.07)	1.71 (1.53–1.91)	0.436			
RB10 lumen area mm <sup>2</sup>	19.2 (14.6–24.1)	16.2 (12.0–19.2)	0.056			

Data are presented as median (interquartile range) or n (%), unless otherwise stated. Bold font indicates p<0.05. FEV<sub>1</sub>: forced expiratory volume in 1 s; DLco % pred COHb: diffusing capacity of the lung for carbon monoxide corrected for haemoglobin and carboxyhaemoglobin, as a percentage of the predicted value; FeNO<sub>50</sub>: exhaled nitric oxide fraction measured at a flow rate of 50 mL·s<sup>-1</sup>; RB10: right bronchial division 10. #: comparison of never-smokers with asthma and smokers with asthma; †: comparison of healthy never-smokers and healthy smokers.

never-smokers with severe asthma (0.19 (0.09–0.27) versus 0.13 (0.06–0.18), p<0.001 and 0.27 (0.12–0.38) versus 0.13 (0.05–0.18), p=0.046, respectively) (online supplementary table E1).

**Correlation of sputum MMP-9 protein and enzyme activity and TIMP concentrations with lung function, sputum neutrophil percentage and CT scan measures of airway dimensions**

*Lung function*

In smokers with asthma sputum MMP-9 protein activity/TIMP-1 and TIMP-2 ratios were positively associated with post-bronchodilator FEV<sub>1</sub> % pred (r=0.359 (95% CI 0.065–0.599) and 0.334 (0.008–0.587), respectively) and FEV<sub>1</sub>/forced vital capacity (FVC) ratio (0.365 (0.070–0.626) and 0.386 (0.105–0.614), respectively) (table 4). There were no significant associations with DLCO, except for sputum MMP-9/TIMP-2 ratio in never-smokers with asthma (-0.344 (-0.621– -0.028)).

*Sputum neutrophil percentage*

In never-smokers with asthma, MMP-9 activity/TIMP-1 and MMP-9 concentration/TIMP-1 were positively associated with sputum neutrophil percentage (0.373 (95% CI 0.020–0.697) and 0.579 (0.251–0.797), respectively) (table 4). Associations of MMP-9 concentration and activity/TIMP-2 were not significant in never-smokers with asthma, nor were any associations with sputum neutrophil percentage significant in smokers with asthma.

*CT measure of airway wall dimensions*

Sputum MMP-9 activity/TIMP-1 and TIMP-2 and sputum MMP-9 protein concentration/TIMP-1 and TIMP-2 ratios were positively associated with RB10 airway lumen area in smokers with asthma (r=0.450 (95% CI 0.138–0.717), 0.410 (0.102–0.668), 0.499 (0.189–0.728) and 0.320 (0.000–0.579), respectively) (fig. 2 and table 4). Sputum MMP-9/TIMP ratios were not significantly associated with RB10 airway wall thickening. There were no significant associations between the ratios and RB10 CT measures in

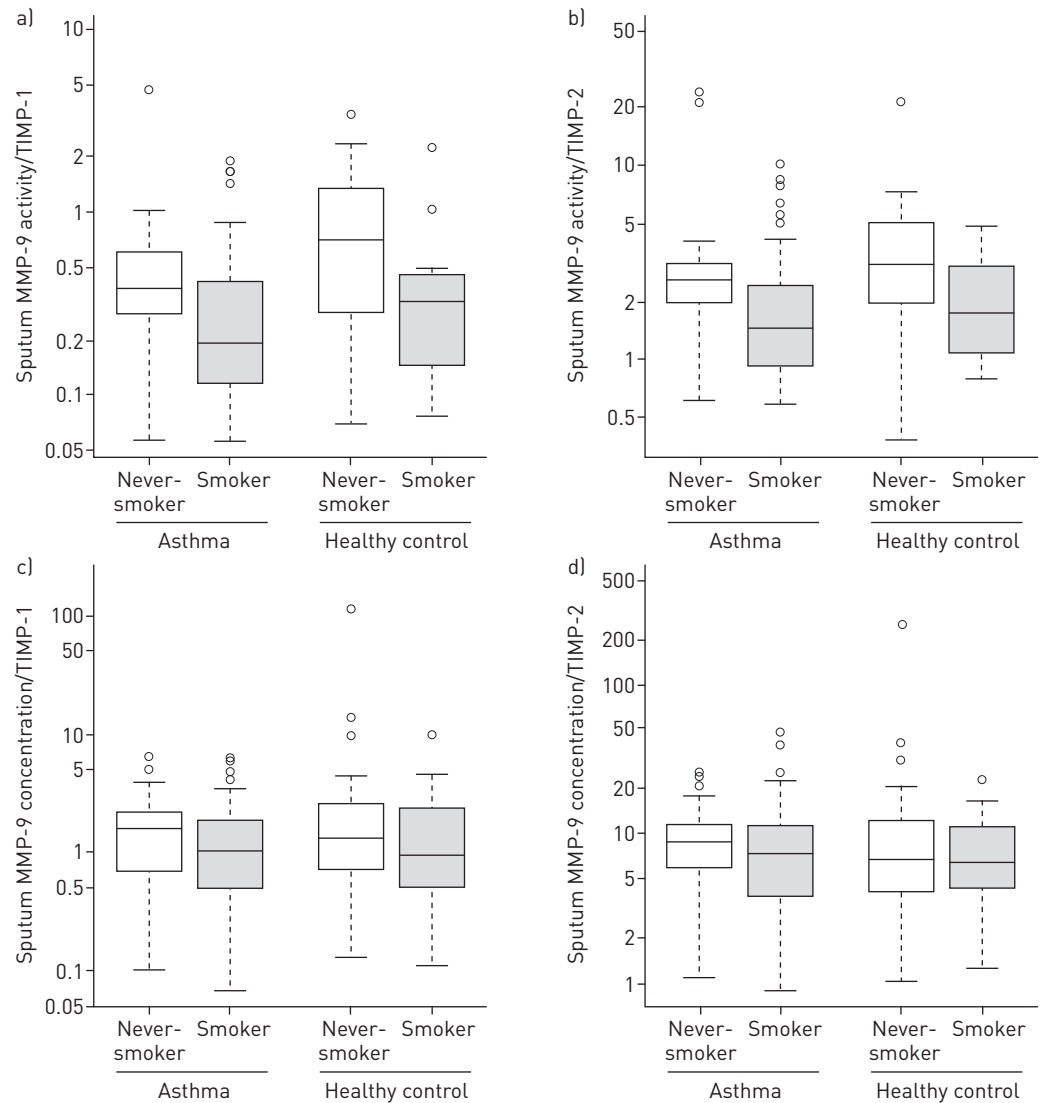


FIGURE 1 a) Sputum matrix metalloproteinase (MMP)-9 activity/tissue inhibitor of metalloproteinase (TIMP)-1 concentration ratio, b) MMP-9 activity/TIMP-2 concentration ratio, c) MMP-9 protein concentration/TIMP-1 concentration ratio, and d) MMP-9 protein concentration/TIMP-2 concentration ratio in patients with asthma and healthy controls. The ratio of sputum MMP-9 activity with sputum TIMP-1 concentration or sputum TIMP-2 concentration was lower in smokers with asthma compared with never-smokers with asthma ( $p=0.002$  and  $p=0.001$ , respectively) and in healthy smokers compared with healthy never-smokers ( $p=0.018$  and  $p=0.024$ , respectively).

never-smokers with asthma. RB10 airway lumen area in smokers with asthma and never-smokers with asthma did not correlate with body surface area ( $r=0.208$ ,  $p=0.170$  and  $r=0.066$ ,  $p=0.680$ , respectively).

**Discussion**

A reduced ratio of MMP-9/TIMPs in nonsmokers with asthma has been implicated in airway remodelling [8, 10–12]. We sought to determine if the ratio of sputum MMP-9/TIMPs is associated with lung function or CT measures of airway dimensions in smokers and never-smokers with asthma. Particular strengths of our study were recruitment of smokers with asthma, who account for up to a third of all adults with asthma, and yet in whom there are limited published data describing the balance between MMP-9 and TIMPs, or CT imaging to assess airway wall dimensions. We also describe the use of functional assays for MMP-9 activity, which adds information about the proportion of the enzyme that becomes activated.

Our main new findings were that sputum MMP-9 activity/TIMP ratios were reduced in smokers with asthma compared with never-smokers with asthma and that reduced ratios in smokers with asthma were associated with reduced post-bronchodilator FEV1/FVC ratio and reduced airway lumen area as measured

TABLE 2 Sputum MMP-9 activity and concentrations, TIMP-1 and TIMP-2 concentrations, and MMP/TIMP ratios in healthy controls and patients with asthma

	Asthma			Healthy control			p-value <sup>‡</sup>
	Never-smoker	Smoker	p-value <sup>#</sup>	Never-smoker	Smoker	p-value <sup>†</sup>	
<b>MMP-9 activity ng·mL<sup>-1</sup></b>	27.1 (13.1–41.2)	14.5 (12.5–26.1)	<b>0.035</b>	16.6 (12.5–28.4)	18.9 (13.4–46.4)	0.285	0.178
<b>MMP-9 ng·mL<sup>-1</sup></b>	102.5 (43.5–147.9)	73.4 (49.2–148.6)	0.826	34.0 (24.4–131.0)	69.0 (38.6–184.0)	0.160	0.891
<b>TIMP-1 ng·mL<sup>-1</sup></b>	60.5 (46.7–116.5)	96.7 (55.0–158.0)	<b>0.022</b>	35.3 (14.8–67.4)	101.8 (60.8–149.2)	<b>0.001</b>	0.825
<b>TIMP-2 ng·mL<sup>-1</sup></b>	10.6 (6.3–18.5)	13.1 (9.2–16.8)	0.355	8.0 (4.1–11.2)	13.9 (10.4–18.5)	<b>0.004</b>	0.592
<b>MMP-9 activity/TIMP-1</b>	0.38 (0.28–0.61)	0.19 (0.12–0.42)	<b>0.002</b>	0.72 (0.30–1.33)	0.33 (0.14–0.46)	<b>0.018</b>	0.402
<b>MMP-9 activity/TIMP-2</b>	2.6 (2.0–3.1)	1.4 (0.9–2.3)	<b>0.001</b>	3.1 (2.0–5.0)	1.7 (1.1–3.0)	<b>0.024</b>	0.402
<b>MMP-9/TIMP-1</b>	1.6 (0.7–2.2)	1.0 (0.5–1.8)	0.185	1.3 (0.8–2.4)	0.9 (0.5–2.4)	0.490	0.915
<b>MMP-9/TIMP-2</b>	9.0 (6.0–11.6)	7.4 (3.8–11.4)	0.345	6.7 (4.3–11.2)	6.4 (4.3–11.2)	0.744	0.791

Data are presented as median (interquartile range), unless otherwise stated. Bold font indicates p<0.05. MMP: matrix metalloproteinase; TIMP: tissue inhibitors of metalloproteinase. #: comparison of never-smokers with asthma and smokers with asthma; †: comparison of healthy never-smokers and healthy smokers; ‡: comparison of healthy smokers and smokers with asthma; §: comparison of healthy never-smokers and never-smokers with asthma.

TABLE 3 Sputum MMP-9 activity and concentrations, TIMP-1 and TIMP-2 concentrations, and MMP/TIMP ratios and induced sputum cell cytology in patients with asthma, subdivided by severity and smoking

	Never-smokers with asthma			Smokers with asthma		
	Mild	Moderate	Severe	Mild	Moderate	Severe
<b>Subjects n</b>	12	11	12	14	13	19
<b>MMP-9 activity ng·mL<sup>-1</sup></b>	29.4 (17.5–40.5)	21.8 (16.5–32.0)	31.6 (12.5–55.8)	18.9 (13.0–47.4)	14.3 (12.5–44.0)	13.5 (12.5–23.2)
<b>MMP-9 ng·mL<sup>-1</sup></b>	106 (69–156)	90 (40–143)	97 (42–140)	67 (48–144)	71 (38–355)	80 (55–123)
<b>TIMP-1 ng·mL<sup>-1</sup></b>	63.8 (42.3–110)	54.4 (51.2–90.7)	69.3 (52.5–117)	96.7 (50.6–157)	111 (53.4–143)	85.9 (63.1–170)
<b>TIMP-2 ng·mL<sup>-1</sup></b>	9.9 (6.0–16.3)	10.6 (7.4–16.0)	14.0 (8.9–19.9)	11.7 (8.3–16.4)	14.1 (8.0–25.0)	13.2 (9.5–15.6)
<b>MMP-9 activity/TIMP-1</b>	0.4 (0.4–0.6)	0.3 (0.2–0.5)	0.4 (0.2–0.8)	0.2 (0.1–0.7)	0.3 (0.2–0.4)	0.1 (0.1–0.3)
<b>MMP-9 activity/TIMP-2</b>	2.9 (2.4–3.1)	2.0 (1.8–2.6)	2.5 (1.5–3.5)	1.4 (1.2–3.8)	1.9 (1.3–2.7)	1.3 (0.9–1.6)
<b>MMP-9/TIMP-1</b>	1.7 (1.1–2.2)	1.6 (0.7–2.1)	1.3 (0.6–1.8)	1.3 (0.4–2.0)	1.2 (0.5–1.4)	0.9 (0.6–1.7)
<b>MMP-9/TIMP-2</b>	10.2 (9.2–13.0)	8.5 (6.0–11.8)	7.4 (4.3–9.3)	7.3 (3.5–11.2)	6.8 (3.8–13.6)	7.4 (4.6–10.0)
<b>Sputum cytology %</b>						
Eosinophils	0.7 (0.0–1.0)	0.5 (0.0–1.4)	1.3 (0.6–4.0)	0.5 (0.0–1.0)	0.0 (0.0–1.0)	1.0 (0.5–3.5)
Neutrophils	45.0 (32.2–61.5)	44.5 (25.5–74.0)	46.5 (25.0–71.6)	66.5 (50.0–71.0)	59.5 (36.5–71.5)	48.8 (34.1–71.8)
Macrophages	33.5 (24.8–53.4)	30.5 (9.9–54.4)	28.0 (14.9–45.5)	24.0 (14.0–28.0)	23.0 (14.5–48.0)	23.2 (18.5–41.5)

Data are presented as median (interquartile range), unless otherwise stated. MMP: matrix metalloproteinase; TIMP: tissue inhibitors of metalloproteinase.

using CT. These findings suggest that airway narrowing is affected by the functional balance between MMP-9 and its inhibitors, which appears crucial in the dynamic process of airway remodelling and collagen deposition in asthma [8, 9, 21]. We also found reduced ratios of *MMP9/TIMP1* and *MMP9/TIMP2* mRNA expression levels in nasal respiratory epithelium in the subset of smokers with severe asthma, suggesting that these changes occurred in the sub-epithelial compartment where MMPs have to be activated and their interactions with TIMPs are locally regulated [22] and where the airway remodelling in asthma is most damaging.

Acute exposure of bronchial epithelial cells from patients with asthma to cigarette smoke *in vitro* reduces the MMP-9/TIMP-1 ratio [16]. Based on this finding, it was postulated that a reduction in the MMP-9/TIMP ratio may contribute to remodelling in smokers with asthma due to increased deposition of ECM and airway wall thickness [16]. However, our findings did not support this hypothesis, in that we could not demonstrate any association between MMP-9/TIMP ratio and increased CT airway wall thickening in smokers with asthma. Instead, an important finding in the smokers with asthma group were the positive correlations between sputum MMP-9 activity/TIMP-1 and TIMP-2, MMP-9 concentration/TIMP-1 and TIMP-2 ratios, and CT measurements of segmental airway lumen area. The link between reduced MMP-9/TIMP ratios and a reduced airway lumen in smokers with asthma is not explained by our data. Lower sputum MMP-9 activity/TIMP-1 and TIMP-2 ratios were associated with reduced FEV1 % pred in smokers with asthma, an association reported by others in nonsmokers with asthma [13, 14, 23]. In addition, we showed that lower ratios were associated with reduced post-bronchodilator FEV1/FVC, implying persistent airflow obstruction in subjects with lower ratios. Possibly a low MMP-9/TIMP ratio is associated with the bronchoconstrictor effects of cigarette smoke, increased secretions or epithelial inflammation, although the latter might also be expected to increase airway wall thickness. Body surface area could influence airway dimensions, but we found no association with airway lumen area. The excess of TIMP over MMP-9 may be an exaggerated attempt to protect the bronchi against metalloproteinase degrading activity, leading to increased extracellular matrix deposition [12].

Unlike the results obtained by VIGNOLA *et al.* [8] in mild, steroid naïve asthmatics, we did not observe a difference in asthmatic nonsmokers compared with healthy controls, possibly as the laboratory techniques were different and majority of our subjects were on inhaled corticosteroids. Inhaled corticosteroids have been shown to reduce the expression of MMP-9 and increase the expression of TIMP-1 in bronchial biopsy tissue from patients with asthma [24]. In the present study, the dose of inhaled corticosteroid did not have a significant effect on MMP-9 or TIMP measurements in sputum.

We found that the proportion of sputum neutrophils correlated positively with sputum MMP-9 activity/TIMP-1 and MMP-9 concentration/TIMP-1 ratios in never-smokers with asthma, but not in smokers with

**TABLE 4** Association between log sputum MMP-9/TIMP ratio and computed tomography RB10 airway dimensions, sputum neutrophil percentage and lung function in patients with asthma

Ratio	Asthma	RB10 % wall area	RB10 wall thickness	RB10 lumen area	Sputum neutrophil %	Post-broncho-dilator FEV1 % pred	Post-broncho-dilator FEV1/FVC	DLco % pred COHb
<b>MMP-9 activity/TIMP-1</b>	Smokers	-0.600 [-0.798-0.297]*	0.023 [-0.288-0.403]	0.450 [0.138-0.717]*	-0.107 [-0.406-0.233]	0.359 [0.065-0.599]*	0.365 [0.070-0.626]*	0.190 [-0.159-0.497]
	Never-smokers	0.076 [-0.277-0.399]	0.080 [-0.304-0.409]	0.271 [-0.059-0.544]	0.373 [0.020-0.697]*	0.226 [-0.141-0.533]	0.261 [-0.092-0.559]	-0.258 [-0.585-0.114]
<b>MMP-9 activity/TIMP-2</b>	Smokers	-0.590 [-0.760-0.312]*	-0.036 [-0.356-0.321]	0.410 [0.102-0.668]*	-0.261 [-0.561-0.089]	0.334 [0.008-0.587]*	0.386 [0.105-0.614]*	0.188 [-0.163-0.481]
	Never-smokers	0.071 [-0.273-0.393]	0.063 [-0.328-0.416]	0.250 [-0.118-0.555]	0.070 [-0.282-0.455]	0.219 [-0.152-0.558]	0.154 [-0.193-0.487]	-0.291 [-0.598-0.054]
<b>MMP-9/TIMP-1</b>	Smokers	-0.338 [-0.611-0.010]*	0.170 [-0.167-0.478]	0.499 [0.189-0.728]*	0.164 [-0.173-0.473]	0.237 [-0.053-0.506]	0.050 [-0.273-0.355]	0.197 [-0.161-0.501]
	Never-smokers	0.025 [-0.296-0.350]	-0.079 [-0.429-0.289]	0.203 [-0.149-0.508]	0.579 [0.251-0.797]*	0.242 [-0.075-0.521]	0.192 [-0.145-0.484]	-0.178 [-0.529-0.217]
<b>MMP-9/TIMP-2</b>	Smokers	-0.210 [-0.521-0.131]	0.084 [-0.267-0.416]	0.320 [0.000-0.579]*	0.107 [-0.198-0.408]	0.128 [-0.173-0.428]	-0.037 [-0.369-0.282]	0.148 [-0.185-0.464]
	Never-smokers	0.091 [-0.259-0.438]	-0.235 [-0.592-0.152]	0.110 [-0.253-0.426]	0.341 [-0.028-0.643]	0.192 [-0.161-0.522]	0.096 [-0.251-0.432]	-0.344 [-0.621-0.028]*

Data are presented as Pearson correlation coefficient (bootstrap 95% CI). MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; RB10: right bronchial division 10; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; DLco % pred COHb: diffusing capacity of the lung for carbon monoxide, corrected for haemoglobin and carboxyhaemoglobin as a percentage of the predicted value. \*: p<0.05.



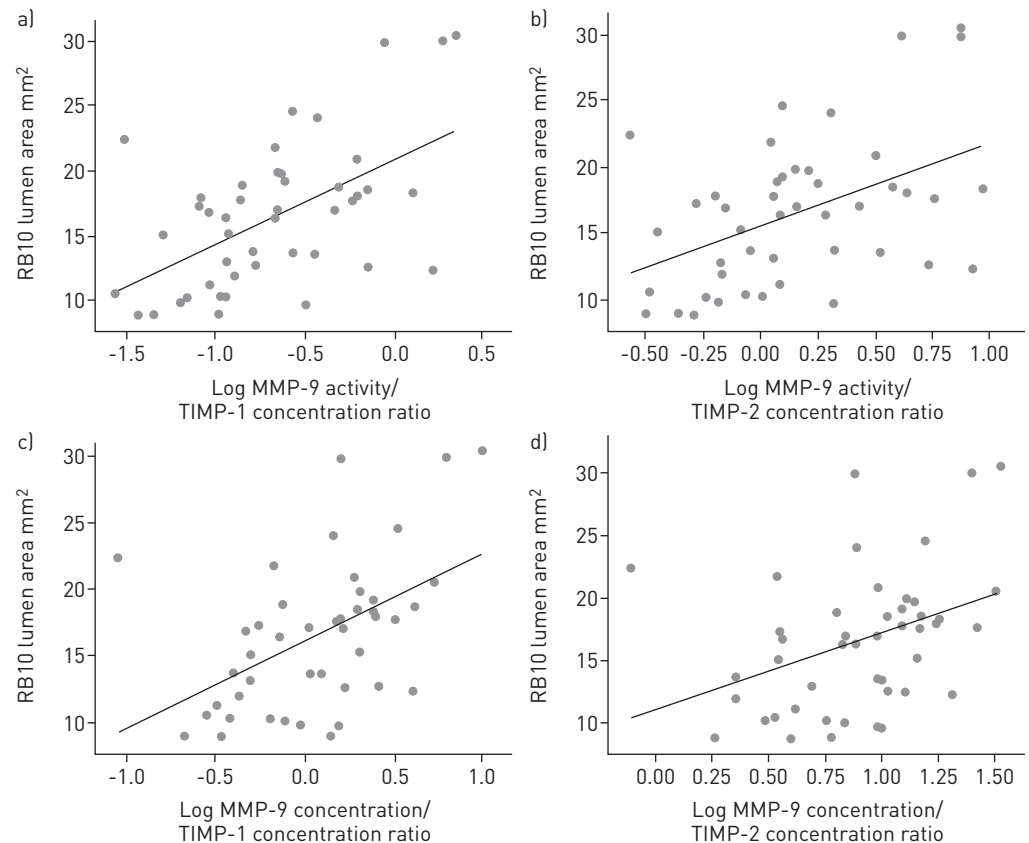


FIGURE 2 Association between a) sputum matrix metalloproteinase (MMP)-9 activity/tissue inhibitors of metalloproteinase (TIMP)-1 concentration ratio, b) MMP-9 activity/TIMP-2 concentration ratio, c) MMP-9 protein concentration/TIMP-1 concentration ratio, and d) MMP-9 protein concentration/TIMP-2 concentration ratio, with computed tomography (CT) airway lumen area in smokers with asthma. The ratios of sputum MMP-9 activity/TIMP-1 concentration, MMP-9 activity/TIMP-2 concentration, sputum MMP-9 protein concentration/TIMP-1 concentration, and MMP-9 protein concentration/TIMP-2 concentration correlated with right bronchial division 10 (RB10) airway lumen area in smokers with asthma ( $r=0.450$  (95% CI 0.138–0.717),  $r=0.410$  (0.102–0.668),  $r=0.499$  (0.189–0.728) and  $r=0.320$  (0.000–0.579), respectively).

asthma. Previous studies using sputum and bronchoalveolar lavage samples have reported a similar association in nonsmokers with asthma [13, 14, 25]. MMP-9 and TIMP-1 are known to be secreted by a variety of cells, including bronchial epithelial cells, eosinophils, mast cells and alveolar macrophages, and these may contribute more, relative to neutrophils, in orchestrating the changes in MMP-9 and TIMP-1 levels in smokers with asthma.

We recognise that there were limitations in the study, such as the fact that we did not perform bronchial biopsies to confirm the sputum, nasal and CT findings, and that the cross-sectional study design precludes collection of data on longitudinal changes to the balance between sputum MMP-9 and TIMPs. The inclusion of different severities of asthma reduces the numbers for conclusive statistical sub-analysis; however, it provides useful pilot data for larger studies of the effects of asthma severity.

In conclusion, these results show, for the first time, that the balance of MMP-9 and TIMPs is lower in smokers with asthma compared with never-smokers with asthma. A reduced sputum MMP-9 activity/TIMP ratio is associated with worse post-bronchodilator FEV<sub>1</sub> and FEV<sub>1</sub>/FVC ratio and decreased sub-segmental CT measurements of airway lumen in smokers with asthma. This result suggests that an imbalance of MMP-9 and TIMPs may contribute to structural changes to the airways in this group.

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