AN OBSERVATIONAL STUDY OF MATRIX METALLOPROTEINASE (MMP)-9 IN CYSTIC FIBROSIS.

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Abstract

Background

In Cystic Fibrosis (CF), cross–sectional studies have reported sputum matrix metalloproteinase (MMP)-9 to be elevated and negatively correlated with FEV_1. This longitudinal study examined the association between MMP-9 and tissue inhibitors of metalloproteinases (TIMP) to prognostic parameters in CF.

Method

A cross-sectional survey of CF and control subjects, CF patients were followed-up for a median of 49months. MMP-9 and TIMP-1&2 were quantified in sputum and plasma.

Results

Seventy-three patients with CF, median age 22 years and 40 controls were recruited. Fifty-three of these CF patients were followed up. Prospectively, in CF subjects, plasma MMP-9 activity was adversely associated with FEV_1 (β -1.15 (95% CI -2.10, -0.20), p=0.019 and rate of FEV_1 decline, plasma TIMP-1 was adversely associated with mortality: hazard ratio 3.66 (1.91-7.04), p<0.001.

Conclusions

These associations further justify investigation of MMP-9 and TIMP-1 as biomarkers for short- to medium-term FEV_1 decline and mortality in patients with CF.

Introduction

In cystic fibrosis (CF) the major causes of morbidity and mortality are the pulmonary manifestations of progressive airflow obstruction and bronchiectasis. Neutrophilic release of proteolytic enzymes is believed to be central to the pathogenesis of CF airway damage and remodelling [1]. The identification of the mediators contributing to the pathophysiological processes of CF airway disease could provide biomarkers with the potential to predict clinical prognosis, and assess response to therapy. Several studies have highlighted human neutrophil elastase in the pathophysiology of CF airway disease and increasing evidence suggests a role for matrix metalloproteinases (MMP) [2,3,4].

MMPs are zinc dependent enzymes implicated in extracellular matrix turnover, tissue degradation and repair [5]. MMP-9 is constitutively expressed by neutrophils, but inflammatory stimuli can induce MMP-9 expression by other airway cells [6,7,8]. MMPs are secreted in an inactive pro-form, activated extracellularly and inactivated by specific tissue inhibitors of metalloproteinases (TIMP) released by cells that often co-secrete MMPs [6]. MMP-9 can degrade extracellular matrix, and interacts with other substrates, including pro-IL-8 pro-TNF-α, and pro-IL-1β. MMPs have been implicated in the pathophysiology of asthma and COPD [6].

Several small single-centre studies have investigated MMP-9 in CF [4]. In children with mild/moderate lung impairment MMP-9 has been shown to be elevated in sputum and bronchoalveolar lavage fluid (BALF) [1,9,10]. In adults with CF, sputum and serum MMP-9 is elevated when compared with controls and increases further during infective exacerbations [11,12]. Two paediatric studies have reported associations between BALF MMP-9 and FEV_1 [1,9].

To test the hypothesis that sputum and plasma MMP-9 levels are associated with recognised clinical indicators of prognosis in CF, we conducted a study of children and adults with CF. MMP-9, TIMP-1 & 2 were measured and related to clinical parameters. A follow-up study was subsequently conducted to collect longitudinal clinical data and we present here the results of both studies.

Methods

Subjects

A cross-sectional study of patients aged ≥8 years attending paediatric and adult CF clinics in Aberdeen (Royal Aberdeen Children's Hospital, Aberdeen Royal Infirmary) and Dundee (Ninewells Hospital), Scotland was conducted in 2008. As in previous studies of MMP-9, all CF participants were symptomatically and spirometrically clinically stable, free of acute respiratory infections for ≥4 weeks [9,13,14]. Participants unable to spontaneously expectorate had sputum induced using 3.0% saline [15]. Blood samples were collected. The clinical data collected for CF participants included: age, sex, height, weight, and FEV_1. Also recorded were the bacterial species persistent in the sputum [16]. As described in detail elsewhere [17] healthy control subjects were recruited from the local population as part of contemporaneous studies of sputum MMP-9 in asthma and COPD. Healthy controls were non-smokers; without respiratory symptoms/disease or recent chest infection; had normal spirometry and no recent environmental tobacco smoke exposure. Longitudinal data subsequent to the cross-sectional study were collected in 2012 by accessing clinical annual review data. The number of infective exacerbations in the year after the initial study was recorded, along with annual body mass index (BMI) and FEV_1 measurements. For deceased patients, survival was noted.

The cross-sectional and follow-up studies were approved by Tayside Committee on Medical Research Ethics and the North of Scotland Research Ethics Service, respectively. All adults gave written informed consent and for children, parental written informed consent and the child's assent was obtained.

Samples, MMP-9, TIMP quantification

The methods used to process samples, the immunoassays, assay ranges and intra and inter assay precision are described in detail in the supplemental materials. Quantification of sputum and plasma MMP-9 and TIMP and sputum supernatant protein was performed at TMRC Core Laboratory, University of Dundee, UK.

Statistical considerations

MMP-9 and TIMP values above and below the assay range were recorded as 1 unit above the upper quantification limit and as half the lower quantification limit, respectively. Plasma MMP-9 activity and TIMP-1 values were all within their assay range. Sputum and plasma MMP-9 was expressed per millilitre supernatant/plasma, as absolute values and as ratios to TIMP-1, TIMP-2 and sputum protein. Visual inspection of MMP and TIMP data (confirmed by Lillifors corrected Kolmogorov-Smirnov test) indicated that they approximated to log-normal distributions and the data were logarithmically transformed. FEV_1 data were converted to standard deviation (z) scores, adjusting for age, height and sex [18]. BMI data were analysed as absolute values and for children as z scores [18]. Characteristics of controls and CF patients (similarly CF followed-up versus CF not followed-up) were compared using chi-squared tests, t-tests or Mann-Whitney as appropriate. Sputum and plasma MMP-9 and TIMP values were related to clinical parameters using t-tests and partial correlation (adjusting

for CF centre). Linear mixed effects models were used with random slopes and intercepts to model longitudinal FEV_1 and BMI data [19]. This modelling enabled MMP-9 and TIMP to be related to FEV_1 and BMI and rate of decline of these outcomes (interaction terms with age). Survival data were related to MMP-9 and TIMP values dichotomised above/below the median value using Kaplan-Meier plots and logrank tests. Proportional hazards models were used to relate survival to log-transformed MMP-9, TIMP values. Analyses were performed using IBM SPSS v21.0 (Armonk, NY) and SAS v9.3 (SAS Institute Inc., Cary, USA). The pre-study considerations of statistical power and sample size are provided in the supplemental material.

Results

84 adults and 73 children were attending the two CF clinics at the time of the study, with 104 fulfilling the study inclusion criteria (patients were mainly excluded because aged <8 years, no CF lung disease, or post lung transplantation). 73 CF patients (53 adults, 20 children) volunteered to participate in the cross-sectional study. 70 (96%) provided blood samples, 59 (81%) provided sputum samples (85% adults, 70% children), four after sputum induction. Forty healthy control subjects were recruited as part of contemporaneous studies of sputum MMP-9 in asthma and COPD [17]. Table 1 details the control and CF subjects. For the follow-on study, longitudinal data were available for 53 (72.6%) of the patients with CF who had participated in the cross-sectional study; 15 had died, 38 provided further consent, median follow-up was 49 months (IQR 37-54). When compared with CF subjects not followed up, CF subjects in the follow-on study were older, had a greater BMI, lower FEV_1 and higher sputum MMP-9 protein (MMP-9p), and higher plasma MMP-9p and TIMP-1 (Table 1).

Sputum MMP-9 in CF and controls

Compared with controls, CF subjects had significantly higher mean concentrations of sputum MMP-9p and MMP-9 activity (MMP-9a) and mean ratios of MMP-9 to TIMP-1, TIMP-2 and sputum protein, (Figure 1).

Correlations between sputum and plasma MMP-9 in CF

The geometric mean (95% CI) ratio of MMP-9a to MMP-9p in sputum was 0.33 (0.27-0.41) and in plasma 0.21 (0.18-0.25). Sputum MMP-9p and MMP-9a were correlated with plasma MMP-9a, (partial correlation coefficient 0.33, p=0.012 and 0.39, p=0.003 respectively). Sputum and plasma MMP-9p were not correlated.

Associations between MMP-9 and FEV_1 and BMI in CF

Cross-sectional study:

FEV_1 was weakly/moderately negatively correlated with sputum MMP-9p (correlation coefficient 0.32, p=0.015) and plasma MMP-9a but not correlated with sputum MMP-9a or plasma MMP-9p (table 2). BMI was not correlated with sputum MMP-9 but was weakly/moderately negatively correlated with plasma MMP-9 protein and activity (table 2). The associations between plasma MMP-9a and FEV_1 and BMI appeared to be stronger in adults, being of moderate strength.

Follow-on study:

FEV_1 and BMI measurements for the annual reviews in the five years subsequent to the initial study were available for 51 subjects (2 subjects died within a year of the cross-

sectional study). Linear mixed-effects modelling demonstrated no significant associations between plasma MMP-9a and prospective BMI measurements.

Linear mixed-effects modelling (Table 3) demonstrated significant negative associations between annual FEV_1 measurements subsequent to the initial study and plasma MMP-9a suggesting that high plasma MMP-9a is associated with lower FEV_1 in the years following MMP-9 measurement. Significant negative associations were demonstrated between sequential annual FEV_1 measurements and multiplicative interaction terms between age and plasma MMP-9a suggesting that high plasma MMP-9a is associated with a more rapid subsequent decline in FEV_1. The FEV_1 of subjects with plasma MMP-9a in the highest tertile declined faster at a rate of 0.12 (0.01, 0.22, p=0.032) z score units more per year increase in age than subjects with plasma MMP-9a in the lowest tertile.

Exacerbations

Sputum/plasma MMP-9 or TIMP were not associated with exacerbation rate in the year after measurement.

Bacterial infection in CF

Persistent lung bacterial infection rates were 88% (Staphylococcus aureus (SA)), 75% (Pseudomonas aeruginosa (PA)) and 68% (both organisms). SA infection was associated with elevated MMP-9p (but not MMP-9a) in sputum and plasma (supplemental figure S1). PA infection was associated with increased plasma (but not sputum) MMP-9 protein and activity (supplemental figure S2). Two-way analysis of variance was used to relate plasma MMP-9p to infection with both organisms, demonstrating that plasma MMP-9p concentrations were independently associated with these organisms (SA coefficient β 0.1.17, 95% CI 0.38-1.95,

p=0.004, PA β 1.06, 0.57-1.55, p<0.001). There was no statistical interaction between SA and PA infection.

CF survival

Of the 73 CF subjects participating in the cross-sectional study, 15 died during the follow-up period. Although no MMP-9 parameters were associated with survival, higher levels of plasma TIMP-1 were associated with increased mortality (figure 2): Mean (95% CI) survival of subjects with plasma TIMP-1 greater than median (47.0 (40.0-53.9) months) was less than those with TIMP-1 less than the median (58.4 (56.1-60.6) months), p=0.005. Multivariate modelling demonstrated that after adjustment for age, sex, centre, FEV_1, and BMI, an SD increase in plasma TIMP-1 was associated with a 3.66 (95% CI 1.91-7.04) fold increased likelihood of death in the subsequent 4-5 years, p<0.001.

Discussion

We have confirmed the previous reports that sputum MMP-9 protein and activity is elevated in CF and negatively correlated with FEV_1. Novel observations are that, in the short to medium term, plasma MMP-9a is adversely associated with subsequent rate of FEV_1 decline, and that plasma TIMP-1 is adversely associated with CF survival. A third novel finding is that plasma MMP-9p is associated with SA and PA infection in CF lung disease.

Several studies have reported increased sputum/BALF MMP-9p in CF adults [12] and children [1,8,9], however there have been no studies of sputum MMP-9a in children [9,10]. One study reported serum MMP-9 protein and activity to be elevated in CF adults, but there are no studies of serum/plasma MMP-9 in CF children. Whilst previous studies have reported sputum/serum MMP-9 to be elevated in CF, few have related MMP-9 to clinical prognosis.

FEV_1 in children has been reported to be negatively correlated with sputum MMP-9 [9] and BALF MMP-9:TIMP-1 ratio [1]. However no association has been reported between FEV_1 and sputum or serum MMP-9 protein/activity in adults. This study confirms that FEV_1 is negatively correlated with sputum MMP-9p and plasma MMP-9a but for the first time also demonstrates that the association is present and probably stronger in CF adults, perhaps reflecting age related changes in the microbiological and/or immunological stimuli to airway inflammation. In addition, we demonstrate that elevated plasma MMP-9a is associated with reduced FEV_1, and with an accelerated rate of decline of FEV_1A similar association between serum MMP-9 and rate of FEV_1 decline has been reported in COPD [20].

In a longitudinal study similar to ours Sagel et al related a single measurement of sputum biomarkers, in children with CF, to rate of lung function decline in the subsequent three years [13]. However, the children with CF studied by Sagel et al (mean age 11.1, mean FEV_1 95% predicted, and 17% lung infection with pseudomonas) differed somewhat from the current study that was predominantly adults (median age 24years), with impaired lung function (mean FEV_1 z score -3.20) and 82% lung infection with pseudomonas. Sagel et al reported no association between sputum MMP-9 activity and subsequent FEV_1 decline, and considered human neutrophil elastase (HNE) to be the most informative biomarker predicting subsequent FEV_1 decline. Notably Sagel et al did not quantify plasma HNE or MMP-9 activity. Although we did not quantify HNE, we similarly report no association between sputum MMP-9 activity and FEV_1 decline. Sagel et al reported a $\log_{10}$ increase in sputum HNE to be associated with a 1% increase in the rate of FEV_1 decline, whereas in the current study a $\log_{10}$ increase in plasma MMP-9 activity was associated with 5.6% increase in in the rate of FEV_1 decline.

The association we report between plasma MMP-9 activity and FEV_1 may be a consequence of intravascular HNE activation of pro-MMP-9 by cleavage at two sites between Valine[38] and Alanine[39],and between Alanine[39] and glutamic acid[40] [14]. These sites correspond with appropriate molecular weight for the activated MMP-9 isoform in CF sputum [14]. Further work will be required to confirm that the plasma MMP-9 activity associated with FEV_1 in the current study originates from HNE acting in the intravascular space, rather than reflecting 'leakage' from the airways or secretion from airway epithelial, endothelial or smooth muscle cells into the intravascular space [21,22]

Only one study appears to have related MMP-9 to CF severity parameters other than FEV_1. Hilliard et al [1] reported that BALF MMP-9p was not associated with lung PA infection or F508del homozygosity. Our demonstration that MMP-9 indices, particularly plasma MMP-9a, are associated with recognised parameters of CF severity (FEV_1, rate of FEV_1 decline, BMI, and PA/SA infection) implicate MMP-9 in the airway inflammatory processes, airway tissue damage and the systemic inflammatory processes of CF.

The association between FEV_1 and MMP-9a rather than MMP-9p is biologically plausible, reflecting the biological activity of the activated MMP-9. MMP-9 has several actions pertinent to the CF bronchial inflammation and tissue damage. It degrades extra-cellular matrix proteins and could plausibly contribute to bronchial wall destruction especially in the relative absence of TIMP as evidenced by the associations between the increased ratios of MMP-9: TIMP-1/2 and parameters of severity. In addition to tissue destruction, MMP-9 could also contribute to CF airway inflammation. For example MMP-9 has been implicated in neutrophil migration through collagen [23], and MMP-9 is a potent activator of pro-IL-1β

[24] and has been reported to increase neutrophil activity and chemotaxis by truncating IL-8 [25].

A novel finding of our study was that in patients with CF plasma TIMP-1 was adversely associated with survival and that this association was independent of FEV_1 and BMI. Similar adverse associations with TIMP-1 have been reported in COPD [26,27,28]. TIMPs inactivate MMPs in equimolar ratio [6] so it is somewhat counter-intuitive that TIMP-1 was adversely associated with survival given that TIMPs 'protect' tissues from degradation by MMPs. It is possible that TIMP-1 may not be exerting a 'protective' effect because of inactivation by HNE that has been shown to inactivate TIMP-1 by cleavage at a site between $Valine^{69}$ and $Cysteine^{70}$ [14]. Whatever the mechanism underlying the association with TIMP-1, the association we report is clinically significant, half of the CF subjects with plasma TIMP-1 in the highest quartile died in the subsequent 4-5 years and an SD increase in plasma TIMP-1 almost quadrupled mortality. Further studies are required to confirm that plasma TIMP-1 is a prognostic biomarker for survival and whether serial TIMP-1 measurements could be used to guide referral for lung transplantation (identifying patients with anticipated survival <50% in the next two years) and/or identify patients with a poor prognosis requiring intensification of treatment.

The current study has a number of strengths. The subjects comprised about 70% of the local adult CF population and were representative of the local CF population. We were able to follow-up a substantial proportion of the subjects for up to five years. A further strength was the measurement of both MMP-9 protein and activity in sputum and plasma. Limitations of the current study were not being able to measure plasma MMP-9 in the control subjects and only measuring MMP-9 once. The sputum MMP-9 measurements in normal subjects reported

here were from control subjects participating in local contemporaneous studies of MMP-9 in asthma and COPD that did not take blood samples. Although we have not directly demonstrated that plasma MMP-9 is elevated in CF, the positive correlations between sputum MMP-9 protein/activity and plasma MMP-9a suggest that the elevated sputum MMP-9 protein and activity levels in our CF patients are likely to be reflected by elevated plasma levels. MMP-9 and TIMPs were only measured once and therefore we cannot comment on the intra-subject variability of these measurements, however it is more likely that high intra-subject variability will generate type II errors rather than generating a type I error. An additional limitation was that longitudinal clinical data were not available for all the patients with CF who participated in the cross-sectional study. The ethical approval for the follow-up study required that participants were reconsented to access clinical notes and not all patients reconsented. The patients with CF not participating in the follow-up study differed from those who did, appearing to be in better health, (less likely to die, better lung function, fewer CF related complications, less likely to be colonised with PA, lower plasma MMP-9 and TIMP-1 levels). Considerations of these biases suggest that they are unlikely to have generated the observed longitudinal association with FEV_1, instead these biases are likely to have reduced the strength of the observed association. The association between TIMP-1 and survival was not influenced by incomplete follow-up because survival status was available for all those participating in the cross-sectional study. A further limitation was that many tests of statistical significance were conducted and it is possible that the reported associations are a consequence. However given the strength and consistency of the associations between cross-sectional and longitudinal data this seems unlikely.

In summary we measured sputum/plasma MMP-9 protein/activity in CF children and adults, and in a cross-sectional study confirmed that sputum MMP-9 levels are elevated in CF and

negatively correlated with FEV_1. Plasma MMP-9a is associated with bacterial infection, and negatively correlated with BMI. In a prospective 4-5 year follow-up plasma MMP-9a was adversely associated with FEV_1 decline, and plasma TIMP-1 was adversely associated with mortality. The magnitude of the report associations are clinically significant and justify further investigation into the roles of plasma MMP-9a and TIMP-1 in CF and their potential utility as short to medium term prognostic biomarkers.

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Conflict of interest statement

During the course of the cross –sectional study DM, JB, and JTJH were employed by Wyeth Pharmaceuticals, DM also held stocks/stock options with the Wyeth/Pfizer Pharmaceuticals. The remaining authors had no perceived or actual conflicts of interest.

References

1. Hilliard TN, Regamey N, Shute JK, Nicholson AG, Alton EWF, Bush A, Davies JC. Airway remodelling in children with cystic fibrosis. Thorax 2007; 62: 1074-80.

2. Mayer-Hamblett N, Aitken ML, Accurso FJ, Kronmal RA, Konstan MW, Burns JL, Sagel SD, Ramsey BW. Association between pulmonary function and sputum biomarkers in cystic fibrosis. Am J Respir Crit Care Med 2007; 175: 822-8.

3. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. J Pediatr 2002;141:811–7.

4. Gaggar A, Hector A, Bratcher PE, Mall MA, Griese M, Hartl D. The role of matrix metalloproteinases in cystic fibrosis lung disease. Eur Respir J 2011; 38: 721–727.

5. Nagase H, Woessner JF. Matrix metalloproteinases. J Biol Chem 1999; 274:21491-4

6. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. Exp Lung Res 2005;31:599–621.

7. Dagnell C, Kemi C, Klominek J, Eriksson P, Skold CM, Eklund A, Grunewald J, Olgart Hoglund C. Effects of neurotrophins on human bronchial smooth muscle cell migration and matrix metalloproteinase-9 secretion. J Lab Clin Med 2007; 150:303-10.

8. Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, Ferreira DS, Mulder A, Gomes HA, Fernezlian SM, James A. Mauad T. Extracellular matrix components and regulators in the airway smooth muscle in asthma. Eur Respir J 2008; 32:61-9.

9. Ratjen F, Hartog C-M, Paul K, Wermelt J, Braun J. Matrix metalloproteases in BAL fluid of patients with cystic fibrosis and their modulation by treatment with dornase alpha. Thorax 2002; 57: 930-4.

10. Sagel SD, Kapsner RK, Osberg I. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. Ped Pulm 2005; 39: 224-32.

11. Roderfeld M, Rath T, Schulz R, Seeger W, Tschuschner A, Graf J, Roeb E. Serum matrix metalloproteinases in adult CF patients: Relation to pulmonary exacerbation. J Cystic Fibrosis 2009; 8: 338-47.

12. Gaggar A, Li Y, Weathington N, winkler M, Kong M, Jackson P, Blalock JE, Clancy JP. Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients. Am J Physiol Lung Cell Mol Physiol 2007; 293: 96-104.

13. Sagel SD, Wagner BD, Anthony MM, Emmett P, Zemanick ET. Sputum biomarkers of inflammation and lung function decline in children with Cystic Fibrosis. Am J Respir Crit Care Med 2012; 186,: 857–865.

14. Jackson PL, Xu X, Wilson L,Weathington NM, Clancy JP, Blalock JE, Gaggar A. Human Neutrophil Elastase-Mediated Cleavage Sites of MMP-9 and TIMP-1: Implications to Cystic Fibrosis Proteolytic Dysfunction. Mol Med 2010; 16:159-66.

15. Henig NR, Tonelli MR, Pier MV, Burns JL, Aitken ML. Sputum induction as a research tool for sampling the airways of subjects with cystic fibrosis. Thorax 2001; 56: 306-11.

16. Lee TW, Brownlee KG, Conway SP, Denton M, Littlewood JM. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J Cyst Fibros 2003;2:29–34.

17. Huang JTJ, Chaudhuri R, Albarbarawi O, Barton A, Grierson C, Rauchhaus P, Weir CJ, Messow M, Stevens N, McSharry C, Feuerstein G, Mukhopadhyay S, Brady J, Palmer CNA, Miller D, Thomson NC. Clinical validity of plasma and urinary desmosine as biomarkers for chronic obstructive pulmonary disease. Thorax 2012;67:502-508.

18. Stanojevic S, Wade A, Stocks J, Hankinson J, Coates AL, Pan H, Rosenthal M, Corey M, Lebecque P, Cole TJ. Reference ranges for spirometry across all ages. Am J Respir Crit Care Med 2008;177: 253–60.

19. Brown H and Prescott R. Applied mixed models in medicine. John Wiley & Sons, Ltd, Chichester, UK, 2001.

20. Higashimoto Y, Iwata T, Okada M, Satoh H, Fukuda K, Tohda Y. Serum biomarkers as predictors of lung function decline in chronic obstructive pulmonary disease. Resp Med 2009; 103:1231-8.

21. Sack R, Sathe S, Beaton AR, McNamara N, Fleiszig S, Ni M. Protein array characterization of bioactive proteins secreted by immortalized human corneal epithelium in response to pseudomonas constituents. Curr Eye Res 2009;34:92-8.

22. Perez A, van Heeckeren AM, Nichols D, Gupta S, Eastman JF, Davis PB. Peroxisome proliferator-activated receptor-gamma in cystic fibrosis lung epithelium. Am J Physiol Lung Cell Molec Physiol 2008;295:L303-13.

23. Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A, Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. Am J Respir Cell Molec Biol 1996; 14:288-95.

24. Schonbeck U, Mach F, Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 1998; 161:3340-6.

25. Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood 2000; 96:2673-81.

26. Engstrom G, Lindberg C, Gerhardsson de Verdier M, Nihlen U, Anderson M, Svartengren M, Forsman-Semb K. Blood biomarkers and measures of pulmonary function--a study from the Swedish twin registry. Respir Med 2012; 106:1250-7

27. Olafsdottir IS, Janson C, Lind L, Hulthe J, Gunnbjornsdottir M, Sundstrom J. Serum levels of matrix metalloproteinase-9, tissue inhibitors of metalloproteinase-1 and their ratio are associated with impaired lung function in the elderly: a population-based study. Respirology 2010; 15:530-5.

28. Kwiatkowska S, Noweta K, Zieba M, Nowak D, Bialasiewicz P. Enhanced exhalation of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with COPD exacerbation: a prospective study. Respiration 2012; 84:231-41.

Figure and Table legends

Table 1: Characteristics of control and CF subjects participating in the cross-sectional study and the CF subjects who did or did not participate in the follow up study.

Table 2: Partial correlation coefficients (adjusted for CF centre) between plasma MMP-9 and FEV_1 , in all CF subjects and adult CF subjects only.

Table 3: Results of linear mixed effects modelling (random coefficient model) relating FEV_1 z score to plasma MMP-9 activity, age and plasma MMP-9 activity age interaction.

Figure 1: Box and whisker plot of MMP-9 and TIMP concentrations in the sputum supernatants of CF and control subjects.

($p<0.001$ for all differences between CF and control values unless indicated).

*ratio is to sputum protein. MMP-9p = MMP-9 protein. MMP-9a = MMP-9 activity

Figure 2: Kaplan Meier plot of survival of subjects with CF with plasma TIMP-1 concentrations above or below the median value.

	Controls (n=40)	CF initial study (n=73)	p value*	CF followed up (n=53)	CF not followed up (n=20)	p value+
Male	45.0%	54.2%	0.37	55.8%	52.4%	0.79
Median age (IQR)	23 (20-47)	22 (16-30)	0.067	24 (17-33)	18 (15-24)	0.030
Mean height cm (SD)	171 (8.58)	163 (9.74)	<0.001	163 (10.2)	161 (11.9)	0.54
Mean BMI kg/m^2 (SD)	25.1 (4.02)	20.3 (3.60)	<0.001	20.9 (3.18)	18.7 (3.78)	0.013
Mean FEV_1 z-score (SD)	0.07 (1.00)	-3.91 (1.92)	<0.001	-4.20 (1.80)	-3.20 (2.03)	0.057
%CF liver disease		29.2%		35.3%	14.3%	0.075
%CF diabetes mellitus		29.2%		31.4%	23.8%	0.521
Sputum infection						
% S aureus		88%		94.1%	81.0%	0.087
% P aeruginosa		75%		82.4%	61.9%	0.063
% Burkholderia		8%		9.8%	4.8%	0.482
Sputum						
MMP-9 protein ng/ml				2800 (2328-3367)	1479 (816-2680)	0.001
MMP-9 activity ng/ml				826 (646-1058)	670 (468-958)	0.089
TIMP-1 ng/ml				72.9 (39.8-134)	122 (97.8-153)	0.33
TIMP-2 ng/ml				88.8 (69.2-114)	132 (70.2-113)	0.87
Plasma						
MMP-9 protein ng/ml				158 (118-210)	71.4 (46.2-110)	0.005
MMP-9 activity ng/ml				32.8 (26.3-41.0)	21.9 (13.8-34.6)	0.19
TIMP-1 ug/ml				98.8 (89.6-109)	79.2 (70.6-88.9)	0.001
TIMP-2 ug/ml				148 (134-165)	140 (127-155)	0.36

Table 1: Characteristics of control and CF subjects participating in the initial cross-sectional study and the CF subjects who did or did not participate in the follow up study.

*: p value CF subjects initial study vs control subjects

+: p value CF subjects followed up vs CF not followed up (n=20).

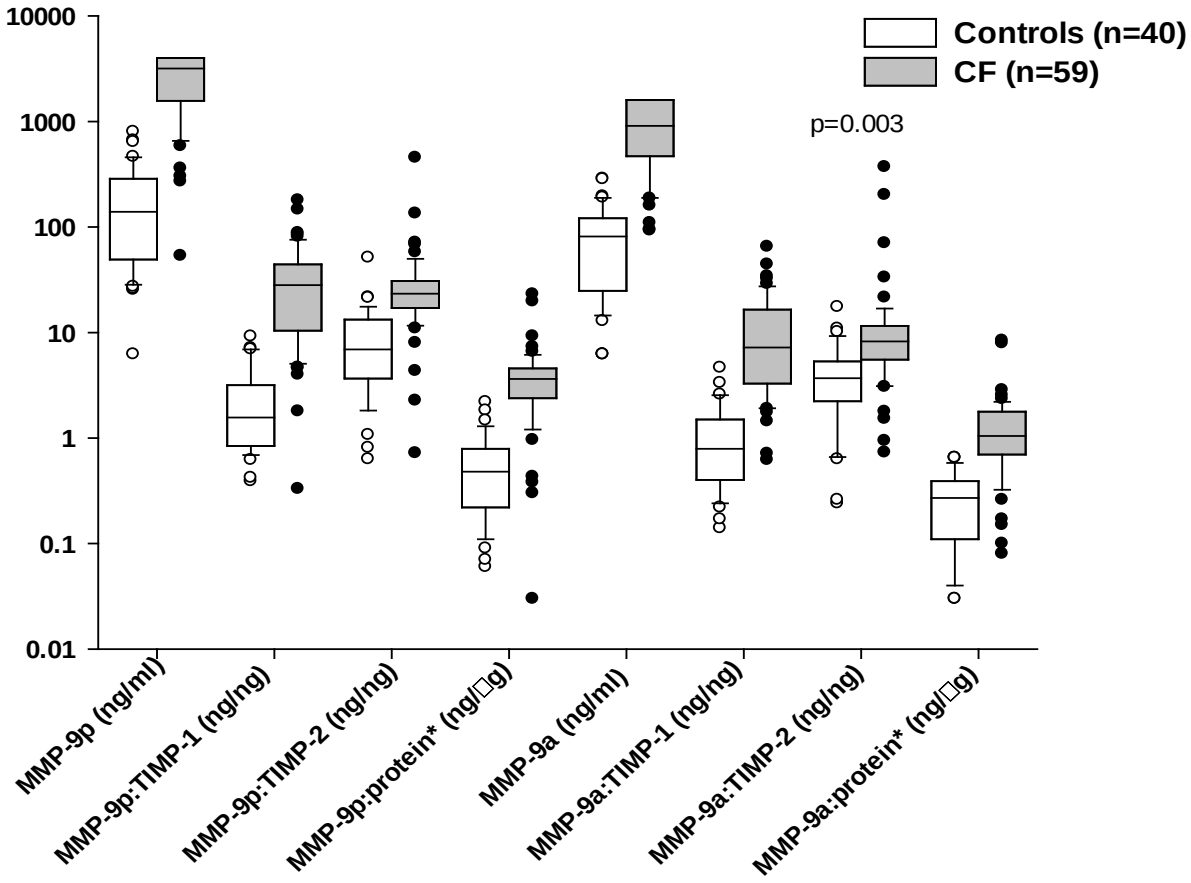

Figure 1 Box and whisker plot of MMP-9 and TIMP concentrations in the sputum supernatants of CF and control subjects.

($p<0.001$ for all differences between CF and control values unless indicated).

*ratio is to sputum protein. MMP-9p = MMP-9 protein. MMP-9a = MMP-9 activity

	FEV_1 z score					BMI			
Plasma	all subjects	p value	adults only	p value		all subjects	p value	adults only	p value
MMP-9 protein ng/ml	0.17	0.902	-0.19	0.188		-0.27	0.027	-0.13	0.370
MMP-9 protein:TIMP-1 ng/ng	0.07	0.623	-0.13	0.369		-0.26	0.035	-0.13	0.365
MMP-9 protein:TIMP-2 ng/ng	-0.09	0.535	-0.24	0.088		-0.24	0.044	-0.19	0.188
MMP-9 activity ng/ml	-0.29	0.015	-0.51	<0.001		-0.37	0.002	-0.47	0.001
MMP-9 activity:TIMP-1 ng/ng	-0.24	0.048	-0.45	0.001		-0.37	0.002	-0.46	0.001
MMP-9 activity:TIMP-2 ng/ng	-0.39	0.001	-0.53	<0.001		-0.33	0.005	-0.49	<0.001

Table 2: Partial correlation coefficients (adjusted for CF centre) between plasma MMP-9 and FEV_1 in all CF subjects and adult CF subjects only.

Model	*FEV_1: subsequent to MMP measurement*	
	Coefficient (95% CI)	p-value
Age	-0.70(-0.11, -0.03)	0.003
MMP-9a$^+$	-1.15 (-2.10, -0.20)	0.019
Age	-0.07 (-0.11, -0.02)	0.004
MMP-9a:TIMP-1	-0.94 (-1.72, -0.17)	0.019
Age	-0.06 (-0.10, -0.02)	0.006
MMP-9a:TIMP-2	-1.30(-2.01, -0.58)	<0.001
Age	0.14 (-0.08, 0.35)	0.216
MMP-9a	0.53 (-1.21, 2.27)	0.547
Age*MMP-9a	-0.063 (-0.13, 0.000)	0.050
Age	-0.52 (-0.96, -0.08)	0.022
MMP-9a:TIMP-1	0.51 (-0.94, 1.97)	0.485
Age*MMP-9a:TIMP-1	-0.055 (-0.11, -0.001)	0.046
Age	0.38 (-0.90, 0.14)	0.145
MMP-9a:TIMP-2	-0.31 (-1.94, 1.33)	0.710
Age*MMP-9a:TIMP-2	-0.037 (-0.10, 0.02)	0.219

Table 3: Results of linear mixed effects modelling (random coefficient model) relating FEV_1 z score to plasma MMP-9 activity, age and plasma MMP-9 activity age interaction.

+MMP-9a: plasma MMP-9 activity. MMP-9 and TIMP parameters transformed to $\log_e$

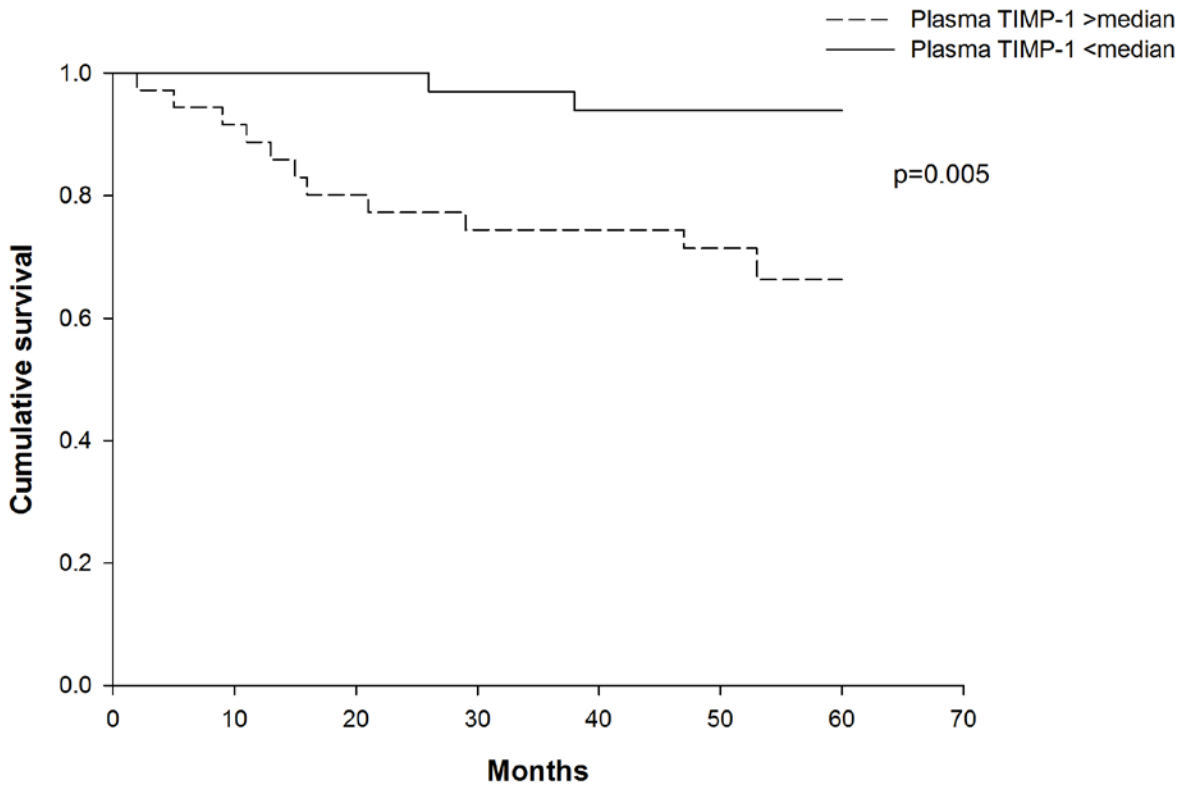

Figure 2: Kaplan Meier plot of survival of subjects with CF with plasma TIMP-1 concentrations above or below the median value.

	Controls (n=40)	CF initial study (n=73)	p value*	CF followed up (n=53)	CF not followed up (n=20)	p value+
Male	45.0%	54.2%	0.37	55.8%	52.4%	0.79
Median age (IQR)	23 (20-47)	22 (16-30)	0.067	24 (17-33)	18 (15-24)	0.030
Mean height cm (SD)	171 (8.58)	163 (9.74)	<0.001	163 (10.2)	161 (11.9)	0.54
Mean BMI kg/m^2 (SD)	25.1 (4.02)	20.3 (3.60)	<0.001	20.9 (3.18)	18.7 (3.78)	0.013
Mean FEV_1 z-score (SD)	0.07 (1.00)	-3.91 (1.92)	<0.001	-4.20 (1.80)	-3.20 (2.03)	0.057
%CF liver disease		29.2%		35.3%	14.3%	0.075
%CF diabetes mellitus		29.2%		31.4%	23.8%	0.521
Sputum infection						
% S aureus		88%		94.1%	81.0%	0.087
% P aeruginosa		75%		82.4%	61.9%	0.063
% Burkholderia		8%		9.8%	4.8%	0.482
Sputum						
MMP-9 protein ng/ml				2800 (2328-3367)	1479 (816-2680)	0.001
MMP-9 activity ng/ml				826 (646-1058)	670 (468-958)	0.089
TIMP-1 ng/ml				72.9 (39.8-134)	122 (97.8-153)	0.33
TIMP-2 ng/ml				88.8 (69.2-114)	132 (70.2-113)	0.87
Plasma						
MMP-9 protein ng/ml				158 (118-210)	71.4 (46.2-110)	0.005
MMP-9 activity ng/ml				32.8 (26.3-41.0)	21.9 (13.8-34.6)	0.19
TIMP-1 ug/ml				98.8 (89.6-109)	79.2 (70.6-88.9)	0.001
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On-Line supplemental material

AN OBSERVATIONAL STUDY OF MATRIX METALLOPROTEINASE (MMP)-9 IN CYSTIC FIBROSIS.

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Supplemental material: methods

Plasma and sputum samples were processed within two hours.

Sputum supernatant processing.

Sputum was processed in dithiothreitol (Sputolysin) as described by Pavord et al, 1997 [1]; Briefly, sputum plugs were selected, removed and weighed, ensuring a minimum amount of saliva was associated with the plugs before incubation with 4 volumes of Sputolysin for 15 minutes at room temperature. Total cell numbers and viability were assessed before separation of the supernatant from the cell pellet. All supernatant aliquots were stored at -80^{o}C and sputum samples were assayed after a single freeze-thaw cycle. A small sample from the cell pellet was used to generate slides which were stained with Rapidiff (Bio Technology Sciences Ltd) to allow assessment of differential white cell counts. The remaining cell pellet was processed further for isolation of RNA.

MMP-9 Assays

MMP-9 Elisa and MMP-9 activity kits were supplied by R&D systems (Abingdon, UK) and validated for use in sputum supernatant and plasma by the TMRC. Assays were performed in accordance with the kit inserts provided, however the activity assay was read kinetically over 4 hours as this showed better reproducibility than an end-point read. Samples were not chemically activated with APMA to allow estimation of endogenous enzyme activity. Sample dilutions for MMP-9 ELISA were 1:40 or 1:200 for sputum supernatant and 1:40 for plasma. Sputum was diluted 1:50 or 1:100 for MMP-9 FRET and plasma was diluted 1:50. Intra, inter assay precision and detectable range of the assays are outlined in Table S1.

TIMP Assay

An X-Map multiplex kit (Cat # LKT003) supplied by R&D systems (Abingdon, UK) was used to simultaneously determine the concentrations of TIMPs 1 and 2 on a Bioplex analyser as per manufacturer's instructions. Sputum and plasma samples were diluted 1:50. The Bioplex® analyser employs a dual laser and a flow based sorting and detection platform. The assays are based on the sandwich immunoassay principle with pre-coated analyte specific antibodies bound to colour coded microparticles and respective detection antibodies are biotinylated for detection with Streptavidin. One laser determines the analyte being detected (colour coded microparticles) and the other measures the phycoerythrin derived signal which is proportional to the concentration of analyte. Intra, inter assay precision and detectable range of the assays are outlined in Table S1.

Protein assay

Sputum supernatant protein was measured colorimetrically using the Pierce 660nm Protein Assay Product no. 1861426. (ThermoFisher Scientific, Cramlington, UK).

Statistical considerations

MMP-9: comparison of CF vs normal controls.

Sputum MMP-9 concentrations in the 70 CF patients recruited to the study were compared with MMP-9 concentrations in 40 normal control subjects recruited and characterised in a parallel project. With a mean sputum MMP-9 concentration of 44 ng/ml (SD 104) in normal control subjects [1] the current study had 80% power to detect a minimum 80% difference in MMP-9 levels in the sputum of CF patients and controls at the 5% level of significance. i.e

normal 44ng/ml, CF 79ng/ml. It is likely that the difference will be far greater; Sagel et al [2] reported a mean MMP-9 level in the sputum of children with mild CF of about 4000ng/ml.

MMP-9 and rate of decline of FEV_1

The median rate of decline of FEV1 in 30 adult CF patients in Grampian in five years prior to the reported study was 2.6%/year (mean 3.4, SD 2.6). Based on Sagel et al [2] with a mean MMP-9 sputum concentration of about 4000 ng/ml (SD 1700), if 70 patients were followed up the study would have had 80% power (at the 5% level of significance) to detect a 30% difference in the MMP-9 levels (ie 4000 vs 5200 ng/ml) in the sputum of the 35 CF patients with rates of decline of FEV1 above the median and the 35 patients with rates of decline below the median rate of decline (approximately 2.7%/year).

The percentage and absolute differences with 70, 60 and 50 patients with CF followed up are detailed in table S1.

Number followed up	Power	α	% difference detectable	Absolute difference detectable
70	80%	0.05	30%	4000 vs 5200 ng/ml
60	80%	0.05	31%	4000 vs 5250 ng/ml
50	80%	0.05	34%	4000 vs 5375 ng/ml

Table S1: Pre study consideration of statistical power, percentage and absolute differences with 70, 60 and 50 patients with CF followed up

References

1. Beeh KM. Beier J. Kornmann O. Buhl R**.** Sputum matrix metalloproteinase-9, tissue inhibitor of metalloprotinease-1, and their molar ratio in patients with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and healthy subjects. Resp Med2003; 97:634-9.

2. Sagel SD, Kapsner RK, Osberg I. Induced sputum metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. Ped Pulm 2005; 39:224-232

Assay with matrix	**Supplier**	**Cat #**	**Intra-assay imprecision (%)**	**Inter-assay imprecision (%)**	**Reportable Range**
MMP-9 ELISA sputum	R&D Systems	DMP900	<7.5	<5.0	12.5-4000 ng/mL
MMP-9 ELISA plasma	R&D Systems	DMP900	<4.5	<4.0	12.5-800 ng/mL
MMP-9 FRET sputum	R&D Systems	F9M00	<10.0	<9.0	12.5-1600 ng/mL
MMP-9 FRET plasma	R&D Systems	F9M00	<10.0	<9.0	12.5-800 ng/mL
TIMP-1	R&D Systems	LKT003	<9.0	nd	61.6-452000 pg/mL
TIMP-2	R&D Systems	LKT003	<9.0	nd	588-1680000 pg/mL

Table S2: Details of MMP-9 and TIMP assays.

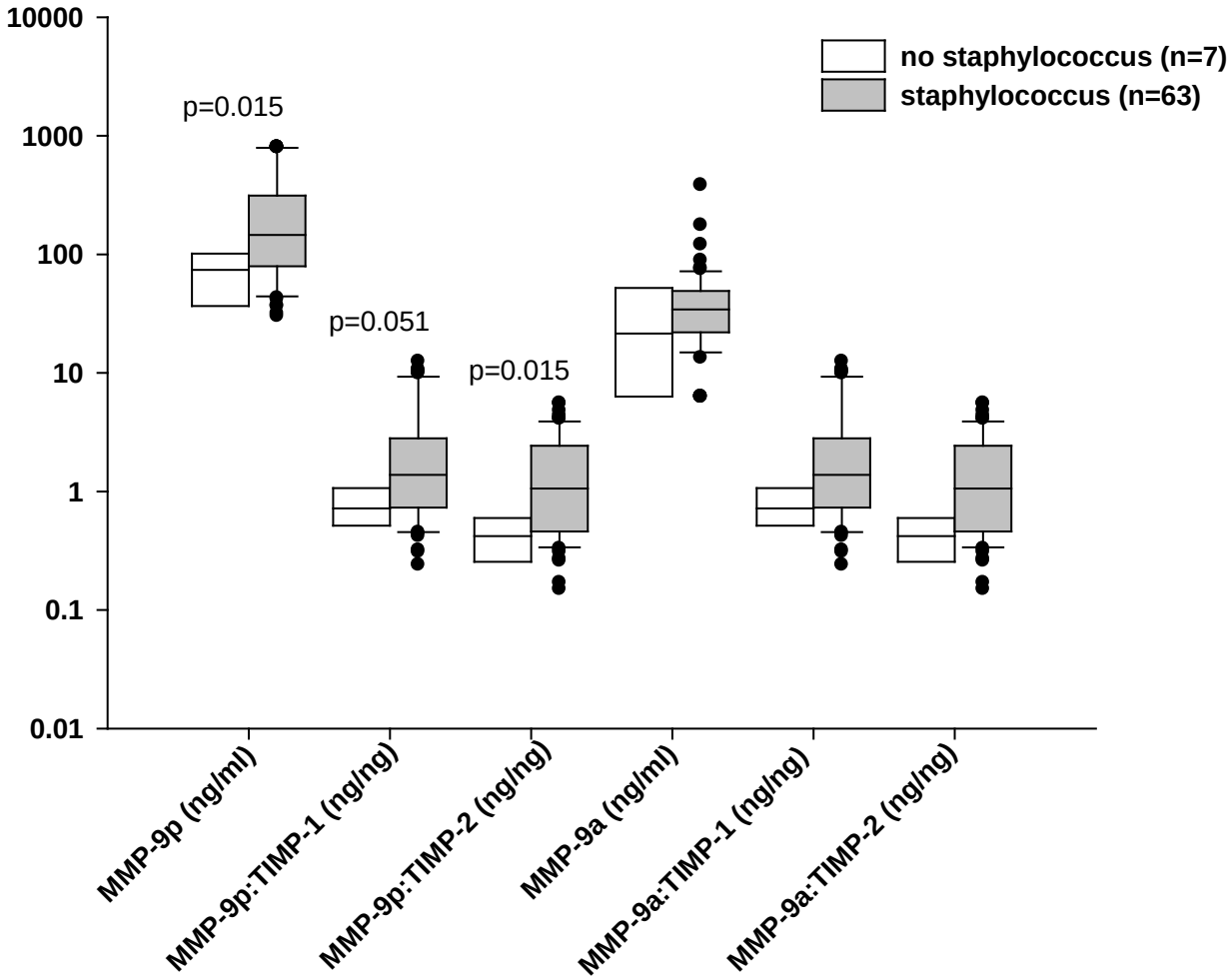

Figure S1: Box and whisker plot of plasma MMP-9protein and MMP-9 activity in CF subjects with and without chronic sputum colonisation with staphylococcus aureus. ($p>0.1$ unless indicated). MMP-9p = MMP-9 protein. MMP-9a = MMP-9 activity

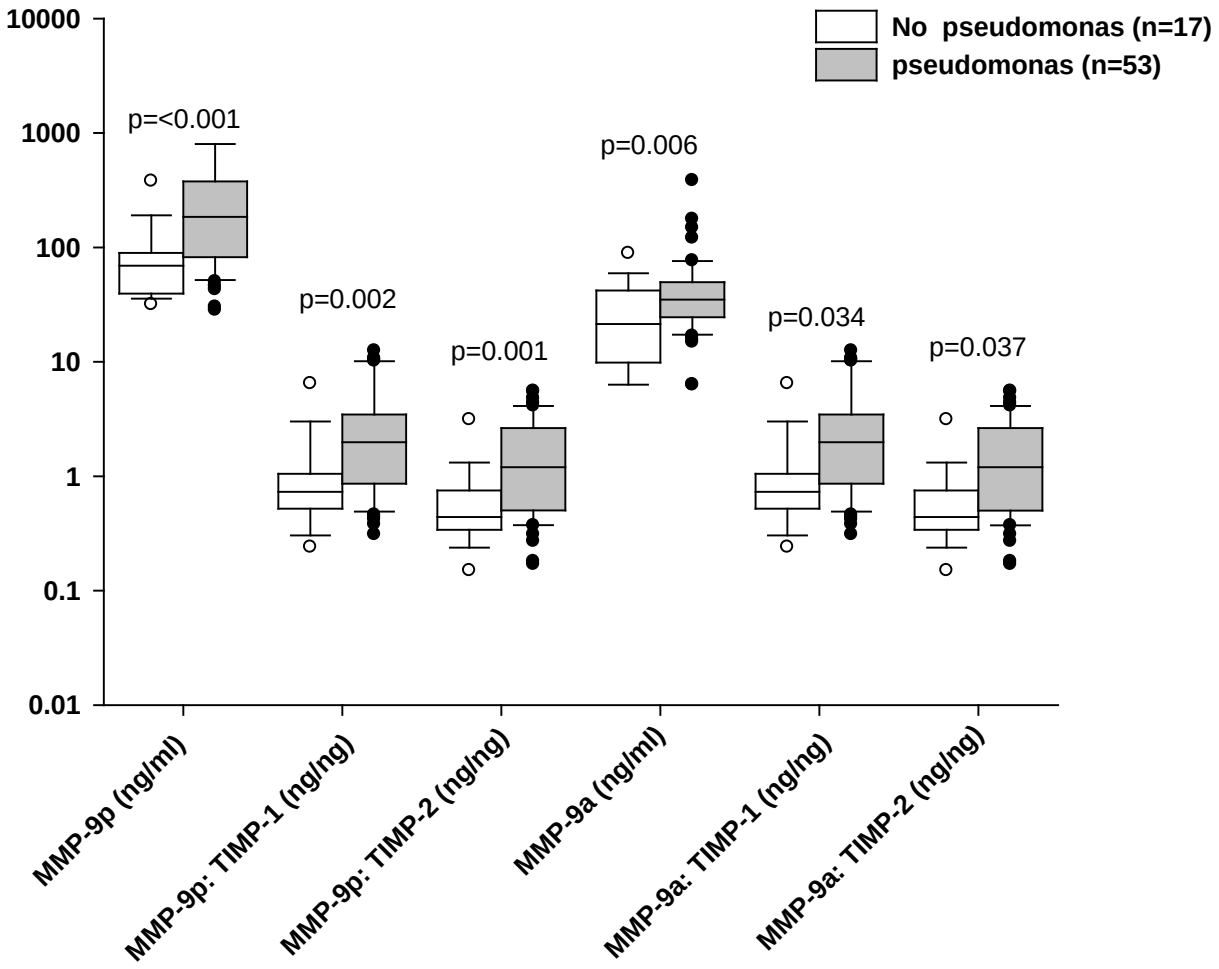

Figure S2: Box and whisker plot of plasma MMP-9protein and MMP-9 activity in CF subjects with and without chronic sputum colonisation with pseudomonas aeruginosa. MMP-9p = MMP-9 protein. MMP-9a = MMP-9 activity