Alveolar macrophage proteinase/antiproteinase expression and lung function/emphysema

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Short title: Macrophage proteinases and emphysema

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ABSTRACT (word count = 193)

Alveolar macrophages play an important role in chronic obstructive pulmonary disease (COPD) via production of matrix metalloproteinases (MMPs) and cathepsins as well as their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) and cystatin C (CST3). We hypothesized that expression levels of these molecules by alveolar macrophages at baseline and after stimulation would be influenced by genotype and associated with COPD phenotypes.

Quantitative PCR and enzyme-linked immunosorbent assays / gelatin zymography were used to investigate expression levels of mRNA and protein, respectively. The relationships of expression with genotype, pulmonary function and emphysema were analyzed.

The results showed that basal expression level of MMP12 mRNA was inversely related to DLco/VA and to FEV1/FVC after correction for multiple comparisons. The expression level of MMP12 protein stimulated with LPS was also inversely related to DLco/VA and was positively related to the extent of emphysema. The basal expression of MMP1 mRNA was positively correlated with the extent of emphysema. Cathepsin L protein level was positively associated with FEV1% predicted.

We conclude that increased MMP12 and MMP1 expression may play a role in the pathogenesis of emphysema. Cathepsin L and MMP9 may be involved in the development of airflow limitation.

Keywords: airflow limitation; cathepsins; emphysema; matrix metalloproteinases
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible and is a major cause of chronic morbidity and mortality throughout the world [1]. The hallmark of this disease is an inflammatory process within the lungs in response to noxious particles originating mainly from cigarette smoke, with concomitant destruction and repair of the lung architecture, leading to tissue remodeling. Although the major trigger for the development of COPD is cigarette smoke, only a minority of chronic heavy smokers develop symptomatic COPD [2]. The clustering of COPD in families suggests that there is a genetic component [3] and may explain why only a subset of smokers develops this disease.

A proteinase:antiproteinase imbalance is thought to play a central role in the pathogenesis of COPD [4]. An important source of both proteinases and antiproteinases within the lung is the alveolar macrophage, which produces matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases – TIMPs), as well as cathepsins and a potent inhibitor of cathepsins, cystatin C (CST3).

MMPs are a family of proteolytic enzymes that degrade collagen or elastin and play an essential role in tissue remodeling and repair associated with inflammation [5]. MMP1, MMP9, and MMP12 are produced by alveolar macrophages, and several studies in animals and humans have provided evidence that these MMPs are important in airway inflammation and the development of emphysema [6, 7]. Cathepsin L (CTSL) and cathepsin S (CTSS) are lysosomal cysteine proteinases produced by alveolar macrophages [6] that may also be involved in COPD through degradation of elastin [8].

Since chronic inflammatory processes are thought to be important in the pathology of COPD, cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)–α may play a
critical role in the disease process [9]. Microbial infections induce exacerbations through bronchial inflammation in COPD patients, which causes tissue damage and could contribute to the development of emphysema [10]. Thus, it is important to also examine the expression change of proteinases and antiproteinases induced by inflammatory cytokines or lipopolysaccharide (LPS), a potent secretagogue for a variety of inflammatory cytokines [10].

We hypothesized that higher expression levels (basal and stimulated by IL-1β, TNF-α or LPS) of proteinases (MMP1, MMP9, MMP12, CTSL and CTSS) and/or lower levels of antiproteinases (TIMP1, TIMP2, TIMP3 and CST3) in human alveolar macrophages would be associated with lower lung function (airflow limitation and diffusion capacity) and greater extent of emphysema. We also hypothesized that polymorphisms of these genes would alter the level of expression of their mRNA and protein.
MATERIALS AND METHODS

Study subjects

One hundred and nine subjects who underwent lung resection for a small (<3 cm diameter), Stage I or II, peripheral tumors at Vancouver General Hospital were recruited for this study. Partial results from fifty-four of these subjects were included in a previous study [11]. Pre-operative pulmonary function tests were performed including forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), and single breath diffusing capacity (DLCO) / alveolar volume (VA), in accordance with ATS guidelines [12]. Computed tomography (CT) scans were available for assessment of emphysema in 75 subjects. The characteristics of the subjects are shown in Table 1.

This study was approved by the University of British Columbia/Providence Health Care and Vancouver Hospital and Health Sciences Research Ethics Boards. All subjects provided written informed consent for the study.

Computed tomographic analysis

Details of a semi-quantitative emphysema visual score, and a quantitative computerized analysis of the percentage of lung with emphysema, are given in the supplemental Materials and Methods.

Bronchoalveolar lavage and cell culture

The resected lung or lobe was obtained immediately post-operatively and bronchoalveolar lavage was performed. Details of the lavage, macrophage separation and subsequent culture are given in the supplemental Materials and Methods.
RNA extraction, cDNA synthesis and quantitative real-time PCR

Details of cDNA preparation and quantification by real-time PCR (Table E1) are given in the supplemental Materials and Methods.

Protein quantification

Details of the protein quantification by enzyme linked immunosorbent assays or zymography are given in the supplemental Materials and Methods.

Genotyping

We selected polymorphisms (Table E2) in MMPs, cathepsins, TIMPs, and cystatin C that have been previously associated with COPD and/or with regulation of gene expression (rs1799750 in MMP1, D20S838 and rs3918242 in MMP9, rs2276109 in MMP12, and rs2277698 in TIMP2 [13-17]). In addition, we selected single nucleotide polymorphisms (SNPs) from the HapMap Project (http://www.hapmap.org/) to identify a haplotype block covering the promoter region of each gene (details are given in the supplemental Materials and Methods).

Statistical analysis

Statistical analyses were performed using JMP software version 5.1 (SAS Institute Inc., Cary, NC, USA). Linear regression analysis was used to evaluate mRNA or protein expression versus pulmonary function tests and quantitative CT scan data. We also used linear regression to evaluate genotype versus mRNA or protein expression with an additive model, and p-values were calculated in the linear model based on a Wald test statistic. Ordinal regression was used to
examine the association between semi-quantitative CT scores and gene expression levels. Adjustment was performed for age, sex, race and smoking history (pack years). If the values were not normally distributed, they were log-transformed before analyses.

P values <0.05 were considered nominally significant. Due to the nature of the analysis and the multiple-testing of independent and dependent variables, modified Bonferroni correction for multiple comparisons was done as described by Nyholt [18] and details of this correction procedure are provided in the supplemental Materials and Methods (Tables E3 and E4). The power of this study design was estimated using the many proportions function and linear regression function in PASS 2005 [19].
RESULTS

Proteinase and antiproteinase mRNA expression

The mRNA expression levels of the proteinases (MMP1, MMP9, MMP12, CTSL, and CTSS) and antiproteinases (TIMP1, TIMP2, TIMP3, and CST3) in alveolar macrophages under the different experimental conditions (basal i.e. uncultured macrophages, cultured without stimulation, cultured with stimulation by IL-1β, TNF-α or LPS) are shown in Figures E1-E9. The alveolar macrophages cultured without stimulation showed a significant increase in expression of all of the genes (except TIMP3 and cystatin C) relative to the basal gene expression level. With LPS stimulation, the expression levels of all the proteases (except cathepsin S) as well as TIMP1 were significantly increased. Conversely, the expression levels of TIMP2 and cystatin C were significantly decreased after LPS stimulation. Following stimulation with either IL-1β or TNF-α the expression levels of MMP1, MMP9, and TIMP1 were significantly increased. Stimulation with either IL-1β or TNF-α resulted in significantly decreased TIMP2 expression.

There were no significant effects of age, gender, or race on the basal expression of the genes. The effect of smoking status on basal gene expression is shown in Table E5. MMP12 basal expression was higher and TIMP2 basal expression was lower in current smokers compared with former smokers. TIMP3 basal expression was positively correlated with pack-years while CTSS expression was inversely correlated with pack-years.

Proteinase and antiproteinase genotypes versus basal and stimulated gene expression

Since genetic variation could affect COPD phenotypes through modulation of the proteinase and antiproteinase mRNA and protein expression profiles, we investigated whether polymorphisms
altered expression levels of their respective gene products. All of the polymorphisms we investigated were in Hardy-Weinberg equilibrium (p ≥ 0.18) in the Caucasian subgroup (n=87) as shown in Table E2.

A summary of the results of association between genotypes and basal gene expression and expression change is shown in Table E6. MMP1 mRNA basal expression and protein expression induced by LPS were both inversely associated with increasing number of the 2G allele of MMP1 rs1799750 (Figures E10 and E11, respectively). CTSL protein expression without stimulation was inversely associated with increasing number of the T allele of CTSL rs2274611 (Figure E12). The most significant association of genotype with gene expression was observed for the basal expression of CST3 mRNA, which was inversely associated with increasing number of the A allele of CST3 rs6036478 (Figure E13). All of these associations remained significant after correction for multiple comparisons.

**Proteinase and antiproteinase mRNA expression and COPD phenotypes**

We analyzed the relationship of COPD phenotypes and basal mRNA expression levels using linear regression analyses (Tables 2 and E7). The basal expression level of MMP12 in alveolar macrophages showed a significant inverse association with DLco/VA% predicted that remained significant after correction for multiple comparisons. A scatter plot of the association between MMP12 basal mRNA expression and DLco/VA% predicted is shown in Figure 1. There was also an inverse association between basal MMP12 and FEV1 / FVC % (Figure 2). The data also suggest that there was a positive association (p=0.0052, uncorrected for multiple comparisons) between basal expression of MMP1 and the semi-quantitative visual assessment of extent of emphysema of all CT scans (total n=75); this association was no longer significant after
correction for multiple comparisons, since the level of significance required was $p<0.0012$. There was no significant association (uncorrected $p=0.120$) with the computerized quantitative assessment of emphysema by CT scanning ($n=45$), which may be due to the lower number of subjects with these data available.

We also tested for associations between changes in mRNA expression induced by LPS, TNF-α and IL-1β and COPD phenotypes (Tables 3 and E8). The most significant result was that expression change of $MMP9$ mRNA stimulated by TNF-α was inversely associated with FEV$_1$% predicted ($p=0.0005$) but this did not survive correction for multiple comparisons, since the level of significance required was $p<0.00042$.

**The relationship between mRNA and protein expression**

Since we postulated that gene expression would be associated with COPD phenotypes through the level of protein expression, we investigated the association between mRNA and protein expression for the genes that showed at least nominally significant associations with the clinical outcomes. We compared the mRNA levels at 24 hours to the protein concentrations of MMP1, MMP9, MMP12, CTSL, TIMP2, and CST3 in media after 48 hours of culture. The levels of protein are shown in Table E9. We observed significant correlations between the mRNA and protein levels for MMP1 stimulated with LPS, MMP9 with and without LPS, and MMP12 with and without LPS (Table E10). The correlations between $MMP12$ mRNA and protein expression of MMP12 in alveolar macrophages are shown in Figure 3. The basal mRNA expression of $MMP12$ was also positively correlated with its protein expression level stimulated with LPS ($p=1\times10^{-5}$).
We also investigated the correlation between the fold change in mRNA and protein levels after culture with the various stimuli (Table E11). Significant relationships were observed for changes in MMP1 level after stimulation with LPS and IL-β. The changes in expression of MMP9 mRNA and protein after culture with all the stimuli were also significantly correlated.

Protein expression of proteinases and antiproteinases and COPD phenotypes

The protein expression of the proteinases and antiproteinases was investigated in relation to COPD phenotypes (Tables 4 and E12). The protein expression of MMP12 stimulated with LPS was inversely associated with DLco/VA% predicted and positively associated with quantitative score for emphysema. The protein expression of CTSL with or without LPS was also positively associated with FEV₁% predicted. However, none of these associations with protein expression were significant after correction for multiple comparisons.
DISCUSSION

In this study, we found that the genotypes of MMP1, CTSL and CST3 showed significant associations with their expression at the mRNA and/or protein level. The MMP1 1G/2G polymorphism (rs1799750) has previously been associated with gene expression [20] but this is the first description of an effect on transcription in alveolar macrophages. There are no previous studies of the rs2274611 CTSL polymorphism but a CTSL promoter SNP (rs3118869) has been associated with reporter gene expression in vitro [21]. The C allele of rs3118869 showed greater expression than the A allele (p=3.36×10^{-6}) in luciferase assays and was associated (p=0.0143) with higher level of CTSL protein in our study (Table E6). The C allele of rs3118869 is in high linkage disequilibrium (r^2 = 0.8648) with the C allele of rs2274611 which was associated (p=0.0007) with higher CTSL protein in our study (Table E6). Thus, this is the first study to confirm the functional effect of rs3118869 on transcription and we also demonstrated a novel association with CTSL protein level. There are no previous studies of the CST3 rs6036478 polymorphism in the literature but this SNP is part of a haplotype of SNPs in high linkage disequilibrium (all r^2 > 0.92) that has been shown to influence serum / plasma cystatin C levels [22-25]. Our observation that the A (minor) allele of rs6036478 was associated with lower basal cystatin C mRNA (Table E6) is consistent with these previous data and is novel in the context of COPD.

The basal level of MMP12 mRNA expression was inversely related to DLco/VA% predicted and FEV1/FVC % (Table 2). MMP12 protein level after stimulation with LPS was also inversely related to DLco/VA% predicted and was positively associated with the quantitative score for emphysema (Tables 4 and E12). Although these latter associations were not significant
after correction for multiple comparisons, the concordance of direction of association suggests a true pathophysiological role for this proteinase.

Our novel findings of associations between *MMP12* expression and lung diffusing capacity as well as the extent of emphysema, suggest that MMP12 has a role in the development of emphysema in humans. It has been reported that genetic variation in *MMP12* is associated with COPD and asthma [26]. However, there are no reports on the direct association between emphysema and the expression of *MMP12* from alveolar macrophages in humans. *MMP12* knockout mice do not develop emphysema following exposure to cigarette smoke [27], suggesting that the presence of MMP12 is critical in smoke-induced emphysema in mice. Our study further suggests a role for MMP12 in the pathogenesis of emphysema in humans. The underlying mechanism for this role may relate to MMP12 stimulation of neutrophils through activation of TNF-α, resulting in the production of MMP9, neutrophil elastase, and cathepsin G, all of which degrade elastin and may lead to emphysema [28]. In addition, MMP9 and MMP12 degrade specific collagens [29, 30]. MMP12 is increased in the lungs [31] and sputum [32] of COPD patients.

We also showed that the basal level of *MMP1* mRNA was positively correlated with a semi-quantitative assessment of the extent of emphysema (p=0.0052) as in our previous report, based on the first 44 cases with CT scans [11]. Thus, we have extended our previous observations by demonstrating that not only MMP12 mRNA but also protein level is associated with DL_{CO}/VA and emphysema.

Cigarette smoke and other irritants stimulate alveolar macrophages to produce MMPs [33, 34] and cathepsins [35]. MMP1 degrades collagen whereas MMP9, MMP12, cathepsin L and S degrade elastin [36]. Although human MMP1 is a collagenase, its expression in the lungs
of transgenic mice caused emphysema [37] and expression of MMP1 was increased in the lungs of patients with emphysema [38]. Smokers who have airway obstruction show increased expression of MMP1 and MMP9 compared with smokers without COPD and non-smokers [39].

We observed a novel association of TNF-α induced MMP9 mRNA expression with FEV₁% predicted (Table 3). This observation is consistent with previous data that demonstrated MMP9 expression increased in the lung parenchyma surrounding small airways as FEV₁% predicted decreased [40]. There is also supportive evidence that MMP9 contributes to the development of emphysema from mouse models [41, 42]. Our data suggest that it is the level of MMP9 in response to specific pro-inflammatory stimuli that plays a critical role in the development of COPD, in agreement with studies showing that increases in TNF-α are associated with reductions in lung function [40] and that TNF-α plays an important role in cigarette smoke-induced emphysema [43].

CTSL protein level was associated with FEV₁% predicted, which suggests that this proteinase has a role in development of COPD. However, individuals whose macrophages secreted more CTSL had better lung function. We did not observe this relationship in our previous study [11] but the same trends were present (i.e. higher CTSL with or without LPS associated with higher lung function) with similar estimates from the regression analysis. However, the p values were not significant due to the smaller sample size. It is unclear what mechanism underlies this “paradoxical” relationship. However, CTSL expression in thymocytes is essential for natural killer T cell development [44], and natural killer cells, which have an essential role in immunity against viral infection [45], are decreased in the peripheral blood in COPD [46]. Similarly, CTSL is involved in antigen processing [47, 48] and T helper cell development [49, 50]. Thus, CTSL protein expression could reinforce immunological capacity,
reduce exacerbations, and prevent progression of COPD. CSTL also plays a prominent role in the production of neuropeptides [51] such as neuropeptide Y which is decreased in the airway epithelium in COPD patients compared with unaffected smokers [52] suggesting a protective role for this molecule. In addition, CSTL is involved in the production of adrenocorticotropic hormone and upregulation of this hormone is attenuated in COPD patients following exercise [53].

In this study, there were significant differences in gene expression in macrophages cultured without stimulation compared with primary macrophages, as we have previously shown [11]. This phenomenon has also been observed in murine macrophages [54] and other cell types following culture [55, 56]. While it was not surprising that non-physiological conditions (i.e. cell culture) altered gene expression in our study, whether this was due to adherence of the cells to the plastic substrate in the cell culture dishes or to components of the culture medium is not known.

A recent study investigated changes in gene expression following instillation of LPS into the lung in healthy individuals (n=8) compared with instillation of saline into the contralateral lung [57]. In that in vivo study, TIMP1 was upregulated 2.4 fold (p=3×10^{-4}) following challenge with LPS, mirroring the increase in TIMP1 mRNA that we observed after culture with LPS (Figure E6). Similarly, TIMP2 mRNA was downregulated following instillation of LPS (p=3×10^{-3}), and we also observed a significant in TIMP2 mRNA decrease following culture with LPS (Figure E7).

In comparison with our previous study [11], we have doubled the sample size to increase the power of the analyses and have assessed additional genes (i.e. the antiproteinases TIMP1,
TIMP2, TIMP3 and CST3). We also measured the levels of the proteins corresponding to six of the genes whereas our previous manuscript only included mRNA data [11].

There are some limitations of our study with respect to gene expression and COPD or emphysema phenotypes. With a total sample of 109 subjects we have 80% power to detect associations between gene expression and COPD phenotypes only where the $r^2$ value is $\geq 0.3$. Since we have a smaller number of subjects for DLco / VA% predicted and the CT scan data, the power associated with these phenotypes is less. In addition, there was a small proportion of severe COPD patients in our study (59% of our subjects had FEV$_1$ values within the normal range). In addition, the use of alveolar macrophages from patients with lung cancer adds a potential bias to our study. Specifically, several MMP polymorphisms have been associated with lung cancer [58] and therefore the risk alleles may have been selected for in our study group. However, all SNPs in our study showed good agreement with expectations under Hardy-Weinberg equilibrium (Table E2) suggesting that there was no strong selection bias. In addition, the allele frequencies are generally similar to the HapMap data (Table E2). Expression levels of proteinase and antiproteinase genes may also have been affected by the presence of tumors in the lungs. However, as noted above our results are similar to those of Reynier et al. [57] in subjects without cancer suggesting that the observations in this study are applicable to non-cancer populations.

In conclusion, among proteinases and antiproteinases, our data further support previous studies demonstrating that MMP12 and MMP1 may have an important role in the pathogenesis of emphysema. In addition, CTSL may positively influence the development of airflow limitation through protein expression and is affected by genetic variation.
ACKNOWLEDGEMENTS

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REFERENCES


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TABLES

Table 1. Characteristics of the study subjects (n=109).

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>67 ± 10</td>
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<tr>
<td><strong>Sex, n (%)</strong></td>
<td>49 men (45%)</td>
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<tr>
<td><strong>Race, n (%)</strong></td>
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<tr>
<td>Current smokers, n (%)</td>
<td>17 (16%)</td>
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<tr>
<td>Former smokers, n (%)</td>
<td>92 (84%)</td>
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<td>Pack years</td>
<td>46 ± 25</td>
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<td><strong>Spirometry</strong></td>
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<td>FEV₁, % predicted pre-bronchodilator (n = 109)</td>
<td>83 ± 20</td>
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<tr>
<td>FEV₁, % predicted post-bronchodilator (n = 97)</td>
<td>87 ± 19</td>
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<tr>
<td>FVC, % predicted pre-bronchodilator (n = 109)</td>
<td>92 ± 17</td>
</tr>
<tr>
<td>FVC, % predicted post-bronchodilator (n = 97)</td>
<td>95 ± 16</td>
</tr>
<tr>
<td>FEV₁/FVC % pre-bronchodilator (n = 109)</td>
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</tr>
<tr>
<td>FEV₁/FVC % post-bronchodilator (n = 100)</td>
<td>70 ± 13</td>
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<tr>
<td>DLco / VA, % predicted (n = 81)</td>
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<tr>
<td>RV/TLC % (n = 73)</td>
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<td>MZ</td>
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<td>ZZ</td>
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</table>

Continuous variables are shown as mean ± SD

* Two subjects were not genotyped because genomic DNA could not be obtained
**Table 2.** Significant results (p<0.05) from linear regression analysis of pulmonary function tests and computed tomography-scan data with mRNA expression at baseline.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Outcome</th>
<th>r²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>Semiquantitative score for emphysema</td>
<td>0.0597</td>
<td>0.0052</td>
</tr>
<tr>
<td>MMP12</td>
<td>FEV₁% predicted</td>
<td>0.2565</td>
<td>0.0265</td>
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<td></td>
<td>FEV₁ / FVC %</td>
<td>0.2393</td>
<td>8 × 10⁻⁵</td>
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<td>DLCO/VA %predicted</td>
<td>0.2112</td>
<td>0.0004</td>
</tr>
<tr>
<td>TIMP2</td>
<td>FEV₁% predicted</td>
<td>0.2547</td>
<td>0.0299</td>
</tr>
<tr>
<td>CST3</td>
<td>FEV₁% predicted</td>
<td>0.2736</td>
<td>0.0087</td>
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</table>

Coefficients of determination ($r^2$) and p values were calculated from regression analysis of each clinical outcome including age, gender, race, pack-years, and mRNA expression as covariates. $r^2$ values are for the whole model, p-values are for the mRNA expression level.

FEV₁ % predicted and FEV₁ / FVC % were measured post-bronchodilator. An ordinal logistic regression was performed for the semiquantitative emphysema scores. DLCO/VA%pred was log-transformed.

Significant results after correction for multiple comparisons are shown in bold. The p-value threshold is $0.05/ (9 \times 4.45) = 0.0012$.

Complete results are shown in Table E7.
Table 3. Significant results (p<0.05) from linear regression analysis of pulmonary function tests and computed tomography-scan data with changes in mRNA in response to stimuli. The mRNA was obtained after 24 hours of culture with the various stimuli, and the change of mRNA from cells cultured with no stimuli was analyzed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stimulus</th>
<th>Outcome</th>
<th>r²</th>
<th>p-value</th>
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<td>MMP9</td>
<td>TNF-α</td>
<td>FEV₁ %predicted</td>
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<td>CTS1</td>
<td>LPS</td>
<td>Semiquantitative score for emphysema</td>
<td>0.0653</td>
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<td>TIMP1</td>
<td>LPS</td>
<td>FEV₁ / FVC %</td>
<td>0.1445</td>
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<tr>
<td>TIMP3</td>
<td>LPS</td>
<td>Semiquantitative score for emphysema</td>
<td>0.0582</td>
<td>0.0407</td>
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</table>

Coefficients of determination (r²) and p values were calculated from regression analysis of each clinical outcome including age, gender, race, pack-years, and mRNA expression as covariates. r² values are for the whole model, p-values are for the mRNA expression level.

FEV₁ % predicted and FEV₁ / FVC % were measured post-bronchodilator. An ordinal logistic regression was performed for the semiquantitative emphysema scores. The p-value threshold to correct for multiple corrections is 0.05/(9 [Number of SNPs] x 3 [Number of conditions] x 4.45 [Number of phenotypes]) = 0.00042.

Complete results are shown in Table E8.
Table 4. Significant results (p<0.05) from linear regression of COPD phenotypes with protein expression of proteinases and antiproteinases.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Condition</th>
<th>Outcome</th>
<th>r²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP12</td>
<td>with LPS</td>
<td>DLCO/VA %predicted</td>
<td>0.2173</td>
<td>0.0449</td>
</tr>
<tr>
<td>MMP12</td>
<td>with LPS</td>
<td>Quantitative score for emphysema</td>
<td>0.4457</td>
<td>0.0326</td>
</tr>
<tr>
<td>CTSL</td>
<td>without LPS</td>
<td>FEV₁ % predicted</td>
<td>0.2163</td>
<td>0.0447</td>
</tr>
<tr>
<td>CTSL</td>
<td>with LPS</td>
<td>FEV₁ % predicted</td>
<td>0.2251</td>
<td>0.0262</td>
</tr>
</tbody>
</table>

The protein was obtained after 48-hour culture with various stimuli in the media, and the associations between protein concentration and COPD phenotypes were analyzed by regression. Adjusted by age, gender, race, and pack-years.

FEV₁ % predicted and FEV₁ / FVC % were measured post-bronchodilator. For the semiquantitative emphysema scores an ordinal logistic regression was performed. DLCO/VA % predicted and quantitative score for emphysema deviated from the normal distribution and were log-transformed.

The p-value threshold after Nyholt's correction is 0.05/(6 [Number of SNPs] x 2 [Number of conditions] x 4.45 [Number of phenotypes]) = 0.00094. Nominally significant values are underlined: MMP12 level after stimulation with LPS was inversely related to DLco/VA % predicted and was positively associated with quantitative score for emphysema. CTSL levels without and with LPS stimulation were both positively related to FEV₁ % predicted.

Complete results are shown in Table E8.
FIGURE LEGENDS

Figure 1. The relationship between basal mRNA expression of MMP12 in alveolar macrophages and diffusion capacity (DLco/VA % predicted).
Figure 2. The relationship between basal mRNA expression of MMP12 in alveolar macrophages and FEV1 / FVC %.
Figure 3. The correlation between *MMP12* mRNA and protein expression of MMP12 in alveolar macrophages. The mRNA expression level of *MMP12* at 24 hours was positively correlated with MMP12 protein in the media in which alveolar macrophages were cultured at 48 hours. A) No stimulation. B) Stimulation with LPS.