

# Matrix metalloproteinase-12 (MMP-12) SNP affects MMP activity, lung macrophage infiltration and protects against emphysema in COPD

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## ABSTRACT

**Background** Recent genetic and animal studies have implicated matrix metalloproteinase-12 (MMP-12) in the pathogenesis of chronic obstructive pulmonary disease (COPD). It has previously been shown that individuals homozygous for the A/A allele of rs652438 in MMP-12 are over-represented among patients with severe COPD (n=1517). A study was undertaken to examine the functional basis of these findings.

**Methods** rs652438 A and G variants were generated by site-directed mutagenesis and transfected into COS7 cells where they were expressed. Casein zymography and a specific FRET activity assay were used to compare MMP-12 activity between alleles. Cell migration was examined using a transwell assay. Patients from two COPD cohorts were genotyped for rs652438 and associated with inflammatory cell number in bronchoalveolar lavage fluid (n=10) and induced sputum (n=262); the emphysema score (n=1428) was assessed by CT scanning.

**Results** Mean MMP activity was 2.95-fold higher by zymography (p=0.0049) and 3.45-fold higher by FRET assay (p=0.0001) for the A allele than the G allele. Mean migration of COS7 cells expressing the A allele was 2.31-fold greater than for those expressing the G allele (p=0.0001). Macrophage numbers were greater in bronchoalveolar lavage fluid (1.28-fold increase, p=0.033) and induced sputum (1.58-fold increase, p=0.083) of A/A individuals compared with A/G heterozygotes. The presence of the A allele was dose-dependently associated with increased emphysema (p=0.016).

**Conclusions** The rs652438 SNP alters MMP-12 activity with the A allele being more active, which is associated with increased macrophage infiltration and emphysema in the lungs of patients with COPD. These findings further implicate MMP-12 and this SNP in COPD.

## INTRODUCTION

It is widely accepted that alveolar destruction in chronic obstructive pulmonary disease (COPD) is the result of an imbalance of proteolytic enzymes and their inhibitors based on the observation that severe  $\alpha_1$ -antitrypsin deficiency predisposes cigarette smokers to the development of pulmonary emphysema. This is thought to be due to uninhibited neutrophil elastase activity degrading elastin, a major component of lung connective tissue.<sup>1</sup>

A number of other proteolytic enzymes are also capable of degrading elastin, including the matrix metalloproteinases (MMPs). These proteases can degrade all the major protein components of the extracellular matrix (ECM). Evidence from genetic

## Key messages

### What is the key question?

- How does a single nucleotide polymorphism in the haemopexin-like domain of MMP-12 protect patients against severe COPD?

### What is the bottom line?

- The protective variant of MMP-12 reduces metalloproteinase activity and cell migration in vitro and is associated with lower lung macrophage numbers and emphysema in patients with COPD.

### Why read on?

- By investigating how this genetic variant in MMP-12 protects against severe COPD, this study addresses one of the factors contributing to the idea of the 'susceptible smoker' in COPD.

studies, animal models and human disease suggest that MMP-12, the major metalloelastase derived from alveolar macrophages, plays a role in lung destruction in COPD.<sup>2–6</sup> Recently, a large multiple cohort association study linked variation in MMP-12 with both lung function in smokers and the risk of developing COPD<sup>2</sup> while, in animal models of COPD, deletion of MMP-12 in mice exposed to cigarette smoke protects these animals from emphysema.<sup>3</sup> In patients with COPD elevated levels of MMP-12 are present in the airways<sup>4 5</sup> and increased numbers of MMP-12 expressing macrophages.<sup>6</sup>

We recently performed a case–control genetic association study into MMPs 1, 9 and 12. This identified haplotypes in MMP-12 of two single nucleotide polymorphisms (SNPs), rs652438 and rs2276109, which were associated with severe and very severe COPD (GOLD stages III and IV). In the study by Hunninghake *et al*<sup>2</sup> rs652438 was not associated, possibly due to different phenotypes tested. This suggests that these SNPs are modifiers of disease severity, and supports the idea that MMP-12 is a mediator of lung damage in COPD.<sup>7</sup> In our previous study about 18% of Europeans were found to have at least one protective allele at these SNP positions, giving a significantly reduced risk of severe disease development.<sup>7</sup>

The SNPs at rs2276109 and rs652438 are located in the promoter region of the gene and haemopexin domain of the enzyme, respectively, and could directly influence MMP-12 activity through altered

expression or substrate binding. The former has been demonstrated for rs2276109, where the A allele in the MMP-12 promoter results in increased MMP-12 transcription.<sup>8</sup> The rs652438 A/G SNP causes a missense change of asparagine to serine at amino acid position 357, located in exon 8, which encodes the haemopexin domain. The haemopexin domain of MMP-12 is involved in substrate specificity and may interact with the enzyme's catalytic domain.<sup>9</sup> We hypothesised that this SNP could alter the function of MMP-12, thereby altering the pathological processes in COPD.

## MATERIAL AND METHODS

Additional data on methods are provided in the online supplement.

### Patient cohorts

Two cohorts were used in this study, a local Nottingham cohort of patients with COPD<sup>10</sup> and ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points).<sup>11</sup>

#### Nottingham cohort

We recruited smokers with COPD defined by GOLD criteria. Patients were aged 40–80 years, had a smoking history of at least 10 pack-years and had not required antibiotics or oral steroids for the previous 6 weeks. Patients with  $\alpha_1$ -antitrypsin deficiency, radiological evidence of interstitial lung disease, previous thoracic surgery or taking inhaled corticosteroids were excluded. Pre- and post-bronchodilator spirometry was performed according to American Thoracic Society guidelines and breathlessness was recorded using the Medical Research Council (MRC) dyspnoea score. Those with reversibility >10% of baseline forced expiratory volume in 1 s (FEV<sub>1</sub>) after inhaling 400  $\mu$ g salbutamol were excluded. Subjects with FEV<sub>1</sub>/forced vital capacity (FVC) of <70% were classified as having COPD.

#### ECLIPSE cohort

Patients with COPD aged 40–75 years with baseline post-bronchodilator FEV<sub>1</sub> <80% of the predicted value, baseline post-bronchodilator FEV<sub>1</sub>/FVC of <0.7 and a smoking history of >10 pack-years were enrolled. Exclusion criteria were related to diagnosis (known respiratory disorders other than COPD and severe  $\alpha_1$ -antitrypsin deficiency), prior medical history (known history of significant inflammatory disease other than COPD), a COPD exacerbation within 4 weeks of enrolment, having undergone lung surgery, recent diagnosis of cancer, having received a blood transfusion in the 4 weeks before the start of the study, inability to walk, taking part in a blinded drug study, treatment with oral corticosteroids at inclusion and participation in studies with radiation exposure.

Information on the cell percentage composition of bronchoalveolar lavage fluid (BAL) was provided for the Nottingham cohort. Absolute cell number and emphysema scores were provided for ECLIPSE, providing some replication for the cell number measure described by the Nottingham cohort. Emphysema scores were obtained using CT scanning performed at full suspended inspiration. A multislice CT scanner was used, taking contiguous images at 1 or 1.25 mm thickness. Scanning was set at 120 kVp and 40 mAs. A low spatial frequency reconstruction algorithm was used for analysis (GE-standard, Siemens Healthcare, Erlangen, Germany). Genotypes of rs652438 were determined in the Nottingham cohort by direct sequencing. ECLIPSE genotypes were provided using methods described elsewhere.<sup>11</sup>

#### Vector creation

MMP-12 (pCMV-XL4 vector) was obtained from Origene (Rockville, USA). The rs652438 SNP G allele was introduced via

the GeneEditor kit (Promega, Southampton, UK) according to the manufacturer's instructions using mutagenic primer 5'-TAATTAGCAGTTTAAAGACC-3'. After DNA preparation (Qiagen, Crawley, UK), inserts were sequenced and aligned using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) to confirm inserts were identical apart from the SNP. The vector without an insert was used as null control in all experiments.

### Cell culture

COS7 cells were grown in DMEM using 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub>. Vectors were transiently transfected using Eugene 6 (Roche, Mannheim, Germany). After 24 h incubation, cells were serum starved, supernatants collected after 24 h and stored at –80°C.

### Analysis of enzymatic activity

Casein zymography was used to perform analysis of activity as detailed elsewhere.<sup>12</sup> Changes to the protocol were 12% casein gels (Invitrogen, Paisley, UK) and quantification against 10 ng recombinant human MMP-12 (R&D Systems, Minneapolis, USA). Images were scanned and analysed using Image J V.1.40 (Wayne Rasband, NIH, USA). Bands were normalised against standard and presented as a ratio.

MMP-12 activity was also measured using the Sensolyte 520 MMP-12 assay kit (Anaspec, San Jose, California, USA) according to the manufacturer's instructions. The assay was run on a Flexstation with Softmax Pro software (Molecular Devices, Sunnyvale, California, USA) at excitation/emission wavelengths of 490/520 nm respectively. Recombinant human MMP-12 (10 ng) was the reference standard. Substrate-only samples were included for background subtraction purposes. All measurements were performed in triplicate and the experiments were repeated three times.

### Western blot

Supernatants and human MMP-12 standard (10 ng) were resolved using 10% SDS page gels followed by blotting onto Hybond-P membrane (GE Healthcare, Buckinghamshire, UK) and probed with IgG anti-human MMP-12 catalytic antibody (R&D Systems, Minneapolis, USA). HRP-conjugated goat anti-mouse IgG (Sigma Aldrich, Gillingham, UK) was used. Visualisation was performed using the ECL western blotting detection kit (GE Healthcare).

### Cell viability assays

Total cell counts and cell viability measurements were performed using a haemocytometer and MTT assay as described elsewhere.<sup>12–15</sup> Quantification of total protein levels in supernatants was performed using a Bradford based method<sup>14</sup> (Bio-Rad Protein Assay, Berkeley, California, USA).

### Real-time PCR

Quantification of MMP-12 mRNA was performed as described elsewhere.<sup>12</sup>  $\beta$ -actin was used for normalisation. Primer sequences are as follows: MMP-12 forward: 5'-CATTTCAGGAGCCACCAACTTGTTC-3', reverse: 5'-CCTTTGGATCACTAGAATGGCC-3';  $\beta$ -actin forward: 5'-GGATGCAGAAGGAGATTACTG-3', reverse: 5'-CGATCCACACAGACTTGTG-3'.

### Minigene analysis of splicing

Exonic splice silencing was analysed using the Minigene technique. Details of the method used are provided in the online supplement.

**Table 1** Basic characteristics of COPD cohorts

	Nottingham	ECLIPSE: cell numbers	ECLIPSE: emphysema score
Male (%)	80.0	63.2	67.0
Age (years)	59.4±3.1	64.0±0.4	63.6±0.2
Smoking (pack-years)	60.6±7.1	50.9±1.6	50.4±0.7
FEV <sub>1</sub> (% predicted)	59.0±7.3	49.2±0.9	47.7±0.4
FEV <sub>1</sub> /FVC (%)	55.45±4.0	43.81±0.7	44.7±0.3
N	10	232	1427

COPD, chronic obstructive pulmonary disease; ECLIPSE, Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity.

**Migration assay**

The transwell migration assay was performed as previously described.<sup>12</sup> Changes to the protocol were use of collagen IV (0.1 mg/ml in serum-free medium) coating and 10% serum as a chemotactic stimulus. Migration was measured at 8 h in three replicates in three independent experiments.

**Statistical analysis**

Data sets were tested for normality using the D’Agostino and Pearson test and analysed using t tests (Prism 4, Graphpad Software, La Jolla, USA). Linear regression of Sensolyte 520 kinetic assay was also performed in Prism 4. Cohort characteristics were investigated using the descriptive statistics function in SPSS V.14 (SPSS Inc). Analysis of the ECLIPSE cohort was performed using a linear regression model adjusted for confounding factors in SPSS V.14. For all tests, p values of <0.05 were considered statistically significant.

**RESULTS**

**Study population characteristics**

The characteristics of the populations used in this study are detailed in table 1.

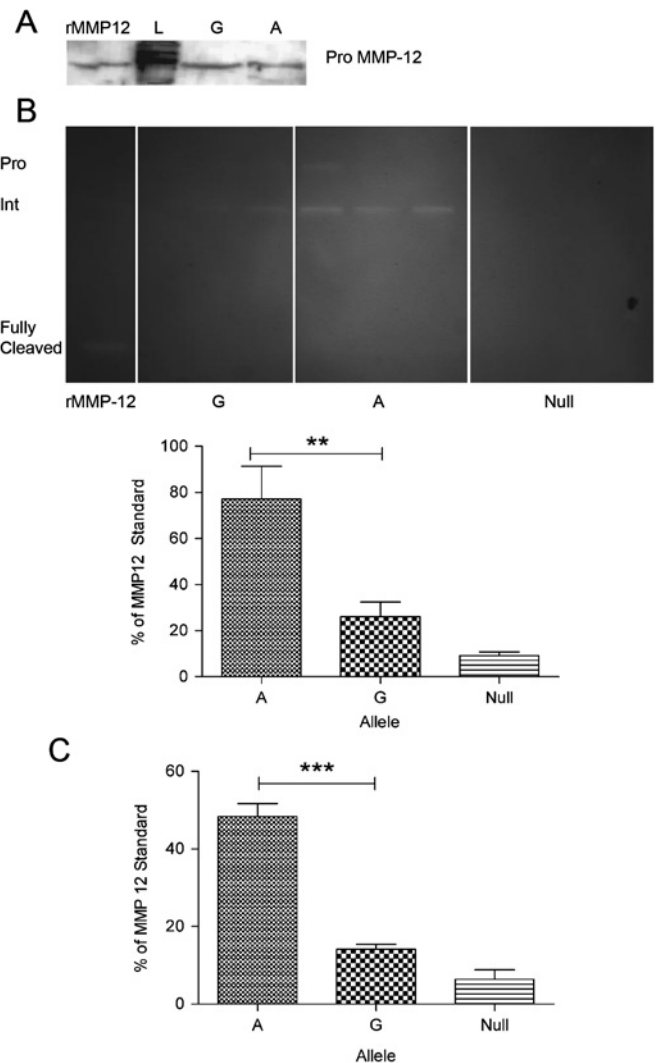
**Analysis of enzymatic activity**

MMP-12 activity was examined using casein zymography of COS7 supernatants from cells expressing the A and G constructs. COS7 cells expressing the null vector had no detectable caseinolytic activity (limit of detection 1 ng per lane). In supernatants from COS7 cells expressing the MMP-12 constructs, caseinolytic activity was present at 54 kDa and 45 kDa, corresponding to the pro- and intermediate-active forms of MMP-12 (figure 1). Using the t test and comparison of the means, densitometry of caseinolytic bands showed that the activity of the A allele was 2.95-fold (p=0.0049) higher than that of the G allele.

We next used the Sensolyte 520 MMP-12 ELISA. Using the t test and comparison of the means, the A allele of rs652438 was 3.42-fold (p=0.0001) more active than the G allele (figure 1). The Sensolyte assay was also used to provide a kinetic read of MMP-12 activity (figure 2). Using the linear regression function in Prism, the slope of substrate cleavage against time for the A allele was 1.6 CI±0.106 relative fluorescence units/min (RFU) (p=0.0001 vs empty vector) and the G allele slope was 0.12 CI±0.096 RFU (p=0.22 vs empty vector). The MMP-12 activity of the G allele was not different (p=0.2153) from the null vector baseline.

**Effect of SNP on cell viability and MMP-12 expression**

Examination of MMP-12 constructs was performed to determine whether the SNP affects MMP-12 mRNA expression, cell number or viability. Real-time PCR and Minigene determined

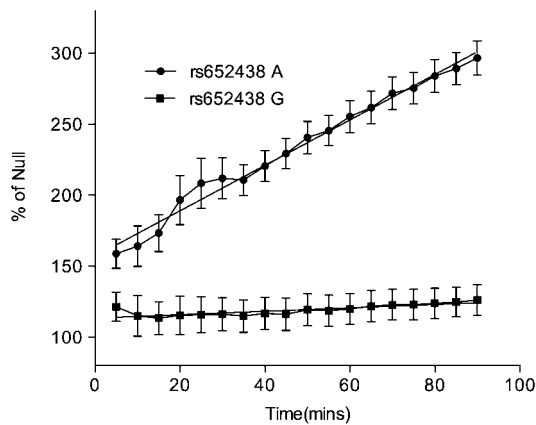


**Figure 1** (A) Western blot of rs652438 A and G paired transfections. Expression levels of matrix metalloproteinase-12 (MMP-12) were found to be equal between the transfection of the different constructs. (B) Representative casein zymogram showing expression of the pro and intermediate forms of MMP-12 from vector-transfected cell supernatants. Below, densitometry of three independent experiments. The A allele has mean±SEM band intensity of 77.11±14.26% compared with the 10 ng MMP-12 standard while the is G allele has a band intensity of 26.11±6.39%. The A allele activity is 2.95-fold higher than the G allele (p=0.0049). (C) Sensolyte 520 MMP-12 assay endpoint read. The A allele has mean±SEM activity of 48.30±3.44% compared with the 10 ng MMP-12 standard while the is G allele has 14.13±1.30% activity. The A allele activity is 3.42-fold higher than the G allele (p=0.0001; n=3).

that the rs652438 polymorphism did not influence mRNA levels (figure 3). Using the t test, no significant differences were seen in cell number, supernatant total protein or viability (figure 4).

**Cell migration**

Transwells were coated with collagen IV, a non-fibrillar collagen expressed in basement membranes which can be degraded by MMP-12.<sup>15</sup> Using t tests and mean values as a comparator, both MMP-12 allele transfected cells had increased migration compared to cells containing the null vector. Further to this, A allele containing cells had significantly increased migration (2.31-fold increase, p=0.0001) when compared to those transfected with the G allele (figure 5).



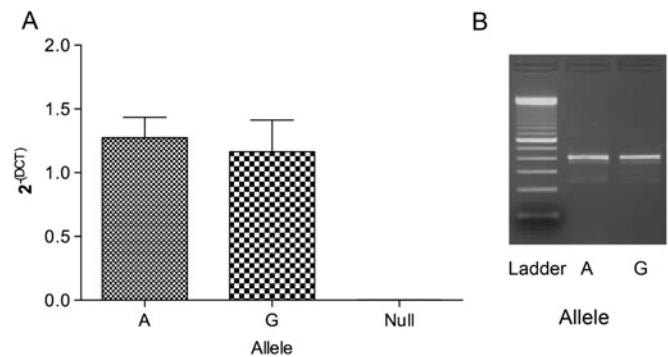
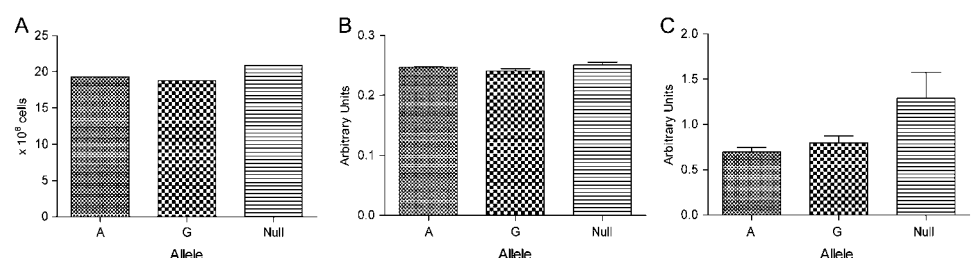
**Figure 2** Sensolyte 520 MMP-12 assay kinetic read. The A allele of rs652438 is significantly different from the null allele baseline slope ( $1.6000 \pm 0.1062$ ,  $p=0.0001$ ) while the G allele is not significantly different from the baseline slope ( $0.1201 \pm 0.0963$ ,  $p=0.2153$ ;  $n=3$ ).

We investigated whether this effect on migration was relevant in patients with COPD. We examined data from the Nottingham cohort using BAL fluid samples in which the percentage composition values of inflammatory cells were available. Patients were genotyped for rs652438 using direct sequencing. Using a t test and comparing the means, we found a 1.28-fold ( $n=10$ ,  $p=0.033$ ) increase in the percentage of BAL fluid macrophages for A/A homozygotes compared with A/G genotypes (figure 6). There has also been evidence that MMP-12 is involved in neutrophil recruitment to the airways<sup>16</sup> but in our study we did not find any significant differences as determined by t test. A/A genotypes had  $4.820 \pm 2.239\%$  composition of neutrophils while A/G genotypes had  $21.040 \pm 9.058\%$  composition ( $p=0.0602$ ).

In order to provide some replication, information from the ECLIPSE cohort was obtained regarding absolute inflammatory cell numbers according to genotype. As the rs652438 SNP is not present on the Illumina 550 genome-wide genotyping platform used in ECLIPSE, we studied the rs651159 intronic SNP which is in full linkage disequilibrium ( $r^2=1$ ) with rs652438 in HapMap CEU, EGP CEPH and AFD EUR submitter populations to the HapMap.<sup>17</sup> Analysis of rs651159 allows 100% inference of rs652438 genotypes.

The focus of the analysis was the association of inferred rs652438 genotypes with absolute cell numbers of macrophages and neutrophils in induced sputum. In this large cohort, BAL fluid samples were not routinely taken and macrophage numbers were examined in induced sputum. Further to this, a comparable cell percentage phenotype in the induced sputum was not available. In the 232 patients for whom genotype and induced sputum data were available, there were 205 A/A homozygotes, 27 A/G heterozygotes but no G/G homozygotes.

**Figure 4** (A) Total cell count and (B) Bradford assay showing no significant differences in total cell number or total secreted protein levels in A and G alleles of matrix metalloproteinase-12 (MMP-12) rs652438 vector-transfected COS7 cells. (C) MTT assay indicating no significant differences in viable cells ( $n=3$  for all experiments).



**Figure 3** (A) Real-time PCR demonstrating robust and equal expression of matrix metalloproteinase-12 (MMP-12) constructs ( $n=3$ ). No endogenous MMP-12 was detected. (B) Real-time PCR of exon trap vector with MMP-12 exon 8 inserted. The 160 bp exon 8 is transcribed along with the vector-specific exons, giving a 417 bp band. Because both bands are 417 bp in size and of equal intensity, exonic silencing does not occur when the G allele is present. If silencing had occurred, a 257 bp band would be formed with only the vector-specific exons transcribed.

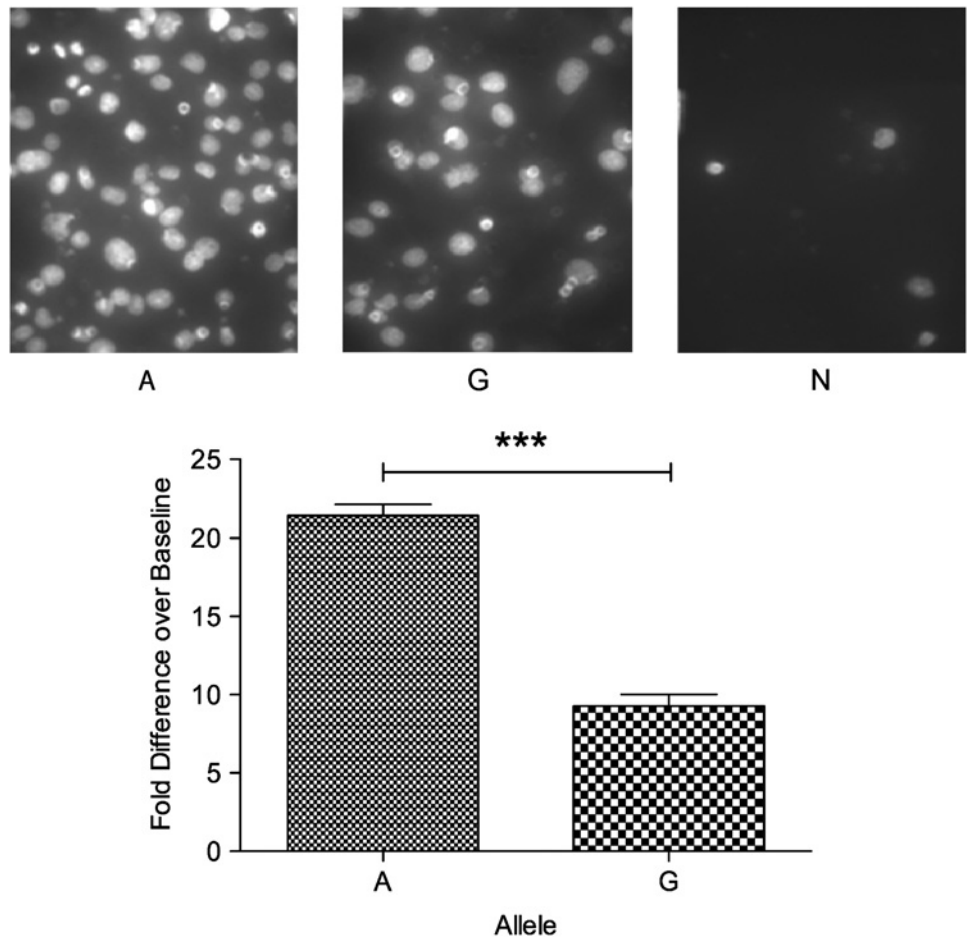
Absolute macrophage number in induced sputum was the dependent variable while genotype is the independent variable of interest. The final linear regression model was adjusted for smoking history, age, sex, pack-years and GOLD stage. Comparing the means showed a 1.58-fold increase in macrophage numbers in induced sputum samples of A/A homozygotes compared with A/G heterozygotes (figure 7), which did not reach statistical significance ( $p=0.083$ , adjusted  $r^2=0.369$ ) using a linear regression test.

The absolute neutrophil number was also investigated using linear regression adjusted for smoking history, age, sex, pack-years and GOLD stage. No significant differences ( $p=0.306$ , adjusted  $r^2=0.493$ ) were found between the A/A group (mean  $\pm$  SE  $2.48 \times 10^6 \pm 0.25 \times 10^6$  cells per count) and the A/G group (mean  $\pm$  SE  $3.25 \times 10^6 \pm 0.71 \times 10^6$  cells per count).

#### Emphysema scores

As in the previous investigation, the rs651159 SNP was used to infer the genotypes of rs652438 in ECLIPSE. There were 1282 A/A homozygous individuals, 141 A/G heterozygotes and four G/G minor homozygotes. The emphysema score determined by CT scanning was used as a quantitative trait in association analysis against genotype. The final linear regression model was adjusted for smoking history, age, sex, pack-years and GOLD stage. There was a dose-dependent effect on emphysema with each G allele ( $p=0.016$ , adjusted  $r^2=0.245$ ), with a reduction in low attenuation areas between the A/A major and G/G minor homozygotes. Comparison of the means of the A/A and G/G homozygotes showed a 1.91-fold increase in the emphysema score (figure 8).

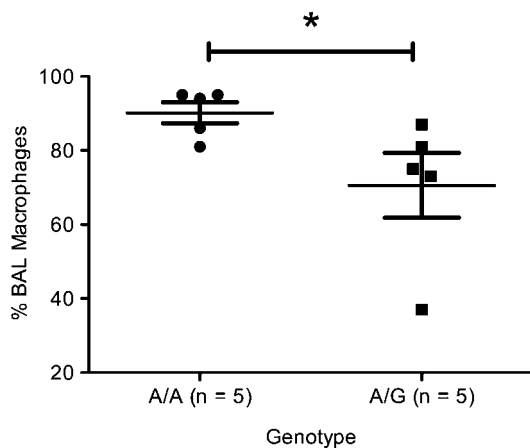
**Figure 5** Chemotactic transwell migration assay (n=3) showing a 2.31-fold (p=0.0001) increase in migration for A allele transfected rs652438 COS7 cells compared with the G allele. Baseline used is the migration of null (N) vector-transfected COS7 cells. Cell migration photographs are representative of three independent experiments.



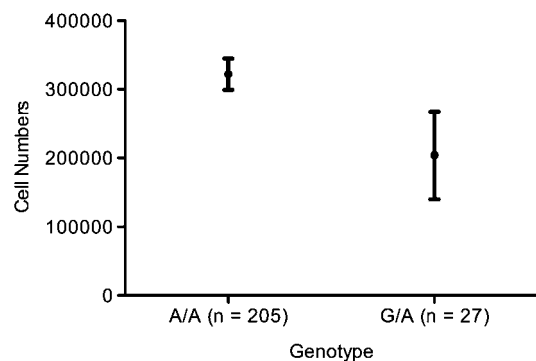
**DISCUSSION**

This study demonstrates that an exonic SNP alters the activity of MMP-12. Our findings provide evidence for the role of the rs652438 SNP in macrophage migration and emphysema severity, with the A allele over-represented in these cases. Consistent with these findings, we found that the A allele significantly increased the activity of MMP-12.

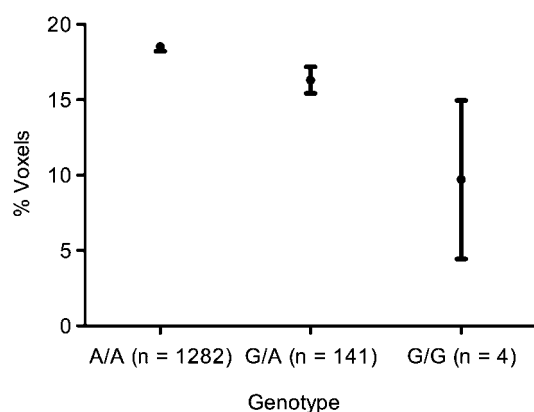
The MMP-12 protein exists in three major forms: an inactive 54 kDa form which has the propeptide domain cleaved to create the 45 kDa active form, and this active 45 kDa form is also processed to form a 22 kDa protein with the haemopexin domain removed. Although processed to the 22 kDa form, we do not think that this will have affected our in vitro findings as, in common with a previous study, we found that, in cell supernatants, the 45 kDa form is stable under storage at -80°C and for the duration of the assays.<sup>18</sup> However, the purified recombinant MMP-12 readily underwent autocatalytic



**Figure 6** Percentage of macrophages in bronchoalveolar lavage (BAL) fluid from the Nottingham cohort. Individuals with A/A genotype had mean±SEM 90.20±2.85% macrophages while A/G individuals had 70.60±8.75% macrophages, a 1.18-fold increase (p=0.033).



**Figure 7** Absolute macrophage number in induced sputum by genotype in the ECLIPSE cohort. A/A homozygotic individuals had a mean±SE of 3.22±0.23×10<sup>5</sup> cells per count compared with 2.04±0.64×10<sup>5</sup> cells per count for A/G heterozygotes, a 1.58-fold increase in cell number (p=0.083, adjusted r<sup>2</sup>=0.369).



**Figure 8** Emphysema score by genotype. The mean  $\pm$  SE values for the different genotypes were: A/A,  $18.52 \pm 0.29$ ; G/A,  $16.31 \pm 0.89$ ; G/G,  $9.70 \pm 5.26$ . The increase between the major and minor homozygotes in emphysema scores is 1.91-fold ( $p=0.016$ , adjusted  $r^2=0.245$ ;  $n=1427$ ).

processing, suggesting the presence of stabilising factors in cell culture supernatants.

The haemopexin domain in MMP-12 has been hypothesised to have a flexible interaction with the catalytic domain compared with other MMPs, suggesting a role for the haemopexin domain in modulating enzymatic activity.<sup>19</sup> The rs652438 SNP causes a neutral-polar mutation of arginine to serine, which suggests that the effect of the SNP seen in our study is through stabilisation of protein flexibility rather than a direct conformational change.

An interesting finding was the investigation of SNP alleles with regard to macrophage migration. The transwell migration assay set up to model macrophage invasion in the airways found significantly increased migration for the A allele. Furthermore, this finding was replicated in humans with statistically significant differences between individuals with A/A and A/G genotypes in the number/proportion of macrophages found in BAL fluid and induced sputum. However, it must be noted that grouped replication rather than direct replication is provided as the phenotypes available to us were not identical for the Nottingham and ECLIPSE populations. Furthermore, the rarity of the G/G genotype resulted in no individuals with this genotype in either dataset used to study inflammatory cells. These findings are consistent with cigarette-induced emphysema as they provide a correlation between the suggested macrophage recruitment role of MMP-12 in mice and humans.<sup>3</sup> It is interesting that activity differences in the protease appear to translate to COPD-specific phenotypes. It would be worthwhile determining whether the SNP has a relationship with other COPD processes such as the levels of pathological agents secreted by alveolar macrophages or increased cleavage of  $\alpha_1$ -antitrypsin, a substrate for MMP-12.<sup>20</sup>

Another finding of this study was the association between the rs652438 SNP and severity of emphysema, with A/A homozygotes having more severe emphysema. Smokers with a less active form of MMP-12 may therefore be partially protected from severe emphysema. However, despite this being a single test of association, it persisted after adjusting for confounding factors ( $p=0.016$ ). Thus, although these associations are consistent, further validation is required through replication in a larger cohort for macrophage infiltration and emphysema.

It has been known for some time that only a proportion of individuals who smoke develop COPD,<sup>21</sup> and our findings contribute some genetic and functional evidence to the concept

of the 'susceptible smoker'. However, the rarity of the A/G and G/G genotypes at MMP-12 rs652438 (around 3.8% of individuals with severe or very COPD possess at least one G allele<sup>7</sup>) suggests that a number of other loci are likely to contribute to COPD susceptibility. It should also be noted that the ECLIPSE populations used in this study were smokers with COPD and thus would be predicted to have a lower frequency of the minor G allele compared with a 'healthy' smoker group.<sup>7</sup> The rarity of these minor homozygotes suggests that, although it confers a protective effect against emphysema, it is negatively selected against. This could be due to the central role of MMP-12 in inflammatory processes and a possible protective role in cancer metastasis.<sup>22</sup> Low MMP-12 activity in healthy individuals could thus be undesirable, resulting in susceptibility to various conditions.

MMP-12 has been implicated in a number of other diseases—specifically, the rs652438 SNP has been implicated in lung cancer metastasis and survival<sup>23, 24</sup>—and our findings may also have implications for these diseases.

Perhaps the most important finding of this study is that it provides evidence that the findings in the MMP-12 knockout mouse model are relevant to the pathogenesis of human COPD. Knockout mice have impaired levels of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) release in response to acute smoke exposure.<sup>25</sup> This is interesting as TNF $\alpha$  receptor knockout mice develop far less emphysema than control mice in response to smoke exposure.<sup>26</sup> MMP-12 has also been shown to be able to cleave  $\alpha_1$ -antitrypsin.<sup>27</sup> Furthermore, investigation of MMP-12 and associated pathways in COPD could lead to an intervention strategy appropriate to individuals with emphysema.

In conclusion, this study provides evidence that an exonic SNP can alter MMP-12 activity and this relates to certain aspects of the COPD phenotype. Together with previous research, this strengthens the case for the role of MMP-12 as a key protease in COPD. It is unusual to obtain correlations of genotype with phenotypic expression to the extent demonstrated in this study.

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**Competing interests** None.

**Ethics approval** This study was conducted with the approval of the Nottingham Research Ethics Committee and all patients gave informed consent. Investigations into ECLIPSE resource were approved by the ECLIPSE steering committee. Informed consent and collection of the cohort was as described by Vestbo *et al.*<sup>11</sup>

**Provenance and peer review** Not commissioned; externally peer reviewed.

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## Journal club

### A new tuberculosis vaccine

The BCG vaccine, designed as a prophylactic vaccine for pre-infection administration, is currently the only tuberculosis vaccine approved for human use.

In this study researchers developed a new multistage tuberculosis vaccine (H56) which can be used before and after exposure, and tested it in a mouse model. To construct the H56 vaccine, the authors purified the recombinant fusion protein (Ag85B-ESAT6-Rv2660c) from *Escherichia coli*. They hypothesised that it is possible to selectively target *Mycobacterium tuberculosis* (Mtb) in the persistent stage of infection by combining early protective antigens such as Ag85B and ESAT-6 (the H1 vaccine) with the latency protein Rv2660c which is involved in stress responses and characterises long-term Mtb adaptation in the immune host.

The authors assessed the effectiveness of H56, H1 and BCG vaccines administered in mice 6 weeks before Mtb exposure. They demonstrated a statistically significant reduction in bacterial load and induction of immune response with the H56 vaccination compared to the H1 and BCG vaccines starting from 12 weeks after the introduction of infection. The H56 vaccine also enhanced the immunological reaction when administered to mice with earlier treated tuberculosis infection as well as improving response in previously BCG-vaccinated mice later exposed to Mtb.

The new vaccine induces vaccine-specific polyfunctional CD4<sup>+</sup> T cells providing efficient containment of early- and late-stage infection in addition to protection against disease reactivation. This emergence of a novel tuberculosis vaccine in mouse models is an important step forward to guide studies in humans.

► **Aagaard C**, Hoang T, Dietrich J, *et al.* A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat Med* 2011;**17**:189–94.

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## **Online Supplement**

**Title:** MMP-12 SNP affects MMP activity, airway macrophage infiltration and emphysema in COPD

**Authors:** Imran Haq<sup>1,2</sup>, Gill E Lowrey<sup>1</sup>, Noor Kalsheker<sup>2</sup>, Simon R Johnson<sup>1</sup>

### **Cell Culture and Vector Transfection**

COS7 cells were cultured in a humidified atmosphere with 10% CO<sub>2</sub> at 37°C with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum and 2mM L glutamine. Passages occurred every 5 – 7 days using 1x Trypsin/EDTA

Cells were plated in six well plates in 3 ml medium. Cells were cultured until 90 – 95% confluence before transfection. Transfection of MMP-12 containing vectors (250ng) was carried out using Fugene 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions.

### **Analysis of enzymatic activity**

MMP-12 constructs and Null pCMV – XL4 (no insert) vector were transiently transfected into COS7 cells. After 24 hours incubation, the medium was replaced with serum free medium before conditioned media was collected in the next 24 time period. The conditioned media was stored at – 80°C until assayed.

Initial analysis of MMP-12 enzymatic activity in supernatants was determined by casein zymography. Zymography was performed as follows; precast sodium dodecyl sulfate - polyacrylamide gels (7.5%), containing 12% casein (Invitrogen, Paisley, UK), were used to separate samples in 2 × nonreducing sample buffer (0.125M Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 0.003% bromphenol blue) at 120V. Sodium dodecyl sulfate was



removed with reanating buffer (2.5% Triton X-100) (Invitrogen, Paisley, UK) for 30, minutes at room temperature. The gels were incubated overnight hours at 37°C (Heraeus Incubator, Langenselbold, Germany) in developing buffer (20mM Tris-HCl, pH 7.6, 10mM CaCl<sub>2</sub> and 0.04% NaN<sub>3</sub>) (Invitrogen, Paisley, UK) and then stained with 0.1% Coomassie blue in 40% methanol and 10% acetic acid and destained until clear proteolytic bands appeared on the contrasting blue background. Bands were visualized where MMP-12 had degraded the casein matrix, leaving a clear band after staining the gel for protein, differential activity or amounts of MMP-12 creates different band intensities. Every gel included as a standard 10 ng of recombinant human MMP-12 (R&D Systems, Minneapolis, USA).

The activity of MMP-12 relative to the standard was determined by densitometry of the bands using ImageJ a Java-based image processing program by the National Institutes of Health. The intensity and width of the bands were taken into consideration.

To provide a specific read out of activities the commercially available Sensolyte 520 MMP-12 Assay Kit (Anaspec, San Jose, CA) was used. It detects MMP-12 activity using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET) peptide. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMP-12, the fluorescence can be monitored at excitation/emission wavelengths = 490 nm/520 nm. Detection was performed using a Flexstation with Softmax Pro software (Molecular Devices, Sunnyvale, CA). Assays were performed in triplicate according to company protocol.

### **Cell Viability**

To monitor cell viability between transfections the MTT assay was used. The MTT assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of pale yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] and forms blue formazan crystals which are accumulated within healthy cells. The number of viable cells is directly proportional to the level of formazan (24).

Transfected cells had the media removed and had MTT containing phenol red free media, prewarmed at 37°C, added at a concentration of 0.5 mg/ml. This was then incubated for 2 – 4 hours.

At the end of the incubation period the medium was removed and the formazan crystals were solubilised with 250µl of DMSO. The sample was then transferred to a 96 – well microtitre plate. Colormetric measurements were made using plate reader (ThermoScientific Multiskan Ex, Waltham, Massachusetts, USA) set at a wavelength of 650 nm, reads for each sample were performed in triplicate, and mean values calculated.

### **Total Protein Levels**

The assay is based on absorbance maximum change for Coomassie Brilliant Blue G-250 (Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Hemel Hempstead, U.K) which shifts from 465nm to 595nm when protein binding occurs.

From each sample to be measured 10µl of the supernatants and taken were added to 500µl of 1:5 diluted Protein Assay Dye Reagent Concentrate with sterile distilled H<sub>2</sub>O. The samples were then incubated for 5 minutes before 200µl were added to two wells of a microtitre plate, providing a duplicate reading from which a mean value was calculated. The optical density was read at 595nm before 1 hour had passed using a plate reader (ThermoScientific Multiskan Ex, Waltham, Massachusetts, USA).

### **MMP-12 Expression Levels**

To produce RNA, the medium was aspirated and washed with PBS and trypsinised as for passage. An RNeasy mini kit (Qiagen, Crawley, U.K) was then used according to manufacturer's instructions.

Superscript II first strand synthesis system for RT-PCR (Invitrogen, Paisley, UK) was used, to produce cDNA from the RNA. All temperature conditions were performed using a DNA Engine Tetrad™ Cyclor system (MJ Research, Massachusetts, USA).

A master mix containing 5µl total RNA, 1µl (100ng) random hexamers, 1µl 10mM dNTPs and 3µl H<sub>2</sub>O per reaction is used. This is then incubated at 65°C for 5 minutes before placing on ice then room temperature.

Another mastermix is then added consisting of 2µl 2X RT buffer, 4µl 25mM MgCl<sub>2</sub>, 2µl 0.1M dithiothreitol and 1µl (40U) RnaseOut. Superscript II Reverse transcriptase (200U) of volume 1µl was also added to RT + samples, whilst 1µl H<sub>2</sub>O was added to RT – controls. Samples were then cycled as follows; 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes. Samples were then stored at -20°C until use in real time PCR reactions.

The Brilliant SYBR® Green QPCR Master Mix system (Agilent Technologies, California, USA) with a Stratagene Mx3005 QPCR light cycler (California, USA) was used to perform real time PCR.

Primers were to the exonic sequence of MMP – 12 and β – Actin which was selected as an internal control. Primers used are as follows (Invitrogen, Paisley, UK):MMP – 12 Forward:

5' – CATTGAGGAGGCACCAACTTGTC – 3' MMP – 12 Reverse: 5' –  
CCTTTGGATCACTAGAATGGCC – 3' β – Actin Forward: 5' –  
GGATGCAGAAGGAGATTACTG – 3' β – Actin Reverse: 5' –  
CGATCCACACAGAGTACTTG – 3'

To perform the real time reaction, each set of forward and reverse primers were mixed to give a 5 $\mu$ M stock. Also a 25 x dilution of cDNA synthesis reaction was made. Every cDNA sample was run in triplicate for each set of primers.

For each sample, the following mastermix is made; 12.5 $\mu$ l of Brilliant SYBR<sup>®</sup> Green QPCR Master Mix, 5 $\mu$ l of diluted cDNA, 6.5 $\mu$ l of diluted cDNA and 1 $\mu$ l of 5 $\mu$ M primer mix.

The samples were then placed in the light cycler, which was set to read SYBR green and set to report fluorescence during both the annealing and extension step of each cycle. The following PCR program was then used 1 cycle at 95 $^{\circ}$ C for 10 minutes, and 40 cycles of 95 $^{\circ}$ C for 30 seconds, 55 $^{\circ}$ C for 1 minute and 72 $^{\circ}$ C for 1 minute.

Triplicate values were averaged and the difference between the  $\Delta$ CT value for housekeeping primers, and the  $\Delta$ CT value for the gene was calculated to give the  $\Delta\Delta$ CT value.

### **Minigene Analysis of Splicing**

Pre-mRNA splicing is when intron sequences are removed and exons are aligned to generate a mature mRNA transcript (25). Spliceosomes, are key to this process. They recognise boundaries through short regulatory motifs of 6-8 nucleotides in both introns and exons which can enhance or silence splicing. Disruption of an enhancer or silencer by polymorphism could result in significantly altered gene function. The effect of the rs652438 polymorphism on possible exonic splice silencing was analysed using the minigene technique.

MMP-12 exon 8 and the flanking intronic sequence representing A and G alleles of rs652438 were amplified from genomic DNA of homozygotic individuals from the EU COPD cohort. The primers used were: forward 5' – tccatgtcgacCCATGGGAACCATAGAAAAGA – 3' and reverse 5' -gcagcccgccgcTCAGAAACCAAAAACACAAAGAA – 3'. The primers were also designed to introduce Sal I and Not I restriction sites (underlined) in the 5' and 3' ends of

the prospective insert. Lowercase indicates genomic DNA sequence complimentary anchors attached to primers to improve annealing.

DNA was amplified in reaction mixtures containing 200  $\mu$ M each of dATP, dCTP, dGTP and dTTP (Fermentas Life Sciences, York, U.K), in a 1x (dilution of 10x) commercial buffer containing; 50mM KCl 10mM tris-HCl pH 8.3 and 1.5mM MgCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>, 1 U *Taq polymerase* (Bioline, London, U.K) on a DNA Engine Tetrad™ Cyclor system (MJ Research, Massachusetts, USA).

The conditions were 94°C for 40 s, and 35 cycles of denaturing at 94°C for 30s, primer annealing at 60°C for 1min, and extension at 72°C for 1 min, with a final extension of 10 min at 72°C. PCR products were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide.

The PCR fragment was 525 bp (166 bp of intron 7, 160 bp of exon 8, and 199 bp of intron 8). This was cloned into TOPO.2.1 vector according to manufacturer's instructions (Invitrogen, Paisley, U.K).

Inserts were sequenced after DNA mini-preparation (Qiagen, Crawley, U.K). Reactions were carried using topo vector forward and reverse primers. 30–50ng of PCR product and 5 p – moles of the primer were used for ABI Big Dye terminator cycle sequencing (Applied Biosystems, Foster City, USA). For the sequencing reaction 2.5 $\mu$ l of purified PCR product was used in a final reaction volume of 10 $\mu$ l. Sequencing was performed as described by the protocol. The products were then outsourced for the gel sequencing reaction. Sequencing alignment was done using EBI-tools ClustalW web-based software (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) to confirm that the inserts were identical apart from the base at the SNP.

Restriction digestion of the TOPO TA constructs and exontrap vector (MoBiTec, Göttingen, Germany) using Sall and NotI in 10µl double digest reactions (Promega, Southampton, U.K). 5 units each was used, 1x digestion buffer 2 µg DNA for 4 hours at 37°C. Followed by inactivation step of 65°C for 10 min.

Digested vectors had gel electrophoresis performed on a 1% gel at 120v before gel extraction (Qiagen, Crawley, U.K). Vector and insert (1:3 ratio) were ligated using T4 DNA ligase and 1x Ligation buffer (Promega, Southampton, U.K) in 10 µl reaction at 4°C overnight. Vectors were transformed into X1 –blue supercompetent cells carried out according to the manufacturer’s instructions (Stratagene, California, USA).

Screened colonies were had DNA mini prep (Qiagen, Crawley, U.K) performed on then sequenced with exontrap vector primers. Successfully cloned exontrap vectors were bulked up for mammalian transfection and plasmid purified using an endotoxin-free midi kit (Qiagen, Crawley, U.K).

COS 7 cells were then transfected with exontrap vectors before having RNA extracted and cDNA synthesis performed as described previously.

The amplification of cDNA was carried out in 10 µl reactions, containing 1 µL of cDNA, 1XTaq buffer with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.5 mM of each vector primer, and 1 unit of Taq polymerase. The conditions for amplification were as follows: 94<sup>0</sup>C for 3 minutes, 20 cycle touchdown of 65<sup>0</sup>C to 55<sup>0</sup>C for 30 seconds per cycle, 20 cycles of 94<sup>0</sup>C for 30 seconds, 55<sup>0</sup>C for 30 seconds, 72<sup>0</sup>C for 60 seconds and a final extension at 72<sup>0</sup>C for 5 minutes.

### **Migration Assay Investigation of MMP-12 Variants**

Transwells, 6.5 mm in size with 8µm pores (Fisher Scientific, Loughborough, U.K) were coated with collagen IV (Sigma, Gillingham, U.K) at 0.1 mg/ml in serum free media with 0.1% bovine serum albumin (BSA) added. A total of 700µl collagen in media is required per well with 100µl for the top well and 600µl for the bottom. This was then incubated at 4°C for 24 hours.

After 24 hours the collagen media was aspirated and replaced with serum free media containing 0.1% BSA in the same manner as the collagen containing media was added previously. Incubation was for 4 hours at 37°C before aspiration.

COS7 cells were transiently transfected with rs652438 constructs before replacing of the media 24 hours after transfection and adding serum free media to perform serum starvation 24 hours prior to the experiment. Cells was then trypsinised as if for passage and resuspended in a small amount of serum free media containing 0.1% BSA.

To ensure a comparable experiment cells were diluted to a density of  $8 \times 10^5$ /ml and 100µl cells added to the top well. The bottom wells were filled with 600µl 10% fetal calf serum containing media which acted as a chemotactic factor. The transwells were then incubated for 8 hours at 37°C and 5% CO<sub>2</sub> in a Heraeus incubator (Thermo-Electron Corporation, Waltham, Massachusetts, USA).

To stop the experiment, media was aspirated from both the bottom and upper well before cells were fixed by replacing with 4% formaldehyde. This was then kept until ready for staining and imaging. After aspirating formaldehyde, remaining cells in the upper chamber were removed using a cotton bud. DAPI at 1µg/ml for 30seconds was then used to stain the cells before washing in H<sub>2</sub>O.

Transwells were then viewed using a wide field fluorescence microscope (Diaphot 400, Nikon, Tokyo, Japan) at 100 x magnification. Six random pictures of each well were taken

using Insight QE camera (Mikron instruments, San Marcos, California, USA), all experiments were blinded and performed to  $n = 3$ .

### **Investigation of Inflammatory Cell Numbers**

Inflammatory cell migration was investigated in a small initial cohort detailed by Lowrey. The Nottingham Research Ethics Committee approved the collection of this resource. Smokers with COPD defined by GOLD criteria and smokers without COPD were recruited. Patients were aged 40–80 years, had  $\geq 10$ -pack year smoking history and had not required antibiotics or oral steroids for the previous 6 weeks. Patients with  $\alpha 1$ -antitrypsin deficiency, radiological evidence of interstitial lung disease, previous thoracic surgery, or taking inhaled corticosteroids were excluded. Spirometry was performed pre and post bronchodilator according to American Thoracic Society guidelines and breathlessness was recorded using the Medical Research Council (MRC) dyspnoea score. Those with reversibility greater than 10% of baseline FEV<sub>1</sub> after inhaling 400 mcg of salbutamol were excluded. Subjects with FEV<sub>1</sub>/FVC of less than 70% were classified as having COPD.

Differential cell counts were performed according to standard methods. Genotypes were determined sequencing of the rs652438 SNP. Collection of cohort and differential cell counts were performed by Gill E Lowrey.

Replication was performed in the ECLIPSE cohort. A total of 2,180 COPD patients aged 40–75 yrs, with baseline post-bronchodilator FEV<sub>1</sub> of <80% of the predicted value, baseline post-bronchodilator FEV<sub>1</sub>/forced vital capacity (FVC) of  $\leq 0.7$  and a smoking history of  $\geq 10$  pack-yrs were recruited. Exclusion criteria included respiratory disorders other than COPD, prior medical history of significant inflammatory disease other than COPD, COPD exacerbation 4 weeks prior to recruitment, lung surgery, diagnosis of cancer, blood transfusion 4 weeks prior to recruitment, inability to walk, taking part in blinded drug studies, therapy with oral



corticosteroids and participation in radiation exposure studies. Information on a number of COPD specific phenotypic endpoints was obtained. For the interest of replicating findings in the Lowrey cohort, absolute cell number counts of inflammatory cells were obtained for those individuals where induced sputum was taken. The Illumina 550 genome wide platform was used to provide genotype information for rs652438 for individuals in the ECLIPSE cohort.

### **Investigation of Emphysema Scores**

SNP investigation with regards to emphysema score was also performed in ECLIPSE. Emphysema scores were obtained using computerised tomography (CT) performed at full suspended inspiration. A multi – slice CT scanner was used, with contiguous images taken at 1 or 1.25 mm slice thickness. Images were obtained at 120kVp and 40mAs. Image analysis was performed using a low spatial frequency reconstruction algorithm (GE-standard, Siemens – b35f).