

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

A disintegrin and metalloprotease 33 and chronic obstructive pulmonary disease pathophysiology

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Background: Chronic obstructive pulmonary disease (COPD) is a respiratory disorder with increasing prevalence and mortality. It is associated with airway obstruction, increased airway hyper-responsiveness (AHR), and ongoing airway and lung inflammation dominated by CD8 lymphocytes and neutrophils. Single-nucleotide polymorphisms (SNPs) in a *disintegrin and metalloprotease 33 (ADAM33)* gene have been associated with AHR and COPD.

Objective: To assess whether SNPs in *ADAM33* are associated with the severity of AHR and airway inflammation in COPD.

Methods: Eight SNPs in *ADAM33* (F+1, Q-1, S_1, S_2, ST+5, T_1, T_2, V_4) were genotyped in 111 patients with COPD (96 males, 69 current smokers, mean (standard deviation (SD)), aged 62 (8) years, median pack-years 42 (IQR 31–55), mean postbronchodilator forced expiratory volume in 1 s (FEV₁)% predicted 63 (9). Provocative concentration of methacholine causing a decrease in FEV₁ of 20% (PC₂₀ methacholine), sputum and bronchial biopsies were collected.

Results: Patients with the ST+5 AA genotype had more severe AHR, higher numbers of sputum inflammatory cells and CD8 cells in bronchial biopsies than patients with the GG genotype ($p=0.03$, 0.05 and 0.01, respectively). CD8 cell numbers were lower in patients carrying the minor allele of SNP T_1 and T_2, and homozygotic minor variants of SNP S_2 compared with the wild type ($p=0.02$, 0.01 and 0.02, respectively).

Conclusions: This is the first study revealing that SNPs in a gene that confers susceptibility to COPD in the general population—that is, *ADAM33*—are associated with AHR and airway inflammation in COPD. These findings constitute an important step forward in linking gene polymorphisms with COPD pathophysiology, thereby possibly contributing to better treatments for this progressive and disabling disease in the future.

Chronic obstructive pulmonary disease (COPD) is highly prevalent and the only disease with increasing mortality rates worldwide.¹ The disease is characterised by irreversible air flow limitation and associated with an influx of neutrophils, macrophages and CD8 T lymphocytes in the airways.^{2–3} Most patients with COPD show airway hyper-responsiveness (AHR),⁴ an exaggerated airway response to non-specific stimuli resulting in airway obstruction. The severity of AHR is positively associated with inflammation in lung tissue⁵ and numbers of CD8 cells in bronchial biopsies in COPD.⁶ One study reported an association of AHR with sputum inflammatory cells in COPD,⁷ whereas another study did not find this association.⁸

Cigarette smoking is by far the most important risk factor for COPD but there is a wide range in disease severity, irrespective of the number of pack-years of smoking. Furthermore, only a minority of smokers develop the disease, suggesting that besides smoking, an underlying genetic constitution has a role in the development and severity of COPD. A disintegrin and metalloprotease 33 (*ADAM33*) gene is a gene of putative interest for COPD. It was first identified as a susceptibility gene for asthma and AHR,^{9–10} and subsequently as a susceptibility gene for COPD in the general population.¹¹ It is furthermore associated with accelerated decline in lung function in the general¹¹ and asthma population.¹²

ADAM33 belongs to the ADAM family of membrane-anchored metalloproteinases that has a role in a variety of processes such as cell fusion, adhesion and signalling.^{13–14}

ADAM33 is expressed in both airways smooth muscle cells and lung fibroblasts. Although the overall substrates and function of *ADAM33* are as yet unknown, it has been shown to be able to cleave α_2 -macroglobulin,^{15–16} which has a role in pulmonary defence. It is suggested that *ADAM33* is involved in tissue remodelling,¹⁷ a physiological process intricately related to airway inflammation, hyper-responsiveness and airway obstruction.

The aim of this study was to test the hypothesis that SNPs in *ADAM33* are associated with the severity of both AHR and airway inflammation in sputum and bronchial biopsies of patients with COPD.

METHODS AND MATERIALS

The extended version of the methods section is available in the online data repository. In all, 114 patients with COPD participating in the GLUCOLD study were included.¹⁸ Patient characteristics have been described in detail previously.¹⁸ In brief, all patients had irreversible air flow limitation and chronic respiratory symptoms, and were current or ex-smokers with at least 10 pack-years of smoking. Patients did not use a course of inhaled or oral corticosteroids within 3 months, or maintenance treatment with these drugs within 6 months

Abbreviations: *ADAM33*, a disintegrin and metalloprotease 33; AHR, airway hyper-responsiveness; COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume; PC₂₀, 20% fall in forced expiratory volume in 1 s; SNP, single nucleotide polymorphism

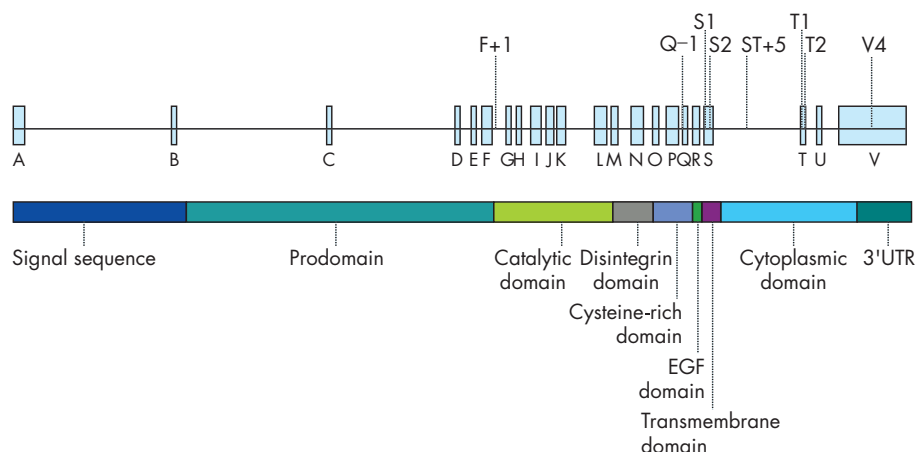


Figure 1 The exon–intron structure of a *disintegrin and metalloprotease 33* (*ADAM33*; the eight genotyped single nucleotide polymorphisms are indicated above the gene) and the domain organisation of *ADAM33*. EGF, epidermal growth factor; Prodomain, proteinase domain.

before randomisation. None of the patients had a history of asthma. The medical ethics committees of the Leiden University Medical Centre (Leiden, The Netherlands) and the Groningen University Medical Centre (Groningen, The Netherlands) approved the study, and all patients gave their written informed consent.

Pulmonary function tests have been described previously.¹⁸ Spirometry and reversibility to salbutamol were measured. Methacholine challenge tests were performed with the 2-min tidal breathing method¹⁹ and expressed as the provocative concentration of methacholine causing a fall in forced expiratory volume in 1 s (FEV_1) of 20% (PC_{20}).

Sputum induction and whole-sample processing were performed according to a validated technique²⁰ as described in detail previously.¹⁸

Details on biopsy collection, processing and immunohistology have been published previously.²¹ In brief, we collected the two best biopsies out of four paraffin-wax-embedded biopsies per patient, and used specific antibodies against T lymphocytes (CD3, CD4 and CD8), macrophages (CD68), neutrophil elastase, mast cell tryptase (AA1) and eosinophils (EG2).

DNA was extracted from peripheral blood. Genotyping was performed as described previously¹¹ using primers and probes from Applied Biosystems TaqMan SNP Genotyping Assays (Nieuwekerk aan de IJssel, Dordrecht, The Netherlands).

Figure 1 shows the eight SNPs in *ADAM33* genotyped: F+1 (G/A), Q-1 (C/T), S_1 (Val-Iso), S_2 (G/C), ST+5 (A/G), T_1 (Met–Thr), T_2 (Pro–Ser), V_4 (C/G). We based the selection of the SNPs on previous associations with AHR, excess decline in FEV_1 , and/or presence of COPD.^{9 11 12 22 23}

We used Arlequin (V.2.000) to test whether SNPs were in Hardy–Weinberg equilibrium and linkage disequilibrium. We investigated whether *ADAM33* SNPs are associated with PC_{20} and the number and subset of inflammatory cells in sputum and bronchial biopsies. Our primary genetic model for all SNPs was a dominant model (homozygotes and heterozygotes for the minor allele being compared as a group with homozygotes for the major allele). In addition, SNPs with a minor allele frequency of ≥ 0.30 were entered in (1) a codominant model (three genotype groups per SNP separately) and (2) a recessive model (homozygotes and heterozygotes for the major allele being compared as a group with homozygotes for the minor allele). PC_{20} and inflammatory cells in sputum and bronchial biopsies were log transformed to obtain a normal distribution. We performed univariate analyses using the t test and ANOVA. Multiple linear regression analyses were performed to investigate

the association of polymorphisms in *ADAM33* with PC_{20} , sputum inflammatory cells and inflammatory cells in biopsies as dependent variables. Independent variables included in the model were sex, smoking status, lung function, genotype and the interaction of smoking status and genotype. To determine whether the regression estimates (with standard errors) and p values were not due to chance, we performed bootstrapping by resampling rows of the original data frame (using $n = 5000$ bootstrap replicates) on the main significant outcome variables.

RESULTS

Results of univariate analyses and multiple regression analyses assuming a recessive model are shown in the online data supplement. Multiple regression analyses assuming a dominant and codominant model are presented below. The low number of patients per haplotype did not allow haplotype analysis.

Prevalence of *ADAM33* SNPs

DNA was available from 111 out of 114 patients with COPD. Table 1 presents the clinical characteristics. All genotyped SNPs were in Hardy–Weinberg equilibrium and in significant linkage disequilibrium. Table 2 shows the prevalence of the eight SNPs. SNPs ST+5, F+1 and S_2 had a minor allele frequency of ≥ 0.30 ; therefore, these SNPs were analysed both in a dominant model and codominant model. The frequency of SNP ST+5 in our group was 50% for each allele. We use the term AA genotype for the wild type and GG genotype for the homozygotic mutant genotype, as published previously.²³

Multivariate association of *ADAM33* SNPs with AHR

Dominant model: patients with a G-allele for SNP ST+5 had a significantly higher PC_{20} compared with the AA genotype (geometric mean (GM) $0.61 \nu 0.27$ mg/ml, $p = 0.04$). SNPs F+1, Q-1, S_1, S_2, T_1, T_2 and V_4 were not significantly associated with the level of AHR.

Codominant model: patients with the GG genotype for SNP ST+5 had a significantly higher PC_{20} compared with the AA genotype (fig 2A). SNPs F+1 and S_2 were not significantly associated with the level of AHR.

Multivariate association of *ADAM33* SNPs with inflammatory cells in sputum

Dominant model: patients with one or more G-alleles of SNP ST+5 had a significantly lower total cell count in sputum than the AA genotype (GM $176 \nu 287 \times 10^4$ cells/ml; $p = 0.04$). Individuals with minor alleles for SNPs F+1, Q-1 and S_2 had

Table 1 Clinical characteristics

	n = 111
Male:female	96:15
Age (years)	61.5 (7.7)
Current smokers, n (%)	69 (62)
Smoking history (pack-years)*	42.0 (31.3–54.5)
FEV ₁ (% pred)	56.4 (9.8)
FEV ₁ /IVC (%)	49.9 (8.5)
Postbd FEV ₁ (% pred)	63.2 (8.8)
Postbd FEV ₁ /IVC (%)	50.7 (8.5)
PC ₂₀ methacholine (mg/ml)*	0.5 (0.2–2.4)

FEV₁, forced expiratory volume in 1 s; IVC, inspiratory vital capacity; PC₂₀ methacholine, provocative concentration of methacholine causing a decrease in FEV₁ of 20%; % pred, percentage of predicted value; Postbd, postbronchodilator.

*Median (25th–75th centile).

Data are mean (SD) unless otherwise specified.

lower numbers of sputum neutrophils compared with the wild type for those SNPs (F+1 GM $103.0 \nu 134.9 \times 10^4$ cells/ml, $p = 0.05$; Q-1 GM $84.5 \nu 122.5 \times 10^4$ cells/ml, $p = 0.01$; S_2 GM $94.0 \nu 127.9 \times 10^4$ cells/ml, $p = 0.02$). SNPs S_1, T_1, T_2 and V_4 were not significantly associated with the number of sputum inflammatory cells.

Codominant model: patients with the GG genotype for SNP ST+5 had a significantly lower total cell count in sputum than the AA genotype, and patients with the AG genotype tended to have a lower total cell count (fig 2B).

Heterozygotic individuals for SNP S_2 had higher numbers of sputum macrophages than the wild type (S_2 GG: GM (95% CI) 27.7×10^4 /ml (20.3 to 37.8); GC: 39.5×10^4 /ml (28.7 to 54.5), $p = 0.03$; CC: 29.6×10^4 /ml (17.6 to 49.7); $p = 0.80$). Heterozygotic individuals for SNP S_2 also had lower numbers of neutrophils (S_2 GG: GM (95% CI) 127.9×10^4 /ml (97.5 to 168.3); GC: 93.3×10^4 /ml (70.5 to 123.9), $p = 0.03$; CC: 97.1×10^4 /ml (61.5 to 153.5); $p = 0.24$).

Multivariate association of ADAM33 SNPs with inflammatory cells in bronchial biopsies

Dominant model: patients with a minor allele for SNPs T_1 and T_2 had a significantly lower number of CD8 cells than the wild type (T_1: $p = 0.02$; T_2: $p = 0.01$). We present only the results of SNP T_2 in fig 3A given the fact that SNPs T_1 and T_2 are in complete linkage disequilibrium ($p = 10^{-16}$; see online data repository, fig E1) and the association of these SNPs with the number of CD8 cells in bronchial biopsies was similar.

Codominant model: patients with the GG genotype for SNP ST+5 had significantly lower numbers of CD8 cells in bronchial biopsies than the AA genotype (fig 2C). Homozygotic individuals for the minor allele of SNP S_2 had significantly lower numbers of CD8 cells in bronchial biopsies compared with the wild type (fig 3B) and lower numbers of plasma cells (S_2 GG: GM (95% CI) $13.5/0.1 \text{ mm}^2$ (8.9 to 20.7); GC: $11.8/0.1 \text{ mm}^2$ (7.7 to 18.2), $p = 0.54$; CC: $6.5/0.1 \text{ mm}^2$ (3.3 to 12.9); $p = 0.04$). Heterozygotic patients for SNP S_2 had significantly lower numbers of CD4 cells compared with the wild type (S_2 GG: GM (95% CI) $28.5/0.1 \text{ mm}^2$ (19.9 to 41.0); GC: $21.8/0.1 \text{ mm}^2$ (16.9 to 28.2), $p = 0.04$; CC: $25.6/0.1 \text{ mm}^2$ (17.0 to 38.3); $p = 0.59$). No significant associations were found with SNP F+1.

We found no significant associations between SNPs in ADAM33 and the number of mast cells or eosinophils in bronchial biopsies.

Results of bootstrapping confirmed the above-presented main outcome results, with comparable and significant p values.

Table 2 Prevalence of a disintegrin and metalloprotease 33 single nucleotide polymorphisms in patients with chronic obstructive pulmonary disease

SNP	COPD % (n)	SNP	COPD % (n)
F+1	GG 34.9 (38)	ST+5	AA 23.7 (26)
	GA 46.8 (51)		AG 53.6 (59)
	AA 18.3 (20)		GG 22.7 (25)
Q-1	TT 67.3 (70)	T_1	TT 80.3 (86)
	TC 28.9 (30)		TC 17.8 (19)
	CC 3.8 (4)		CC 1.9 (2)
S_1	GG 72.9 (78)	T_2	GG 80.8 (88)
	GA 26.2 (28)		GA 17.4 (19)
	AA 0.9 (1)		AA 1.8 (2)
S_2	GG 44.6 (45)	V_4	CC 51.0 (55)
	GC 44.6 (45)		CG 44.4 (48)
	CC 10.8 (11)		GG 4.6 (5)

COPD, chronic obstructive pulmonary disease; SNP, single nucleotide polymorphism.

DNA was available from 111 out of 114 patients with COPD. Different numbers for the SNP genotypes (ranging from 101 to 110) are due to missing genotype data.

DISCUSSION

The important message of this study is that SNPs in ADAM33 are associated with the pathophysiology of COPD. Patients with the AA genotype for SNP ST+5 had more severe AHR, higher numbers of sputum inflammatory cells and higher numbers of CD8 cells in bronchial biopsies than those with the GG genotype. Moreover, individuals with the minor allele of SNP T_1 and T_2, and homozygotic individuals for the minor allele of SNP S_2 had significantly lower numbers of CD8 cells in bronchial biopsies, cells relevant to the pathology of COPD.

Prevalences of the ADAM33 SNPs found in our COPD population are comparable to those recently reported by van Diemen *et al.*¹¹ We also confirm their findings that minor alleles for SNPs F+1, S_1 and S_2 are more prevalent in patients with COPD than in healthy participants. Regarding SNP Q-1 we showed a significantly higher prevalence of the minor allele in patients with COPD compared with healthy controls, whereas van Diemen *et al.*¹¹ showed a trend in the same direction (see online data repository). In addition, we found a higher prevalence for the A-allele in SNP ST+5 in patients with COPD. As far as we know, this is the first study suggesting that a COPD susceptibility gene is also associated with the pathophysiological process in COPD.

ADAM33 is a member of the ADAM family, a group of membrane anchored metalloproteases that contain a disintegrin and a metalloprotease domain and is expressed in multiple tissues, including lung tissue.²⁴ As its function has not been unravelled yet, we can only speculate about its role in COPD. It is conceivable that ADAM33 has a role in both remodelling and inflammation by shedding growth factors, cytokines and their receptors from the cell surface. If a SNP alters ADAM33 function to increase protein production, this might result in enhanced airway inflammation. Alternatively, when ADAM33 has a diminishing effect on the release of proinflammatory cytokines, inflammation would increase by a decrease in ADAM33 production or function. The same could be true for the role of ADAM33 in airway remodelling, in case a change in ADAM33 results in an increase in growth factors resulting in proliferation of airway smooth muscle cells and lung fibroblasts. Whatever the functions of ADAM33 turn out to be, our data suggest that it is associated with inflammation and AHR.

We showed an association between ADAM33 and the severity of AHR in COPD. AHR is important to COPD given its association with accelerated FEV₁ decline²⁵ and increased risk of COPD mortality.²⁶ The exact pathophysiology underlying

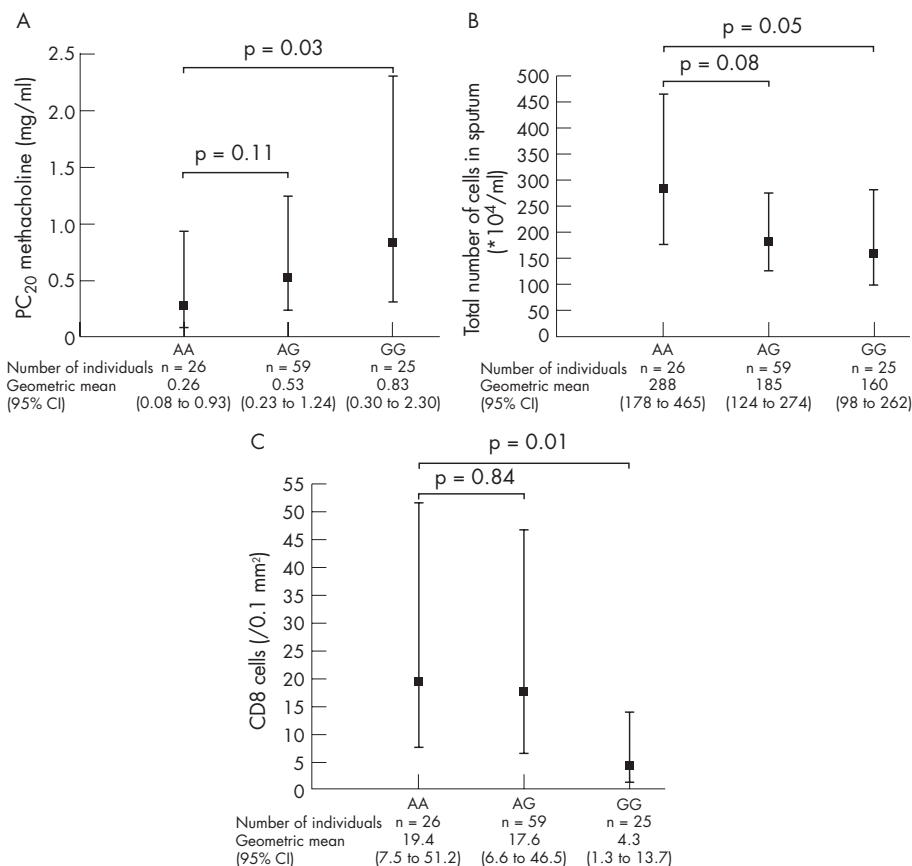


Figure 2 Association of a *disintegrin and metalloprotease 33* single nucleotide polymorphism (SNP) ST+5 with airway hyper-responsiveness (provocative concentration of methacholine causing a decrease in forced expiratory volume in 1 s (FEV₁) of 20% (PC₂₀ methacholine)), sputum inflammatory cells and CD8 cells in bronchial biopsies in patients with chronic obstructive pulmonary disease. (A) PC₂₀ methacholine per SNP ST+5 genotype. (B) Total sputum cell count per SNP ST+5 genotype*. (C) The number of CD8 cells in bronchial biopsies per SNP ST+5 genotype. *Total sputum cell count refers to the total non-squamous cells in induced sputum. Solid squares represent the geometric mean; vertical bars represent the 95% CI. Different numbers for the SNP genotypes are due to missing genotype data.

AHR is unclear, but it is thought to result from an inflammatory process in the airways in addition to geometric changes owing to airway remodelling. As discussed above, it is possible that ADAM33 has a role in both these processes thereby contributing to the severity of AHR.

ADAM33 SNP ST+5 is associated with the total sputum cell count in our COPD population. When we replaced this by numbers of sputum neutrophils or macrophages, we found significant associations with SNPs S₂, F+1 and Q-1 but not with the other SNPs. This suggests that not one specific cell

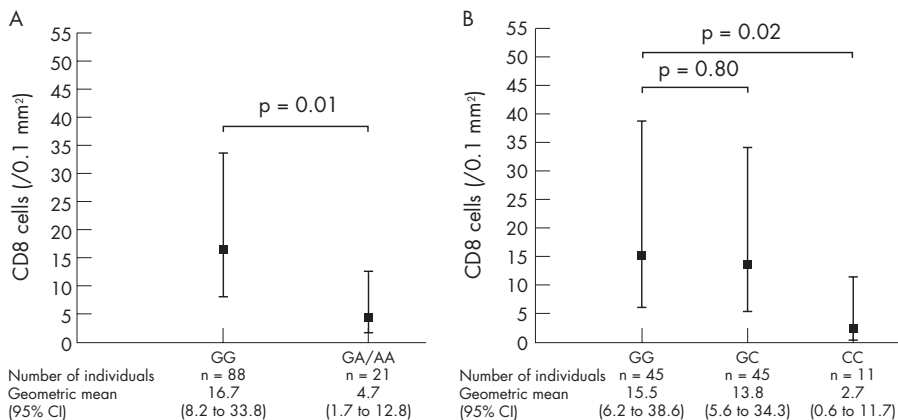


Figure 3 Association of single nucleotide polymorphisms (SNPs) in a *disintegrin and metalloprotease 33* with the number of CD8 cells in bronchial biopsies in chronic obstructive pulmonary disease. (A) Per genotype for SNP T₂. (B) Per genotype for SNP S₂. Solid squares represent the geometric mean; vertical bars represent the 95% CI. Different numbers for the SNP genotypes are due to missing genotype data.

type was predominantly accounting for the association of total cell count with SNP ST+5. Several studies have shown an increase in the number of neutrophils and macrophages and concentrations of proinflammatory cytokines like interleukin 8 and tumour necrosis factor α in induced sputum of patients with COPD.^{27–29} Intuitively, one would think that the severity of AHR is associated with the degree of inflammation and, in fact, we previously did show an independent, positive association between severity of AHR and total sputum cell counts in these patients with COPD³⁰; perhaps *ADAM33* is the missing link.

O'Shaughnessy *et al*³¹ showed an increased number of neutrophils and CD8 lymphocytes in bronchial biopsies of smokers with air flow limitation, and this increase was inversely associated with the level of lung function. We, therefore, hypothesised that individuals with a genetic predisposition for a higher number of CD8 cells were more susceptible to a further increase in CD8 cells, which might finally result in air flow limitation. Interestingly, we found an association of SNP ST+5 with both the presence of COPD (see online data repository) and the number of CD8 cells in our patients with COPD.

ADAM33 has previously been associated with asthma in some,^{9 22 23} but not all studies.^{32 33} We would like to emphasise that we are confident that our patients with COPD genuinely have COPD and no asthma given the fact they all had moderate to severe air flow limitation after bronchodilation, ≥ 10 pack-years of smoking, and no history of doctor-diagnosed asthma. We selected eight SNPs in *ADAM33* based on previous literature. One could argue that we did not genotype all known SNPs in *ADAM33*. However, we did find consistent and significant associations between the genotyped SNPs and the severity of AHR and airway inflammation in our COPD population, indicating linkage of *ADAM33* with pathophysiological features of COPD.

A potential criticism of our study is that the sample size of 111 patients with COPD is relatively small. From a practical point of view, biopsy studies are very demanding and the issue of sample size would especially be of concern in case we did not find any associations. However, we did find significant associations between *ADAM33* and features of COPD. We set out with the *a priori* hypothesis that the same SNPs in *ADAM33* previously shown to be associated with COPD are also associated with its pathophysiology. To investigate our hypothesis, we performed several analyses to assess the association of *ADAM33* with hyper-responsiveness and airway inflammation and found associations of moderate significance. This has affected the interpretation of the results. One could raise the issue of multiple testing being responsible for the current results and that we should have adjusted for this in our analyses. We do not agree with this for several reasons. Firstly, the independent variables in our analyses (eg, sputum total cell count and differential cell count) are mutually related, indicating that a rigid statistical procedure like a Bonferroni correction for multiple testing would not do justice to their biologically linked nature. Secondly, we did not randomly test for associations between *ADAM33* and features of COPD but had a predefined hypothesis based on previous literature.

ADAM33 is a highly polymorphic gene containing at least 58 SNPs of which we investigated eight, based on previous literature.^{9 11 12 22 23} We found an association of SNP ST+5 with the severity of AHR and airway inflammation in induced sputum and bronchial biopsies in COPD. The ST+5 SNP is an intron SNP between the S-exon (transmembrane region) and the T-exon (which includes a SH₃ domain and a phosphorylation site). This may have functional relevance, as non-coding introns can exert their effect by influencing alternative splicing, splicing efficiency or messenger RNA turnover.

We furthermore found an association of SNP S₂ with the inflammatory cell profile in sputum and the number of inflammatory cells in bronchial biopsies. A higher number of airway wall CD8 cells is associated with more severe AHR,⁶ by itself a risk factor for accelerated lung function loss.²⁵ van Diemen *et al*¹¹ showed an association of the minor allele of SNP S₂ with accelerated lung function decline in a general population and of the minor allele of SNP T₂ with the presence of COPD. Yet, in this study the minor alleles of these SNPs were associated with a lower number of CD8 cells. How can we reconcile these seemingly discordant findings? SNP S₂, a silent mutation, is located in the S-exon and SNP T₂ is located in the T-exon. Both SNPs are in close proximity to SNP ST+5. The significant linkage disequilibrium between SNPs S₂, ST+5 and T₂ may suggest that the part of *ADAM33* involved in genetic susceptibility and pathophysiology of COPD lies in the 3' region. Alternatively, it may still be either at a different location within *ADAM33* or at an adjacent gene. Our study was not designed to discuss this question and clearly, it needs further research.

In conclusion, our study confirms *ADAM33* as a COPD susceptibility gene and is the first to extend this observation by revealing an association of *ADAM33* with the severity of both AHR and airway inflammation in individuals affected with COPD. These findings constitute an important step forward in linking gene polymorphisms with COPD pathophysiology, thereby possibly contributing to better future treatments for this progressive and disabling disease.

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LUNG ALERT

Detecting PE in COPD

▲ Monreal M, Munoz-Torrero JF, Naraine VS, *et al*. Pulmonary embolism in patients with chronic obstructive pulmonary disease or congestive heart failure. *Am J Med* 2006;**119**:851–8.

Pulmonary embolism (PE) is a difficult diagnosis to make or exclude and there have been various pretest probability scoring systems developed to aid the clinician in this area. However, these scoring systems do not take into account underlying cardiopulmonary abnormalities. This interesting study by Monreal *et al* looked at the diagnosis of PE in patients with congestive heart failure (CHF) and chronic obstructive pulmonary disease (COPD).

From a total of 4444 patients with proven PE enrolled in a registry, 14% had COPD and 9.5% had CHF. Using the Geneva scoring system, a lower percentage of patients with PE with COPD or CHF fell into the low pretest probability category (RR 0.82, 95% CI 0.66 to 1.02 and RR 0.73, 95% CI 0.56 to 0.95, respectively). The percentage of patients with a high probability of PE was similar among the three patient groups (CHF, COPD and neither). Using the Pisa score, the frequency of patients with COPD and CHF with a high pretest probability for PE were higher (61% and 72%, respectively). The Wells score was not used as the patient registry did not identify “an alternative diagnosis more likely than PE”.

The authors conclude that there are significant differences in the presentation of patients with PE who do and do not have coexistent COPD or CHF and that the performance of the various scoring systems varies. Physicians dealing with suspected PE should consider the underlying cardiopulmonary diagnoses and make necessary allowances when planning investigations and management.

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