A Disintegrin and Metalloproteinase 33 and Chronic Obstructive Pulmonary Disease Pathophysiology

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ABSTRACT

Rationale: Chronic obstructive pulmonary disease (COPD) is a respiratory disorder with increasing prevalence and mortality. It is associated with airway obstruction, increased airway hyperresponsiveness (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 with 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 age 62 years (SD=8), median pack-years 42 (IQR 31-55), mean postbronchodilator FEV1% predicted 63 (SD=9)). 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, p=0.05, p=0.01, respectively). CD8+ cell numbers were lower in subjects carrying the minor allele of SNP T_1 and T_2, and homozygous minor variants of SNP S_2 compared to the wild-type (p=0.02, p=0.01, p=0.02, respectively).

Conclusions: This is the first study demonstrating that SNPs in a gene that confers susceptibility to COPD in the general population, i.e. 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 future treatments for this progressive and disabling disease.
INTRODUCTION
Chronic obstructive pulmonary disease (COPD) is worldwide highly prevalent and the only disease with increasing mortality rates.[1] The disease is characterized by irreversible airflow limitation and associated with an influx of neutrophils, macrophages, and CD8+ T lymphocytes in the airways.[2][3] A majority of COPD patients demonstrates airway hyperresponsiveness (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 tissue[5] 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 smoking. Furthermore, only a minority of smokers develops the disease, suggesting that besides smoking an underlying genetic constitution plays 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 a general population.[11] It furthermore is associated with accelerated lung function decline in a general[11] and asthma population.[12]

ADAM33 belongs to the ADAM family of membrane-anchored metalloproteinases which play 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 yet unknown, it has been shown to be able to cleave α2-macroglobulin,[15] [16] which plays a role in pulmonary defence. It is suggested that ADAM33 is involved in tissue remodelling,[17] a physiologic process intricately related to airway inflammation, hyperresponsiveness and airway obstruction.

The aim of this study was to test the hypothesis that SNPs in ADAM33 are associated with the severity of both airway hyperresponsiveness 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. One-hundred-and-fourteen 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 airflow 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 prior to randomisation. None of the patients had a history of asthma. The medical ethics committees of the Leiden University Medical Centre and the Groningen University Medical Centre 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-minute tidal breathing method[19] and expressed as the provocative concentration of methacholine causing a 20% fall in FEV1 (PC20).

Sputum induction and whole sample processing were performed according to a validated technique[20] 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 embedded biopsies per patient, and used specific antibodies against T lymphocytes (CD3, CD4, and CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1), and eosinophils (EG2).

DNA was extracted from peripheral blood. Genotyping was performed as described previously[11] using primers and probes from Applied Biosystems TaqMan® SNP Genotyping Assays (Nieuwekerk aan de IJssel, The Netherlands). Figure 1 shows the 8 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 airway hyperresponsiveness, excess decline in FEV1, and/or presence of COPD.[9][11][12][22][23]

We used Arlequin (version 2.000) to test whether SNPs were in Hardy Weinberg equilibrium and linkage disequilibrium (LD). We investigated whether ADAM33 SNPs are associated with PC20 and 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). Additionally, 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). PC20 and inflammatory cells in sputum, and bronchial biopsies were log transformed to obtain a normal distribution. We performed univariate analyses using t-test and ANOVA. Multiple linear regression analyses were performed to investigate the association of polymorphisms in ADAM33 with PC20, sputum inflammatory cells, and inflammatory cells in biopsies as dependent variables. Independent variables included in the model were gender, smoking status, lung function, genotype, and the interaction of smoking status and genotype. In order to determine whether the regression estimates (with standard errors) and p values were not due to chance, we performed bootstrapping by re-sampling 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 subjects per haplotype did not allow haplotype analysis.

Prevalence of ADAM33 SNPs

DNA was available from 111 out of 114 COPD patients. Clinical characteristics are presented in table 1. All genotyped SNPs were in Hardy Weinberg equilibrium and in significant LD. Table 2 shows the prevalence of the 8 SNPs. SNPs ST+5, F+1, and S_2 had a minor allele frequency ≥ 0.30, therefore these SNPs were analyzed 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 homozygous mutant genotype, as previously published.[23]
### Table 1: Clinical characteristics *

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>96/15</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.5 ± 7.7</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>69 (62)</td>
</tr>
<tr>
<td>Smoking history (pack-years) ‡</td>
<td>42.0 (31.3-54.5)</td>
</tr>
<tr>
<td>FEV₁ (% pred.)</td>
<td>56.4 ± 9.8</td>
</tr>
<tr>
<td>FEV₁/IVC (%)</td>
<td>49.9 ± 8.5</td>
</tr>
<tr>
<td>Postbd. FEV₁ (% pred.)</td>
<td>63.2 ± 8.8</td>
</tr>
<tr>
<td>Postbd. FEV₁/IVC (%)</td>
<td>50.7 ± 8.5</td>
</tr>
<tr>
<td>PC₂₀ methacholine (mg/ml) ‡</td>
<td>0.5 (0.2-2.4)</td>
</tr>
</tbody>
</table>

Table 1: *Data are presented as mean ± standard deviation or; ‡ median (25th-75th percentile).

Definition of abbreviations: FEV₁ = forced expiratory volume in one second; % pred = percentage of predicted value; Postbd. = postbronchodilator; FEV₁/IVC = forced expiratory volume in one second/inspiratory vital capacity; PC₂₀ methacholine = the provocative concentration of methacholine causing a decrease in FEV₁ of 20%.

### Table 2: Prevalence of ADAM33 SNPs in patients with COPD

<table>
<thead>
<tr>
<th>SNP</th>
<th>COPD % (n)</th>
<th>SNP</th>
<th>COPD % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F+1</td>
<td>GG 34.9 (38)</td>
<td>ST+5</td>
<td>AA 23.7 (26)</td>
</tr>
<tr>
<td></td>
<td>GA 46.8 (51)</td>
<td></td>
<td>AG 53.6 (59)</td>
</tr>
<tr>
<td></td>
<td>AA 18.3 (20)</td>
<td></td>
<td>GG 22.7 (25)</td>
</tr>
<tr>
<td>Q-1</td>
<td>TT 67.3 (70)</td>
<td>T₁</td>
<td>TT 80.3 (86)</td>
</tr>
<tr>
<td></td>
<td>TC 28.9 (30)</td>
<td></td>
<td>TC 17.8 (19)</td>
</tr>
<tr>
<td></td>
<td>CC 3.8 (4)</td>
<td></td>
<td>CC 1.9 (2)</td>
</tr>
<tr>
<td>S₁</td>
<td>GG 72.9 (78)</td>
<td>T₂</td>
<td>GG 80.8 (88)</td>
</tr>
<tr>
<td></td>
<td>GA 26.2 (28)</td>
<td></td>
<td>GA 17.4 (19)</td>
</tr>
<tr>
<td></td>
<td>AA 0.9 (1)</td>
<td></td>
<td>AA 1.8 (2)</td>
</tr>
<tr>
<td>S₂</td>
<td>GG 44.6 (45)</td>
<td>V₄</td>
<td>CC 51.0 (55)</td>
</tr>
<tr>
<td></td>
<td>GC 44.6 (45)</td>
<td></td>
<td>CG 44.4 (48)</td>
</tr>
<tr>
<td></td>
<td>CC 10.8 (11)</td>
<td></td>
<td>GG 4.6 (5)</td>
</tr>
</tbody>
</table>

Table 2: DNA was available from 111 out of 114 COPD patients. Different numbers for the SNP genotypes (ranging from 101 to 110) are due to missing genotype data.
Multivariate association of ADAM33 SNPs with AHR

**Dominant model** Subjects with a G-allele for SNP ST+5 had a significantly higher PC_{20} compared to the AA-genotype (geometric mean (gm) 0.61 versus 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 level of AHR.

**Codominant model** Subjects with the GG-genotype for SNP ST+5 had a significantly higher PC_{20} compared to the AA-genotype (figure 2a). SNPs F+1 and S_2 were not significantly associated with 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 versus 287*10^4 cells/ml; p=0.04). Individuals with minor alleles for SNPs F+1, Q-1, S_2 had lower numbers of sputum neutrophils compared to the wild-type for those SNPs (F+1 gm 103.0 versus 134.9 *10^4 cells/ml; p=0.05; Q-1 gm 84.5 versus 122.5*10^4 cells/ml, p=0.01; S_2 gm 94.0 versus 127.9*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 subjects with the AG-genotype tended to have a lower total cell count (figure 2b). Heterozygous 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 (20.3-37.8); GC: 39.5*10^4/ml (28.7-54.5), p=0.03; CC: 29.6*10^4/ml (17.6-49.7); p=0.80). Heterozygous individuals for SNP S_2 had also lower numbers of neutrophils (S_2 GG: gm (95% CI) 127.9*10^4/ml (97.5-168.3); GC: 93.3*10^4/ml (70.5-123.9), p=0.03; CC: 97.1*10^4/ml (61.5-153.5); p=0.24).

Multivariate association of ADAM33 SNPs with inflammatory cells in bronchial biopsies

**Dominant model** Individuals 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 only present the results of SNP T_2 in figure 3a given the fact that SNPs T_1 and T_2 are in complete LD (p= 10^{-16}) (see online data repository; figure 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 (figure 2c). Homozygous individuals for the minor allele of SNP S_2 had significantly lower numbers of CD8^+ cells in bronchial biopsies compared to the wild-type (figure 3b) and lower numbers of plasma cells (S_2 GG: gm (95% CI) 13.5/0.1 mm^2 (8.9-20.7); GC: 11.8/0.1 mm^2 (7.7-18.2), p=0.54; CC: 6.5/0.1 mm^2 (3.3-12.9); p=0.04). Heterozygous subjects 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 mm^2 (19.9-41.0); GC: 21.8/0.1 mm^2 (16.9-28.2), p=0.04; CC: 25.6/0.1 mm^2 (17.0-38.3); 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.
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 homozygous 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 subjects. Regarding SNP Q-1 we demonstrate a significantly higher prevalence of the minor allele in patients with COPD compared with healthy controls, whereas van Diemen et al. demonstrated 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 member of the ADAM family, a group of membrane-anchored metalloproteinases which contain both a disintegrin and a metalloproteinase domain and is expressed in multiple tissues, including lung tissue. [24] Since its function has not been unravelled yet, we can only speculate about its role in COPD. It is conceivable that ADAM33 plays 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 increased protein production this may thus result in enhanced airway inflammation. Alternatively, when ADAM33 has a diminishing effect on the release of pro-inflammatory 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 an alteration 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 suggests that it is associated with inflammation and AHR.

We demonstrated an association between ADAM33 and the severity of AHR in COPD. AHR is important to COPD given its association with accelerated FEV₁ decline[25] and increased risk of COPD mortality.[26] The exact pathophysiology underlying AHR is unclear, but it is thought to result from an inflammatory process in the airways in addition to geometric changes due to airway remodelling. As discussed above, it is possible that ADAM33 plays 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 S_2, F+1, and Q-1 but not with the other SNPs. This suggests that not one specific cell 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 pro-inflammatory cytokines like IL-8 and TNF-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 demonstrate an independent, positive association between severity of
AHR and total sputum cell counts in these COPD patients[30]; perhaps ADAM33 is the missing link.

O’Shaughnessy et al. demonstrated an increased number of neutrophils and CD8+ lymphocytes in bronchial biopsies of smokers with airflow limitation, and this increase was inversely associated with level of lung function.[31] The authors therefore hypothesized 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 airflow limitation. Interestingly, we found an association of SNP ST+5 with both presence of COPD (see online data repository) and the number of CD8+ cells within our COPD patients.

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 COPD patients genuinely have COPD and no asthma given the fact they all had moderate to severe airflow limitation after bronchodilation, >10 pack-years of smoking, and no history of doctor diagnosed asthma. We selected 8 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. In order to investigate our hypothesis, we performed a number of analyses to assess the association of ADAM33 with hyperresponsiveness and airway inflammation and found associations of moderate significance. This has impact on 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 a number of reasons. Firstly, the independent variables in our analyses (e.g. sputum total cell count and differential cell count) are mutually related, indicating that a rigid statistical procedure like e.g. 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 8, 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, since non-coding introns can exert their effect by influencing alternative splicing, splicing efficiency or messenger RNA turnover.

We furthermore found an association of SNP S_2 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 S_2 with accelerated lung function decline in a general population and of the minor allele of SNP T_2 with presence of COPD. Yet in the present 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 S_2, a silent mutation, is located in the S-exon and SNP T_2 is located in the T-exon. Both SNPs are in close proximity to SNP ST+5. The significant LD between SNPs S_2, ST+5, and T_2 may suggest that the part of ADAM33 involved in genetic susceptibility and pathophysiology of COPD lies in the 3’ region. Alternatively, it still may be either at a different location within ADAM33 or at an adjacent gene. Our study was not designed to address 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 demonstrating an association of ADAM33 with the severity of both airway hyperresponsiveness and airway inflammation in COPD-affected individuals. 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.
APPENDIX

Competing interests
The authors declare no competing interests

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Acknowledgements
FIGURE LEGENDS

Figure 1
The exon intron structure of ADAM33 (the eight genotyped SNPs are indicated above the gene) and the domain organisation of ADAM33

Figure 2
Association of ADAM33 SNP ST+5 with airway hyperresponsiveness (PC_{20} methacholine), sputum inflammatory cells, and CD8* cells in bronchial biopsies in patients with COPD
a. PC_{20} 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 of non-squamous cells in induced sputum. Blue squares represent the geometric mean; vertical bars represent the 95% confidence interval. Different numbers for the SNP genotypes are due to missing genotype data.

Figure 3
Association of SNPs in ADAM33 with the number of CD8* cells in bronchial biopsies in COPD
a. Per genotype for SNP T_2
b. Per genotype for SNP S_2
Blue squares represent the geometric mean; vertical bars represent the 95% confidence interval. Different numbers for the SNP genotypes are due to missing genotype data.
References


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Online Data Repository
EXTENDED METHODS
Statistical analysis
We used Arlequin (version 2.000) to test whether SNPs were in Hardy Weinberg equilibrium and linkage disequilibrium (LD). Figure E1 illustrates the LD patterns with \(D', r\) and \(P\)-values in \textit{ADAM33}. We investigated whether \textit{ADAM33} SNPs are associated with \(PC_{20}\) and 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). Additionally, SNPs with a minor allele frequency of \(\geq 0.30\) 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). \(PC_{20}\), sputum inflammatory cells, and inflammatory cells in bronchial biopsies were log transformed to obtain a normal distribution. We performed univariate analyses using t-test and ANOVA. Multiple linear regression analyses were performed to investigate the association of polymorphisms in \textit{ADAM33} with \(PC_{20}\), sputum inflammatory cells, and inflammatory cells in biopsies as dependent variables. Independent variables included in the model were gender, smoking status, lung function, genotype, and the interaction of smoking status and genotype. We entered sputum total cell count (centred around the mean of the total population) to the model when analyzing the differential cell count in sputum, and the number of CD3\(^+\) cells (centred around the mean of the total population) when analyzing CD4\(^+\) and CD8\(^+\) cells in bronchial biopsies.

EXTENDED RESULTS
Univariate association of \textit{ADAM33} SNPs with AHR
To provide our readership with all information, we show below the results of the dominant, codominant and recessive genetic models.
Dominant model Subjects with a G-allele for SNP ST+5 had a significantly higher \(PC_{20}\) compared to the AA-genotype (geometric mean (gm) 0.74 versus 0.27 mg/ml, \(p=0.02\)). SNPs F+1, Q-1, S\(_1\), S\(_2\), T\(_1\), T\(_2\), and V\(_4\) were not significantly associated with AHR.
Codominant model There was a significant difference in \(PC_{20}\) methacholine between the three genotypes for SNP ST+5 (AA: gm 0.27 mg/ml; AG: 0.64 mg/ml; GG: 1.04 mg/ml; \(p=0.04\)). SNPs F+1 and S\(_2\) were not significantly associated with AHR.
Recessive model Homozygous individuals for the minor allele of SNP S\(_2\) had a significantly lower \(PC_{20}\) than subjects with one or more major alleles (gm 0.26 versus 0.72 mg/ml, \(p=0.008\)). SNPs F+1 and ST+5 were not significantly associated with AHR.

Univariate association of \textit{ADAM33} SNPs with inflammatory cells in sputum
Dominant model Patients with a G-allele for SNP ST+5 had a significantly lower total sputum cell count compared to the AA-genotype (gm 121 versus 204\(\times 10^4\) cells/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 sputum inflammatory cells.
Codominant model SNPs F+1, S\(_2\), and ST+5 were not significantly associated with inflammatory cells in sputum.
Recessive model SNPs F+1, S\(_2\), and ST+5 were not significantly associated with inflammatory cells in sputum.
Univariate association of *ADAM33* SNPs with inflammatory cells in bronchial biopsies

**Dominant model** Individuals with a minor allele for SNP S_2 had lower numbers of CD4⁺ cells than the wild-type for that SNP (gm 41.9 versus 56.1/0.1 mm², p=0.05).

**Codominant model** There was a significant difference between the three genotypes for SNP ST+5 and SNP S_2 in the number of CD8⁺ cells in bronchial biopsies (gm ST+5 AA: 27.7/0.1 mm²; AG: 21.6/0.1 mm²; GG: 8.2/0.1 mm², p=0.003; S_2 GG: 21.8/0.1 mm²; GC: 20.4/0.1 mm²; CC: 6.2/0.1 mm²; p=0.03). No significant associations were found with SNP F+1.

**Recessive model** Homozygous individuals for the G-allele of SNP ST+5 had a significantly lower number of CD8⁺ cells and a significantly higher number of eosinophils, compared to individuals with one or more A-alleles for SNP ST+5 (gm 8.2 versus 23.3, p=0.03 and gm 2.4 versus 0.8/0.1 mm², p=0.04, respectively).

Multivariate association of *ADAM33* SNPs with AHR and inflammatory cells in sputum and bronchial biopsies, assuming a recessive model

For completeness, we present below the outcome of the multivariate linear regression analyses assuming a recessive model.

SNPs F+1, S_2, and ST+5 were not significantly associated with PC20, total sputum cell count, or sputum differential cell counts. The number of CD8⁺ cells in bronchial biopsies was significantly lower in individuals with the GG-genotype for SNP ST+5 compared with individuals with one or more A-alleles in SNP ST+5 (gm (95% CI) 7.7 (3.9-13.5) versus 18.7/0.1mm² (12.3-28.3), p=0.002), lower in individuals with the CC-genotype for SNP S_2 compared with individuals with one or more G-alleles in SNP S_2 (5.3 (2.4-12.0) versus 16.5/0.1 mm² (10.5-26.0), p=0.007), and lower in individuals with the AA-genotype in SNP F+1 compared with individuals with one or more G-alleles in SNP F+1 (8.3 (4.4-15.4) versus 17.0/0.1 mm² (11.1-26.0), p=0.02). The number of eosinophils in bronchial biopsies was significantly higher in individuals with the GG-genotype for SNP ST+5 compared with individuals with one or more A-alleles in SNP ST+5 (3.5 (1.2-9.8) versus 1.2/ 0.1 mm² (0.6-2.5), p=0.04)

Differences in SNP prevalence between patients with COPD and controls

Previously, it has been demonstrated by van Diemen *et al.* that SNPs in *ADAM33* are associated with the presence of COPD in a general population.[1] In addition to the data presented in the current manuscript with regard to the association of *ADAM33* with airway hyperresponsiveness and airway inflammation in patients with COPD, we compared the distribution of *ADAM33* SNPs of our COPD patients with that of a population based control group.

As a control group, we selected 1097 Caucasians of Dutch descent without airflow limitation (FEV₁ > 80% pred, and FEV₁/forced vital capacity > 70%) from the Vlagtwedde-Vlaardingen cohort.[2][3] Genotyping of the control group has been previously described in detail.[1] Differences in prevalence of rare alleles of SNPs between the COPD patients described in the current manuscript and controls were tested using chi-square tests.

DNA was available from 1097 controls. All genotyped SNPs were in Hardy Weinberg equilibrium and in significant linkage disequilibrium. Clinical characteristics of the controls are presented in table E1. Table E2 shows that the prevalence of the minor allele of SNPs F+1, Q-1, S_1, and S_2 was significantly higher in the COPD group than the control group (p=0.04, p=0.03, p=0.003, and p=0.02, respectively),
whereas the prevalence of the minor allele of SNP ST+5 was lower ($p=0.02$). The prevalence of SNPs T_1, T_2, and V_4 was not significantly different between both groups. With these findings, we confirm the findings by van Diemen et al.[1] i.e. minor alleles for SNPs F+1, S_1, and S_2 are more often prevalent in patients with COPD than in subjects without airflow limitation. Regarding SNP Q-1 we found a significantly higher prevalence of the minor allele in patients with COPD compared with healthy controls, whereas van Diemen et al. demonstrated a trend in the same direction. In addition, we found a higher prevalence for the A-allele in SNP ST+5 in patients with COPD.

Table E1: Clinical characteristics of the population based control group*

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1097</td>
</tr>
<tr>
<td>Male/female</td>
<td>535/562</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50.8 ± 9.5</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>378 (34)</td>
</tr>
<tr>
<td>Smoking history (pack-years) ‡</td>
<td>6.6 (0-18.4)</td>
</tr>
<tr>
<td>FEV₁ (% pred.)</td>
<td>94.9 ± 11.3</td>
</tr>
<tr>
<td>FEV₁/IVC (%)</td>
<td>75.8 ± 6.1</td>
</tr>
</tbody>
</table>

Table E1:*Data are presented as mean ± standard deviation or; ‡ median (25th-75th percentile).

Definition of abbreviations: FEV₁ = forced expiratory volume in one second; % pred = percentage of predicted value; FEV₁/IVC = forced expiratory volume in one second/inspiratory vital capacity.

Table E2: Prevalence of genotypes in COPD patients and controls

<table>
<thead>
<tr>
<th>SNP</th>
<th>COPD % (n)</th>
<th>Controls % (n)</th>
<th>P value Df=2</th>
<th>SNP</th>
<th>COPD % (n)</th>
<th>Controls % (n)</th>
<th>P value Df=2</th>
</tr>
</thead>
<tbody>
<tr>
<td>F+1</td>
<td>AA 18.3 (20)</td>
<td>11.8 (128)</td>
<td>0.02</td>
<td>ST+5</td>
<td>AA 22.7 (25)</td>
<td>35.4 (386)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>AG 53.6 (59)</td>
<td>46.9 (511)</td>
<td></td>
<td>ST+5</td>
<td>GG 35.4 (386)</td>
<td>46.9 (511)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG 22.7 (25)</td>
<td>35.4 (386)</td>
<td></td>
<td></td>
<td>GG 22.7 (25)</td>
<td>35.4 (386)</td>
<td></td>
</tr>
<tr>
<td>Q-1</td>
<td>TT 67.3 (70)</td>
<td>77.9 (844)</td>
<td>0.003</td>
<td>TT 80.3 (86)</td>
<td>77.2(795)</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC 28.9 (30)</td>
<td>20.3 (220)</td>
<td></td>
<td>TC 17.8 (19)</td>
<td>21.2 (219)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC 3.8 (4)</td>
<td>1.8 (19)</td>
<td></td>
<td>CC 1.9 (2)</td>
<td>1.6 (17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_1</td>
<td>GG 72.9 (78)</td>
<td>85.3 (934)</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA 26.2 (28)</td>
<td>14.1 (154)</td>
<td></td>
<td>GA 17.4 (19)</td>
<td>21.8 (232)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA 0.9 (1)</td>
<td>0.6 (7)</td>
<td></td>
<td>AA 1.8 (2)</td>
<td>1.5 (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_2</td>
<td>GG 44.6 (45)</td>
<td>58.5 (626)</td>
<td>0.02</td>
<td>V_4</td>
<td>CC 51.0 (55)</td>
<td>58.2 (631)</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>GC 44.6 (45)</td>
<td>34.9 (374)</td>
<td></td>
<td>GC 44.4 (48)</td>
<td>36.4 (394)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC 10.8 (11)</td>
<td>6.6 (71)</td>
<td></td>
<td>GG 4.6 (5)</td>
<td>5.4 (58)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FOOTNOTES

Figure E1
Graph of the LD patterns with D’, r and P-values in ADAM33. Explanation of the color scheme: red indicates a p-value of less than 0.025, orange indicates a p-value between 0.025 and 0.1, and yellow indicates a p-value of greater than 0.1
REFERENCES


Figure 2c:

<table>
<thead>
<tr>
<th>SNP ST+5</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals</td>
<td>26</td>
<td>59</td>
<td>25</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>19.4</td>
<td>17.6</td>
<td>4.3</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(7.4,51.2)</td>
<td>(6.6,46.9)</td>
<td>(1.3,13.7)</td>
</tr>
</tbody>
</table>

CD8+ cells (/0.1 mm²)

P = 0.84

P = 0.01
A Disintegrin and Metalloproteinase 33 and Chronic Obstructive Pulmonary Disease Pathophysiology

Margot M E Gosman, H Marike Boezen, Cleo C van Diemen, Jiska B Snoeck-Stroband, Therese S Lapperre, Pieter S Hiemstra, Nick H Ten Hacken, Jan Stolk and Dirkje S Postma

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