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Linking gene plymorphisms with COPD onset and pathology

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Linking gene polymorphisms with COPD onset and pathology

Simona E. Budulac

CMB

Linking gene polymorphisms with COPD onset and pathology

Simona E. Budulac

Stellingen behorende bij het proefschrift:

Linking gene polymorphisms with COPD onset and pathology

1. Although variations in *MRP1* are involved in COPD severity, they have no further effects on lung function over time in COPD. (This thesis)

2. MRP1 protein expression in bronchial biopsies plays a role in FEV_1 decline occurring selectively in COPD patients with long-term ICS therapy. (This thesis)

3. Polymorphisms in *nAChR* cluster potentially have a causal role in COPD via smoking habits. (This thesis)

4. The tagging polymorphisms in *TLR2* and *TLR4* are important for the severity and progression of COPD. (This thesis)

5. A developmental gene such as *HHIP* may play an important role in protection to accelerated lung function loss in COPD. (This thesis)

6. Luck is where opportunity meets preparation. (Denzel Washington)

7. Ik schrijf je een lange brief, want ik heb geen tijd voor een korte. (Blaise Pascal)

8. A problem well stated is a problem half-solved. (Charles F. Kettering)

9. The greatest power is often simple patience. (E. Joseph Croosman)

10. In science when nothing goes right, go left.

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S.E. Budulac

Linking gene polymorphisms with COPD onset and pathology

Thesis University of Groningen with summary in Dutch

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CONTENTS

CHAPTER 1

General introduction

General introduction

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of death worldwide. The Global Burden of Disease Studies showed that COPD was the sixth most common cause of death in 1990, but the distressing prediction was that COPD would become the third most common cause of death by 2020 [1]. Although tobacco smoking is widely accepted as the most important risk factor for COPD, factors such as other environmental exposures and genetic predisposition are also considered to contribute to COPD development.

Definition of COPD

COPD is a complex disease characterized by poorly reversible airflow limitation that is usually progressive. COPD is associated with an abnormal inflammatory response of the lung and degradation of lung tissue [2]. It is comprised primarily of three conditions, namely chronic bronchitis, bronchiolitis (small airway disease) and emphysema that represent the pathological hallmarks of COPD. Chronic bronchitis is characterized by inflammation of the central airways which is the main site of mucous hypersecretion contributing to airway obstruction and respiratory symptoms like chronic cough and sputum production. Emphysema is characterized by the destruction of lung parenchyma, by which the area available for gas transport is reduced, ultimately leading to insufficient oxygen uptake in the bloodstream, causing symptoms like dyspnea on exertion and in later stages also at rest. Bronchiolitis, i.e. small airway disease is characterized by inflammation of the peripheral airways that contributes to the airflow limitation by narrowing and obliterating the lumen and by actively constricting the airways.

The diagnosis of COPD is usually based on lung function measurements and according to most recommendations a postbronchodilator ratio of forced expiratory volume in one second (FEV_1)/ forced vital capacity (FVC) below 0.70 is taken as cut-off value for the diagnosis [2, 3]. In addition, staging of the disease is based on the lung function assessed as FEV_1 as percentage of predicted value (that is, what is normal given a person's gender, height and age) (Table 1).

Table 1: Stages of COPD according to Global Initiative for Chronic Obstructive Lung Disease [2]

GOLD stage	*Spirometry*
I – Mild COPD	FEV_1/ FVC < 0.70 and $FEV_1 \geq 80\%$ predicted
II – Moderate COPD	FEV_1/ FVC < 0.70 and $50\% \leq FEV_1$ % predicted < 80%
III – Severe COPD	FEV_1/ FVC < 0.70 and $30\% \leq FEV_1$ % predicted < 50%
IV – Very Severe COPD	FEV_1/ FVC < 0.70 and FEV_1 < 30% predicted or FEV_1 < 50% predicted plus chronic respiratory failure

Environmental and host risk factors for COPD

Tobacco smoking is the most important risk factor for the development and progression of COPD. Besides the established role of tobacco smoking in the development of COPD, there are other factors that are associated with development of COPD such as environmental and host factors [4].

Table 2: Summary of known risk factors for COPD

Environmental factors	*Host factors*
Tobacco smoking • active smoking • passive smoking • maternal smoking during pregnancy	Genetic factors Family history α1-antitrypsin deficiency Gender Age
Low socio-economic status	Race
Air pollution	Low birth weight
Respiratory infections	
Poor nutrition	

Low socio-economic status (SES) has an impact on lung function and this association between SES and COPD is consistent across different types of studies in different

populations [4]. Maternal smoking, more frequent respiratory tract infections during childhood, housing conditions, air pollution and other lifestyle factors including passive smoking are contributing risk factors for the development of COPD [4]. Nutrition also plays an important role in the development of COPD. Most of the evidence refers to vitamins C and E, which have an antioxidant effect that supposedly counteracts the oxidative damage produced by exposures like smoking and air pollution [4]. Besides consumption of vitamin C, wine intake has been positively associated with the level of FEV_1 and FVC in the general population [5, 6] and this beneficial effect of wine may be due to resveratrol (a polyphenol present in the skin of grapes) intake that has been observed to exhibit beneficial effects on FVC level in the general population [6].

Family history plays a role in the development of COPD since it has been shown that first-degree relatives of subjects with severe, early onset COPD are at increased risk to develop chronic bronchitis and airflow limitation [7]. The COPDGene Study showed that early-onset COPD may develop in susceptible females [8]. Remarkably, this early-onset COPD in females may be attributed to risk factors such as a decreased maximally attained lung function level or a reduction in lung growth as well as a maternal respiratory history, tobacco smoking, asthma, genetic factors, and hormonal influences [8].

Genetic risk factors

It is established that the level of lung function and the development of COPD is affected by environmental exposures, but genetic factors play an important role as well. Heritability estimates for level of lung function range as high as 30-47% [9, 10]. The strong heritability of COPD is made apparent by familial aggregation of the disease and by the fact that only 10-20% of smokers develop COPD, although 90% of COPD patients smoke [10, 11]. It is of importance to understand why some people are genetically susceptible to develop COPD while others are not. Many genetic studies have been conducted in search for associations between genetic polymorphisms and susceptibility to COPD [12]. Although 99% of the genome has a similar structure in humans, certain base pairs may differ between subjects and these are called Single Nucleotide Polymorphisms (SNPs). While millions of these

SNPs in the human genome are known, many still have an unknown functional role. Some of these polymorphisms result in structural protein changes, altered gene expression or modifications of mRNA stability. Candidate genes studies have been so far the most common approach to identify genes that might be important for the development of COPD and/or play a role in the progression of the disease. In case of a candidate gene approach, a gene is designated for genetic research on basis of a priori knowledge of involvement in the disease, including gene and/or protein expression in patients, implication from animal models, a gene's involvement in disease-related pathways or implication of the genetics of a related phenotype [10].

Genetics of severe early onset COPD

Prior studies have shown that patients with α1-antitrypsin deficiency (AATD) develop early onset emphysema even without cigarette smoke exposure [13]. Since severe AATD is found only in 1-2% of all individuals with COPD, there is a growing interest in the genetic factors involved in severe COPD. Recently, it has been shown that patients with advanced COPD have a reduced expression of the nuclear erythroid 2 p45 related factor-2 (Nrf2) pathway in their lungs, suggesting that loss of this antioxidative protective response is a key factor in the progression of emphysema [14]. Furthermore, genetic disruption of *Nrf2* in mice causes early-onset and severe emphysema [15]. Another gene involved in severe COPD is epoxide hydrolase 1, microsomal (xenobiotic) (*EPHX1*) gene. *EPHX1* was associated with both emphysema and airway wall thickness using CT scans in a severe COPD population [16].

Genetics of common COPD (elderly-age onset COPD)

Several SNPs in the genes related to proteases, antioxidants and inflammation have been found to be related to features of COPD (Table 3).

1. *Oxidant – antioxidant genes*

An imbalance between harmful oxidants such as cigarette smoking and protective antioxidants such as antioxidant enzymes, may lead to oxidative stress that plays an important role in the pathogenesis of smoking-induced COPD. One of these antioxidants that plays a protective role in the lung is *Heme Oxygenase 1* (*HMOX1*).

Two clinical studies have identified a link between the SNPs in *HMOX1* and COPD. In a case-control study it has been indicated that the patients with 30 or more GT repeats in the microsatellite region were more likely to have emphysema as diagnosed by CT scans [19]. Another study observed that the presence of ≥ 33 GT repeats was associated with airflow obstruction and an accelerated lung function decline in smokers in the general population [17]. Moreover, alveolar macrophages of COPD patients contain less HMOX1 protein as compared to controls [18].
The expression of HMOX1 is regulated by the transcription factor NFE2L2 (Nuclear Factor (erythroid-derived 2)-like 2). Disruption of the NFE2L2 complex with its cytosolic repressor Kelch-like ECH-Associated Protein 1 (KEAP1) is caused by oxidative stress [21]. The expression of NFE2L2 and KEAP1 was found to be decreased in COPD patients as compared to healthy never- or ex-smokers [21, 22]. In a general population the SNPs in *NFE2L2* and *KEAP1* were associated with a lower and higher level of FEV_1 respectively, but not with the course of FEV_1 [20].

The *glutathione-S-transferase* (*GST*) genes are encoding a family of enzymes that detoxify some of the harmful contents of tobacco smoke [44]. In the general population, it has been shown that males carrying the *GSTT1* null allele are at risk for accelerated FEV_1 decline [25]. Moreover, the null allele of *GSTM1* has been associated with emphysema [24] and chronic bronchitis [23].

One of the genes involved in the xenobiotic metabolism is *Multidrug resistance-associated protein-1* (*MRP1*) that protects against oxidative stress and toxic compounds generated by cigarette smoking.We have shown previously that MRP1 is less expressed in bronchial epithelium of COPD patients than in healthy subjects [28]. *Mrp1/Mdr1a/1b* triple knock-out mice had a poor ability for smoke-induced IL-8 production compared with wild type mice, which was associated with almost complete absence of inflammatory cell influx in the lung in response to cigarette smoke [29]. An additional study demonstrated that cigarette smoke extract inhibits MRP1 activity in bronchial epithelial cells *in vitro* [30]. In two independent population-based cohorts we observed that two SNPs in *MRP1* were significantly associated with a lower or higher level of FEV_1, two additional SNPs had a significant effect of the same, negative magnitude on the level or decline of

FEV_1 and one SNP was a significant predictor of COPD in the general population [26]. All together these data suggest that *MRP1* may play a role in COPD onset, yet its role in progression of the disease is unknown.

Table 3: Genes related to antioxidants, proteases and inflammation

Gene	*Gene name*	*References*
HMOX1	*heme oxygenase (decycling) 1*	[17-19]
NFE2L2	*nuclear factor (erythroid-derived 2)-like 2*	[20-22]
KEAP1	*kelch-like ECH-associated protein 1*	[20-22]
GSTP1	*glutathione S-transferase pi 1*	[23-25]
GSTM1	*glutathione S-transferase mu 1*	[23-25]
GSTT1	*glutathione S-transferase theta 1*	[23-25]
ABCC1	*ATP-binding cassette, sub-family C, member 1*	[26-30]
GCLC	*glutamate-cysteine ligase, catalytic subunit*	[31]
GCLM	*glutamate-cysteine ligase, modifier subunit*	[31]
SOD3	*superoxide dismutase 3, extracellular*	[32-34]
SOD2	*superoxide dismutase 2, mitochondrial*	[32-34]
MMP1	*matrix metalloprotease 1*	[35, 36]
MMP2	*matrix metalloprotease 2*	[35, 36]
MMP9	*matrix metalloprotease 9*	[35, 36]
MMP12	*matrix metalloprotease 12*	[35, 36]
TIMP1	*tissue inhibitor of matrix meralloprotease 1*	[35, 36]
ADAM33	*a disintegrin and metalloprotease domain 33*	[37, 38]
TGFβ1	*transforming growth factor, beta 1*	[39, 40]
DCN	*decorin*	[39, 40]
TLR2	*toll-like receptor 2*	[41-43]
TLR4	*toll-like receptor 4*	[41-43]

Other genes involved in oxidative stress are *glutamate-cysteine ligase* (*GCL*) genes and *superoxide dismutase* (*SODs*). A previous study provided evidence that the *GCLC* gene is associated with low level of lung function in two independent population-based cohorts and moreover the findings indicated that this is due to an interplay between the *GCLC* SNPs, smoking and low vitamin intake that together

contribute to oxidative burden [45].

Extracellular superoxide dismutase (SOD3) is present at high concentrations in the areas of the lung containing large amounts of type 1 collagen, especially around large airways and also adjacent to alveoli [32]. One SNP in *SOD3* had a higher prevalence in smokers with normal lung function than in smokers who had developed COPD [33]. In a Dutch general population-based cohort, SNPs in *SOD3* showed to have protective effects on lung function level and decline [34].

2. *Protease – anti-protease genes*

Matrix Metalloproteinases (MMP) are a large group of extracellular enzymes with proteolytic activity and MMPs play a role in the protease/anti-protease interaction [44]. It has been proposed that in smokers who develop COPD, the balance between proteases and antiproteases is disturbed with subsequent parenchymal injury. The *MMP1*, *MMP2*, *MMP9*, *MMP12* and *TIMP1* (a tissue inhibitor of matrix metalloprotease 1) genes have been found to play a role in COPD with respect to their relation to the level of lung function in two general population-based cohorts [36]. Studies using knockout mouse models support a role of MMPs in COPD [35].

A Disintegrin and Metalloprotease 33 (*ADAM33*) gene is also a gene of putative interest for COPD development. *ADAM33* was associated with lung function decline and development of COPD in a general population-based cohort [38]. Furthermore, the SNPs in *ADAM33* have been associated with the severity of both airway hyperresponsiveness and airway inflammation in sputum and bronchial biopsies of COPD patients [37].

3. *Other genes*

Decorin and TGFβ1 are regulators of the extracellular matrix and their expression is decreased and increased respectively in lung tissue of COPD patients. SNPs in *TGFβ1* were associated with COPD, but not with accelerated FEV_1 decline in the general population [40]. In a mouse model, mice that were unable to activate latent TGFβ1 developed emphysema via alterations of MMP12, suggesting that disordered activation may play a role in the pathogenesis of COPD [39, 44].

Other genes that play a role in host defence are *Toll-like receptors* (*TLRs*). The innate immune response in the airways involves the detection of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) by recognition receptors such as TLRs [46]. Since TLRs participate in the defence against viral and bacterial infections and infections in the airways amplify the disease, it might be that TLRs are of importance in COPD. Genetic studies showed a potential impact of functional single nucleotide polymorphisms (SNPs) in the *TLR2* and *TLR4* genes on COPD at the population level [41-43], but genetic studies concerning the role of *TLR2* and *TLR4* in subjects with established COPD are lacking.

Accumulating evidence suggests that an interaction between the "host" factors such as genetics, gender and race, and the "environmental" factors such as air pollution, smoking, socio-economic status, bacterial and viral infections and diet, may influence the development of COPD [47]. In addition, certain lung developmental pathways could potentially influence COPD development as can be hypothesized based on the results from animal models and humans [48]. One of the genes involved in lung developmental processes is *Hedgehog-interacting protein (HHIP)*. An increasing number of studies stress the importance of early life events in the development of obstructive lung diseases such as COPD, and *HHIP* might be such a developmental gene since COPD is associated with structural defects in small airways and inappropriate lung growth [49]. Genome wide association studies have identified variants near the *Hedgehog-interacting protein* (*HHIP)* gene to be associated with COPD and lung function [50, 51].

In addition to the genetic influences on lung function, there are also genetic determinants of cigarette smoking behaviour that may influence the development and progression of COPD. Since smoking cessation is beneficial with respect to (the course of) lung function, reduced respiratory symptoms and inflammation in COPD [52], it is important to highlight the role of genetic factors in the variation of the ability to quit smoking, ability to start smoking and even to maintain the smoking addiction. The SNPs in the *nicotinic acetylcholine receptors (nAChRs)* cluster have been identified in genome-wide association studies as a risk for COPD [53]. Beside

their association with COPD, these SNPs in the *nAChRs* have been cross-sectionally associated with nicotine dependency based on the reported number of cigarettes per day [54]. However, since smoking is a risk factor for COPD itself, it is not clear from these cross-sectional studies whether the effect of the *nAChR* variants determine COPD development directly or indirectly via smoking addiction.

The genetics of COPD has been studied both in COPD patients and healthy controls, as well as the general population. Besides the population-based cohort studies that are required to identify candidate genes involved in COPD development, new insights into the genes' pathways involved in COPD are of interest. The identification of genes implicated in the lung development or nicotine addiction puts new insight into the progression of lung function loss in COPD patients as well as in subjects with COPD from the general population. Genes' networks might additionally help understanding the pathogenesis of COPD. Potential functional variants were identified to be associated with measurement of lung function in COPD in patients and in subjects from the general population, but these variants are not covering the entire gene, thus essential information might be lost. Moreover, identifying several SNPs that do not have an effect on their own might have in impact within multiple genes networks on the disease development and progression. Studies investigating jointly multiple genes involved in the same pathways are still lacking, thought they are crucial for a complete overview of the genetics of COPD development, severity and progression.

Aims of this thesis

The main purpose of the studies described in this thesis is to identify SNPs in genes that might affect lung function level and decline in mild-to-moderate COPD patients as well as in two independent general population-based cohorts. These genes are involved in oxidative stress, inflammation, lung development, detoxification of cigarette smoke and nicotine dependency.

Study populations

In the current thesis we used data from 3 study populations. The main study population in this thesis is represented by the COPD patients who participated in the GLUCOLD study. Additionally, we used two independent general population-based Dutch cohorts, namely the Doetinchem study and the Vlagtwedde-Vlaardingen study [38, 55] (Table 4).

GLUCOLD study

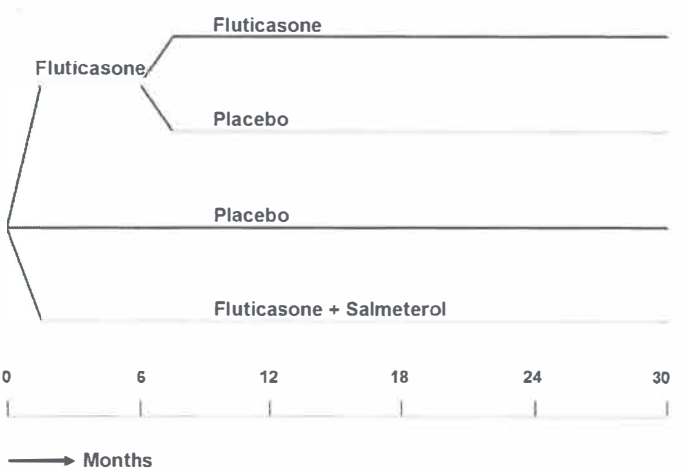
Some of the data described in this thesis involve the GLUCOLD study (Groningen Leiden Universities Corticosteroids in Obstructive Lung disease study). The GLUCOLD study is a two-center, randomised, parallel-group, double-blind, four armed, placebo-controlled study. The GLUCOLD study enrolled 114 patients with moderate to severe COPD that were randomised to either ICS monotherapy (fluticasone 500 μg BID), combination therapy with ICS and LABA (fluticasone/ salmeterol 500/50 μg BID) or placebo twice daily during 30-month treatment. After 6 months of treatment, the fluticasone group was divided in two separate groups: half of the group carried on using fluticasone and half of the group started using placebo (Figure 1).

The GLUCOLD study was designed to investigate the effect of ICS, either or not in combination with LABA in patients with COPD. The COPD patients were aged 45 to 75 years, were current or former smokers, had smoked for 10 or more pack-years, and had lung function levels compatible with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II and III [2].

Table 4: Study populations described in this thesis

Study population	Nr. of subjects	Type of data	Measurements used
GLUCOLD	114	longitudinal (2.5 years)	– lung function (FEV_1&FEV_1/VC) – small airway function ($FEF_{25-75\%}$) air trapping (RV and RV/TLC ratio) – pulmonary biomarkers (induced sputum and bronchial biopsies)
Doetinchem	1152	longitudinal (10 years)	– lung function (FEV_1&FEV_1/VC)
Vlagtwedde/ Vlaardingen	1390	longitudinal (25 years)	– lung function (FEV_1&FEV_1/VC) – changes in smoking habits

Figure 1: Design of the GLUCOLD study



Included patients had neither used a course of oral steroids during the previous 3 months, nor maintenance treatment with inhaled or oral steroids during the previous 6 months. Exclusion criteria were asthma or other diseases likely to interfere with the purpose of the study and receipt of ICS within 6 months before random assignment. The predefined primary outcome was inflammatory cell counts in bronchial

biopsies and induced sputum. Fiberoptic bronchoscopy, biopsy processing, and quantification were performed as described elsewhere [56]. Immunohistochemistry was used by using specific antibodies against T lymphocytes (CD3, CD4, and CD8), macrophages (CD68), neutrophil elastase, mast cell tryptase (AA1), eosinophils (EG2), plasma cells (CD138), and proliferating cells (Ki-67). Secondary outcomes included postbronchodilator spirometry and hyperresponsiveness to methacholine PC_{20} assessed using standardized procedures [56], dyspnea score assessed using the modified Medical Research Council (MRC), dyspnea scale (range, 1 to 5), and health status assessed using the St. George's Respiratory Questionnaire (SGRQ) and the Clinical COPD Questionnaire (CCQ). The follow up procedures involved the measurement of symptoms, health status, self-reported smoking status, medication adherence, and spirometry every 3 months. The adherence was checked by counting the doses in the inhalers. Bronchoscopy, sputum induction, and methacholine challenge were performed at baseline, at 6 and 30 months.

Doetinchem cohort study

The Doetinchem cohort study [55] was a prospective part of the larger MORGEN study [57]. The origin of the Doetinchem Cohort Study lies within the Monitoring Project on Cardiovascular Disease Risk Factors (MP-CVDRF), which was aimed at providing prevalence estimates of cardiovascular disease risk factors, such as smoking, blood pressure and serum cholesterol levels. This cohort study was followed for 10 years and biological risk factors such as blood pressure, total and HDL cholesterol, body weight, height and lung function were measured [55]. Additionally, lifestyle factors such as smoking, dietary intake, physical activity and alcohol intake were assessed [55]. A random sub-sample of 1152 subjects was selected from the total cohort with spirometry tests and DNA available (n=3224) as described elsewhere [45]. In the Doetinchem cohort study subjects were tested for pre-bronchodilator lung function (FEV_1 and FVC) three times with 5-year intervals (1994-2007) [45] according to the European Respiratory Society (ERS) guidelines. In the random sample 100%, 100% and 70.4% subjects participated in the first, second and third survey respectively.

Vlagtwedde-Vlaardingen cohort study

The Vlagtwedde-Vlaardingen cohort study is a general population-based cohort of exclusively white individuals of Dutch descent which started in 1965 and has been followed up for 25 years [38]. Surveys were performed every 3 years, in which information was collected on respiratory symptoms, spirometry, smoking status, age, and sex by the Dutch version of the British Medical Council standardized questionnaire. Blood samples, spirometry tests and DNA were available as described elsewhere [38]. The final surveys were organized in 1989 in Vlagtwedde and in 1990 in Vlaardingen.

Chapters

In **Chapter 2** and **3** Multidrug Resistance-associated Protein-1, *MRP1* (official name *ABCC1*, ABC subfamily C, member 1) is described, one of the genes that is involved in oxidative stress. Previously we investigated 5 SNPs in *MRP1* that were found to be significantly associated with the level of lung function in 2 independent general population-based cohorts. In addition functional studies on MRP1 have been performed [30]. No study focused so far on the relationship of these polymorphisms with the pathophysiology of COPD, i.e. the severity of airway obstruction and airway pathology in COPD. In **chapter 2** we investigated the relation between *MRP1* polymorphisms and the level of lung function, inflammatory markers and MRP1 protein in bronchial biopsies of individuals with established COPD (the GLUCOLD study). Subsequently, **chapter 3** describes the associations of the same *MRP1* SNPs and MRP1 protein expression in airway wall biopsies with the decline of lung function in the same population of COPD patients.

Chapter 4 focuses on genetic variations in *Toll-like receptors* genes like *TLR2* and *TLR4*. Toll-like receptors (TLRs) participate in the defence against viral and bacterial infections and since infections in the airways amplify the disease in the lungs of COPD patients, it might be that TLRs are of importance in COPD. Since so far only the common SNPs have been investigated with respect to COPD at the population level, we investigated the associations of all tagging SNPs in *TLR2* and *TLR4* with the level and decline of FEV_1 as well as with the inflammatory cells in

induced sputum.

Chapter 5 describes one of the genes originally linked to lung developmental processes, *Hedgehog-interacting protein (HHIP)* and its potential role in the general population and COPD patients. Genome wide association studies have identified variants near the *Hedgehog-interacting protein* (*HHIP)* gene to be associated with COPD and lung function. However, these studies can provide information only about the cross-sectional association of the gene with lung function level, not about the natural occurring decline in lung function. Therefore, the main purpose of our study described in **chapter 5** was to investigate the association of the SNPs in the *HHIP* region with level and course of lung function (FEV_1 and FEV_1/FVC) in the general population-based Doetinchem cohort. Additionally we questioned whether these SNPs are also associated with small airway function and lung function level and decline after COPD has developed (the GLUCOLD study). Furthermore, since previous studies suggested a protective effect of the gene in the presence of smoking, we investigated the above mentioned associations also by stratifying our data according to smoking status.

Chapter 6 focuses on other genes identified in the genome-wide association studies, *nicotinic acetylcholine receptors (nAChRs)*. SNPs in the *nicotinic acetylcholine receptors (nAChRs)* cluster were found as a risk for nicotine dependency and COPD. *Nicotinic acetylcholine receptor (nAChR)* is a controversial gene with respect to its association with COPD and smoking addition and it is not clear from the cross-sectional studies performed so far whether these SNPs in the *nAChR* cluster are directly and independently a risk for COPD development. Therefore, we investigated whether the SNPs in the *nAChR* cluster are associated with smoking habits and lung function decline, and if these potential associations are independent of each other in the general population-based Vlagtwedde-Vlaardingen cohort study.

Chapter 7 was set out to investigate gene pathways identification that could be useful for understanding the mechanisms (i.e. lung destruction and repair, protease-antiprotease and oxidant-antioxidant imbalance) of a complex disease like COPD. To this purpose we used 2 different methods to assess which of these genes that

are linked to COPD belong to the same pathway or cluster together, i.e. DAVID (the Database for Annotation, Visualization and Integration Discovery) and factor analysis respectively. The first part of the study focused on exploration via DAVID of 26 genes previously shown to be significantly associated with lung function level and decline and COPD in the general population-based Vlagtwedde-Vlaardingen cohort. In the second part of the study we investigated how the SNPs in these candidate genes were categorized together by factor analysis in subjects with COPD.

Chapter 8 concludes with a summary, conclusions of the studies described in this thesis and future perspective.

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CHAPTER 2

Multidrug resistance-associated protein-1 (MRP1) genetic variants, MRP1 protein levels and severity of COPD

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Abstract

Multidrug resistance-associated protein-1 (MRP1) protects against oxidative stress and toxic compounds generated by cigarette smoking, which is the main risk factor for chronic obstructive pulmonary disease (COPD). We have previously shown that single nucleotide polymorphisms (SNPs) in *MRP1* significantly associate with level of FEV_1 in two independent population based cohorts. The aim of our study was to assess the associations of *MRP1* SNPs with FEV_1 level, MRP1 protein levels and inflammatory markers in bronchial biopsies and sputum of COPD patients.

Five SNPs (rs212093, rs4148382, rs504348, rs4781699, rs35621) in *MRP1* were genotyped in 110 COPD patients. The effects of *MRP1* SNPs were analyzed using linear regression models.

One SNP, rs212093 was significantly associated with a higher FEV_1 level and less airway wall inflammation. Another SNP, rs4148382 was significantly associated with a lower FEV_1 level, higher number of inflammatory cells in induced sputum and with a higher MRP1 protein level in bronchial biopsies.

This is the first study linking *MRP1* SNPs with lung function and inflammatory markers in COPD patients, suggesting a role of *MRP1* SNPs in the severity of COPD in addition to their association with MRP1 protein level in bronchial biopsies.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease associated with an influx of neutrophils, macrophages and $CD8^+$ T-lymphocytes in the airways and lung tissue.[1] Smoking generates oxidative stress resulting from an oxidant – antioxidant imbalance, and oxidative stress markers are increased in airspaces, blood and urine of smokers and COPD patients.[2] Oxidative stress can be reduced by members of the ATP-binding cassette (ABC) superfamily of transporters. One such a transporter is multidrug resistance-associated protein-1, MRP1, (official name ABCC1, ABC subfamily C, member 1) that plays an important role in normal lung physiology by protecting against toxic xenobiotics and endogenous metabolites.[3]

MRP1 was first detected in small cell lung cancer. It has been shown to be highly expressed in the normal human lung [4,5] and particularly at the basolateral side of human bronchial epithelial cells.[6] Interestingly, we have previously shown that MRP1 is less expressed in bronchial epithelium of COPD patients compared to healthy subjects.[7] *Mrp1/Mdr1a/1b* triple knock-out mice had a poor ability for smoke-induced IL-8 production compared with wild type mice, which associated with almost complete absence of inflammatory cells in response to cigarette smoke. [8] An additional study demonstrated that cigarette smoke extract inhibits MRP1 activity in bronchial epithelial cells in vitro.[9] Thus there is a clear role for MRP1 in COPD.

A total of 51 single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) > 5% are required to tag the entire *MRP1* gene in Caucasians.[10] We have shown that two SNPs in *MRP1* significantly associate with a lower or higher level of FEV_1 in two independent population-based cohorts. Two additional SNPs had a significant effect of the same, negative magnitude on the level or decline of FEV_1. One SNP was a significant predictor of COPD in the general population. [11]

So far, no study has focused on the relation between *MRP1* polymorphisms and the level of lung function, inflammatory markers and MRP1 protein in lung tissue of

individuals with established COPD. We had the unique opportunity to do so in a recently finished, two center trial in COPD that amongst others studies inflammatory markers in bronchial biopsies and induced sputum.[12] Furthermore, we assessed whether MRP1 protein levels in bronchial biopsies of COPD patients are associated with *MRP1* SNPs.

Methods

Study populations

COPD patients

We included 114 patients with COPD who participated in a two-center trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study). Patient characteristics and methods have been described in detail previously.[12] In brief, all patients had irreversible airflow limitation and chronic respiratory symptoms.[13] Included patients had neither used a course of oral steroids during the previous 3 months, nor maintenance treatment with inhaled or oral steroids during the previous 6 months. They were current or ex-smokers with a smoking history of ≥10 packyears, aged between 45 and 75 years without a history of asthma. The study was approved by the medical ethics committees of the University Medical Centers of Leiden and Groningen. All patients gave their written informed consent.

Controls

To verify the differences of MRP1 levels in bronchial biopsies between COPD patients and healthy subjects, we included 37 subjects as controls, of which 28 were previously recruited in order to participate in a smoking cessation program. [14] They were symptomatic and asymptomatic smokers according to the ATS-ERS (American Thoracic Society-European Respiratory Society) guidelines [15] and met the following criteria: 45-75 years of age, >10 pack years of smoking, FEV_1/FVC pre and post bronchodilator > 70%, no use of inhaled or oral corticosteroids in the previous 6 months, no sign of atopy, no respiratory tract infections one month prior to the study and none of the participants had any co-morbidity.[14] The remaining

9 subjects were included as controls with an FEV_1/FVC pre and post bronchodilator > 70% and FEV_1 >80% predicted.

We used an additional control group from the general population-based cohort (Doetinchem) [16] to check for the differences in genotype distributions between COPD patients and general population (data supplement).

Clinical characteristics

Lung function and reversibility to salbutamol were measured as described previously for COPD patients [12] and for controls.[14]

Sputum induction and processing were performed as described previously [12] according to a validated technique.[17] Details on biopsy processing, immunohistology and analysis have been published previously.[18] In brief, we collected the two best morphological 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) (data supplement).

Selection of the *MRP1* tagging SNPs and genotyping

We selected SNPs based on our previous results showing a significant association of 5 *MRP1* SNPs (rs212093, rs4148382, rs35621, rs4781699 and rs504348) with the FEV_1 level and/or annual FEV_1 change in two independent population–based cohorts. [11] The rs504348 SNP results in a significant increase in *MRP1* promoter activity in vitro.[19] Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar).

Biopsies and immunohistochemistry on bronchial biopsies from COPD patients and controls

Details on bronchial biopsy collection and processing are described in the data supplement. Four paraffin-embedded biopsies were cut in 4µm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the best morphological biopsy per subject for analysis (without crush artefacts, large blood clots, or only epithelial scrapings). The staining was performed on one paraffin

section of 4µm per subject with monoclonal antibody MRPr1 for MRP1 (Santa Cruz, California, USA). Details on immunohistochemical staining are described in the data supplement.

Evaluation of immunohistochemistry on bronchial biopsies from COPD patients and controls

Evaluation of different types of epithelium was performed separately (i.e. basal epithelium, squamous metaplasia, intact epithelium). For the current study, intact bronchial epithelium was selected for analysis.

MRP1 protein was scored for staining intensity in intact epithelium of bronchial biopsies with a semi quantitative score: 0=no staining; 1=weak; 2=moderate; 3=strong. MRP1 intensity scores for intact epithelium were available from 80 bronchial biopsies of subjects with COPD and 26 bronchial biopsies of controls. Due to the fact that there were only 3 individuals with no immunohistochemical expression of MRP1, the MRP1 intensity was categorised in 3 groups: 1=weak staining, 2=moderate staining and 3=strong staining. Two observers (S.B. and W.T.) performed all evaluations of bronchial biopsies individually, in a blinded manner. Most sections stained variable for MRP1 in epithelium and parts with the most intense staining were evaluated for scoring.

Statistics

Numbers of inflammatory cells in bronchial biopsies and induced sputum were log transformed to achieve a normal distribution. Linear regression analyses were performed to investigate the association of *MRP1* SNPs with FEV_1 level and inflammatory cells (natural logarithm) in bronchial biopsies and induced sputum. Independent variables included in the model were age, gender, height, packyears and genotypes. To assess the effect of SNPs on FEV_1 level and cell numbers in bronchial biopsies and induced sputum we used the following genetic models:

- General: heterozygote and homozygote variants coded separately as dummy variables and compared to the homozygote wild type
- Dominant: heterozygote and homozygote variants pooled and compared to the homozygote wild type

Differences in MRP1 staining intensity between biopsies of COPD patients and

controls and according to *MRP1* SNPs were analyzed using Chi-square tests. Analyses were performed using SPSS version 16.0 for Windows and values of $p<0.05$ (tested 2-sided) were considered statistically significant.

Results

The clinical characteristics of COPD patients and controls are presented in table 1. DNA was available from 110 out of 114 COPD patients and from 37 controls. All 5 *MRP1* SNPs were in Hardy Weinberg Equilibrium (HWE, $p>0.05$) and were not highly correlated with each other (the highest r^2 in our population is 0.34) (See figure S1 in the data supplement). There were no significant differences in genotype distributions between the COPD patients and the general population-based control cohort (data supplement). Likewise, there were no significant differences in genotype distributions between the COPD patients and controls (Table 2).
Table 3 shows the number and the percentage of inflammatory cells in bronchial biopsies and induced sputum from the COPD patients.

Table 1: Clinical characteristics of COPD patients and controls with airway biopsy available

	COPD patients (n= 114)	Controls (n=37)
Males, n (%)	99 (86.8)	16 (43.2)
Age (years)	61.6 (7.7)	52.3 (5.5)
Height (cm)	175.5 (7.8)	172.8 (10)
Packyears¶	41.8 (31.2 – 54.7)	25.4 (20.2-35.0)
Current smoker, n (%)	72 (63.2)	30 (81.1)
FEV_1/FVC (%)	49.5 (8.8)	77.2 (6.1)
FEV_1 (L)	1.8 (0.4)	3.2 (0.8)
FEV_1 % pred.*	56 (10)	100 (14)
MRP1 level #, n	80	26

*Data are presented as mean ± standard deviation or ¶ median (25^{th} – 75^{th} percentile); FEV_1 = forced expiratory volume in one second; FEV_1/FVC = FEV_1/forced vital capacity; * % pred. = percentage of predicted value; # refers to the number of individuals having bronchial biopsies with available MRP1 levels of intensity; MRP1 = multidrug resistance-associated protein-1*

Table 2: Prevalence of *MRP1* SNPs in COPD patients and controls

		COPD patients n=110 (%)	Controls n=37 (%)	p value
rs212093	AA	37 (33.9)	8 (25.0)	
	AG	50 (45.9)	18 (56.2)	0.55
	GG	22 (20,2)	6 (18.8)	
rs4148382	GG	83 (76.1)	29 (82.8)	
	GA	26 (23.9)	5 (14.3)	0.12
	AA	-	1 (2.9)	
rs504348	CC	78 (72.2)	22 (71.0)	
	CG	27 (25.0)	6 (19.3)	0.23
	GG	3 (2.8)	3 (9.7)	
rs4781699	GG	58 (52.7)	21 (61.8)	
	GT	45 (40.9)	10 (29.4)	0.47
	TT	7 (6.4)	3 (8.8)	
rs35621	CC	89 (80.9)	30 (85.7)	
	CT	20 (18.2)	5 (14.3)	0.74
	TT	1 (0.9)	-	

Different numbers for the SNP genotypes are due to missing genotype data; SNP = single nucleotide polymorphism; MRP1 = multidrug resistance-associated protein-1.

Table 3: The number of inflammatory cells in bronchial biopsies and induced sputum of COPD patients

Bronchial biopsies	Absolute numbers per 0.1 mm^2 sub-epithelial area
CD3	123.5 (69.2 – 182.5)
CD4	48.0 (27.7 – 72.0)
CD8	21.5 (11.0 – 37.2)
Plasma cells	8.5 (3.5 – 14.5)
Mast cells	26.5 (19.0 – 34.5)
Macrophages	8.5 (4.5 – 13.0)
Neutrophils	4.0 (2.0 – 8.4)
Eosinophils	1.5 (0.5 – 4.2)

Induced sputum	Absolute numbers (10^4/ml)	Percentage (%)
Total cell count*	139.7 (77.9 – 311.3)	
Neutrophils	101.6 (46.8 – 228.5)	72.8 (59.9 – 81.7)
Macrophages	31.1 (17.9 – 61.1)	22.1 (14.8 – 33.2)
Eosinophils	1.3 (0.4 – 4.5)	1.1 (0.3 – 2.2)
Lymphocytes	2.2 (1.1 – 6.8)	1.7 (1.2 – 2.3)
Epithelial cells	1.4 (0.6 – 3.4)	1.0 (0.3 – 2.3)

*Data are presented as median (25^{th} – 75^{th} percentile; *Total cell count refers to the number of non-squamous cells in induced sputum.*

MRP1 SNPs and FEV_1 level in COPD patients

In a general model, individuals who were homozygote mutant (GG) for rs212093 had a significantly higher FEV_1 than wild type (AA) individuals, as reflected by a regression coefficient B value (95% CI, confidence interval) of 222 ml (48 ml to 396 ml); p=0.013. Heterozygote (GA) individuals for rs4148382 had a significantly lower FEV_1 than wild type (GG) individuals (-215 ml (-356 ml to -75 ml); p=0.003). None of the other 3 SNPs (rs504348, rs4781699 and rs35621) was significantly associated with the FEV_1 level (Figure 1). Additional adjustment for current smoking status did not change the size or significance of the effect estimates of the genotypes on level of FEV_1.

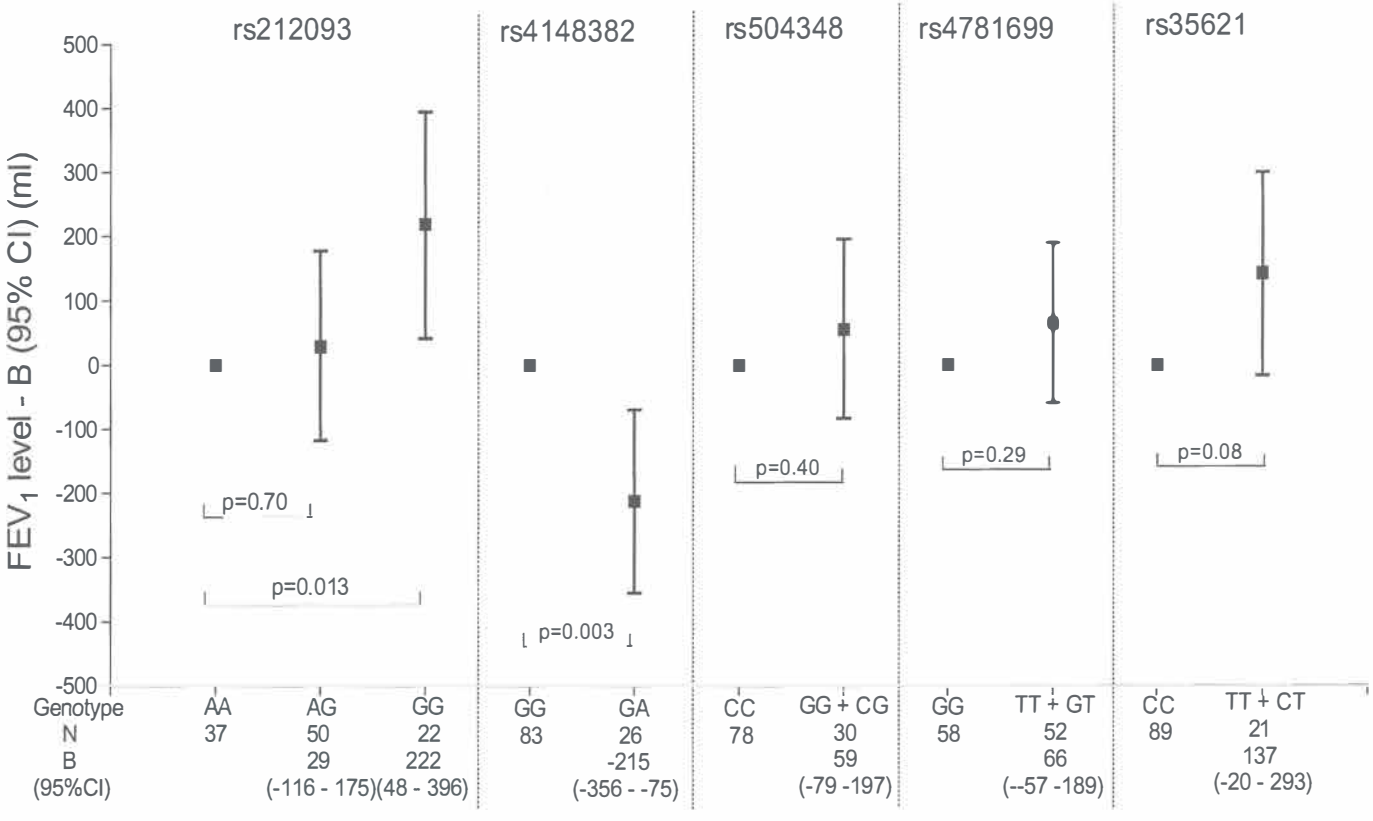
MRP1 SNPs and inflammatory cells in bronchial biopsies in COPD patients

Homozygote mutant (GG) individuals for rs212093 had a significantly lower number of plasma cells (-0.72 (-1.27 to -0.18); p=0.01), neutrophils (-0.63 (-1.16 to -0.09); p=0.02) and macrophages (-0.61(-1.07 to -0.15); p=0.01) in bronchial biopsies than wild type (AA) individuals (Figures 2a, 2b and 2c, respectively). Individuals who were heterozygote (AG) for rs212093 had lower numbers of mast cells than wild type (AA) individuals (-0.25 (-0.47 to -0.03); p=0.02) (Figure 2d).

Minor allele carriers (GT/TT) for rs4781699 had significantly lower numbers of macrophages (-0.34 (-0.67 to -0.02); p=0.04) than wild type (GG) individuals (Figure 3).

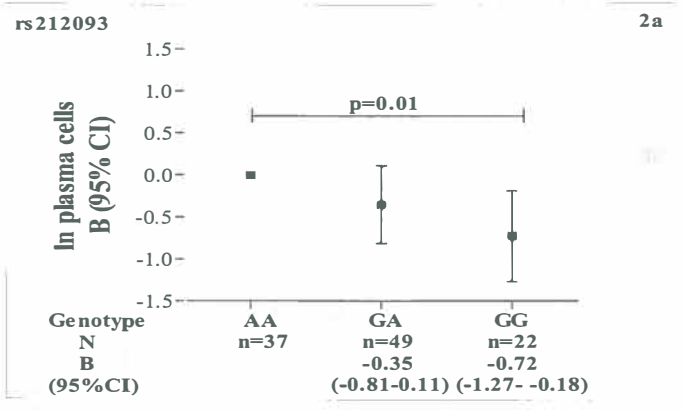
The genotypes for the other two SNPs (rs4148382 and rs35621) were not significantly associated with any of the inflammatory cells in the bronchial biopsies.

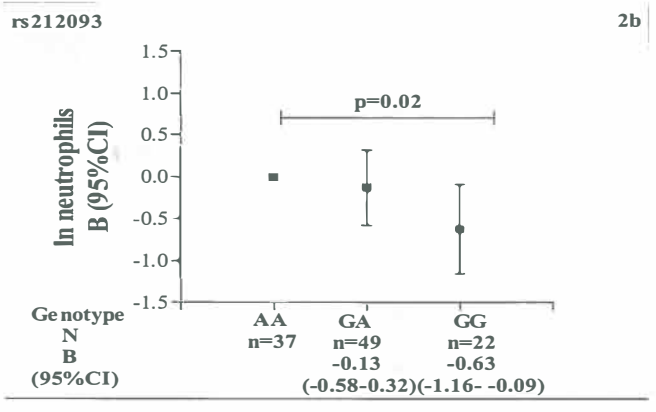
Figure 1: Estimated effects of *MRP1* genotypes on level of FEV_1 in COPD patients

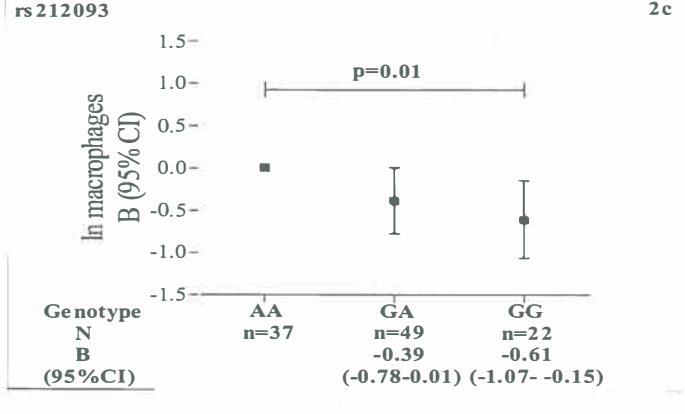


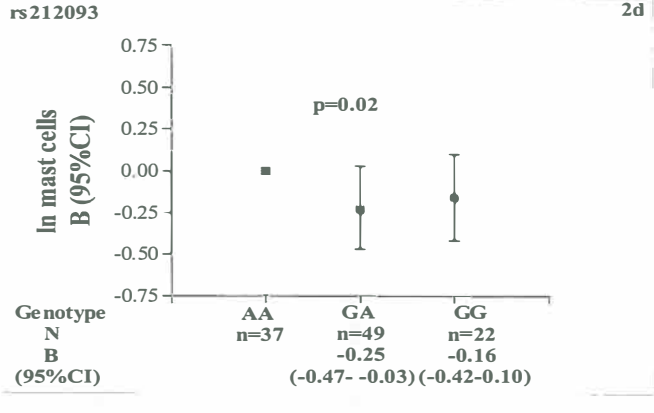
FEV_1 = forced expiratory volume in one second; N= Number of individuals. Squares represent the regression coefficient (B) and vertical bars represent 95% confidence interval (CI); Wild type was set to zero as the reference category. The analyses are adjusted for age, gender, height and packyears.

Figure 2: Estimated effects of *MRP1* genotypes on inflammatory cells in bronchial biopsies of COPD patients



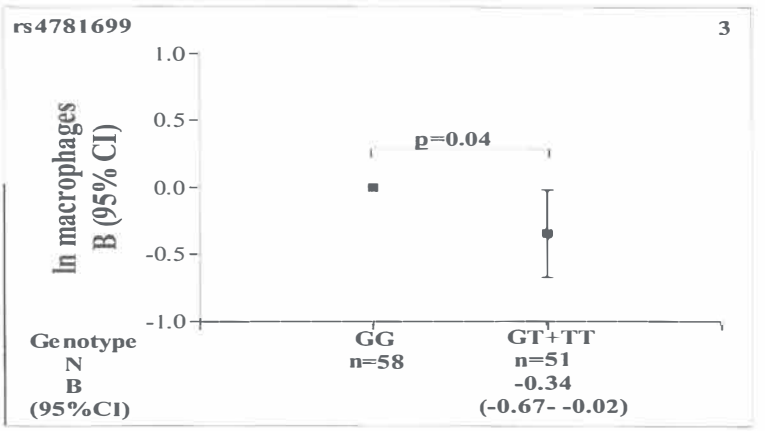






2a: Number of plasma cells according to rs212093 genotype; 2b: Number of neutrophils according to rs212093 genotype; 2c: Number of mast cells according to rs212093 genotype; 2d: Number of macrophages according to for rs212093 genotype. N = number of individuals. Data are presented as natural logarithm of each type of cells in bronchial biopsies. Different numbers for the SNP genotypes are due to missing data on genotype or inflammatory cells. Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI). Wild type was set to zero as the reference category. The analyses are adjusted for age, gender and packyears

Figure 3: Estimated effects of *MRP1* genotypes on inflammatory cells in bronchial biopsies of COPD patients



Number of macrophages according to rs4781699 genotype; N = number of individuals. Data are presented as natural logarithm of macrophages in bronchial biopsies. Different numbers for the SNP genotypes are due to missing data on genotype or inflammatory cells. Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI). Wild type was set to zero as the reference category. The analyses are adjusted for age, gender and packyears

MRP1 SNPs and inflammatory cells in sputum in COPD patients

Heterozygote (GA) individuals for rs4148382 had a significantly higher total cell count (0.59 (0.11 to 1.07) p=0.01) and neutrophils (0.61 (0.06 to 1.16); p=0.03) in sputum compared to wild type (GG) individuals. None of the other SNPs was significantly associated with inflammatory cells in sputum.

Additional adjustment for current smoking status did not change the size or

significance of the effect estimates of the genotypes on inflammatory cells in bronchial biopsies and in induced sputum.
Detailed data on the *MRP1* genotypes and inflammatory cells in bronchial biopsies and induced sputum are presented in the data supplement.

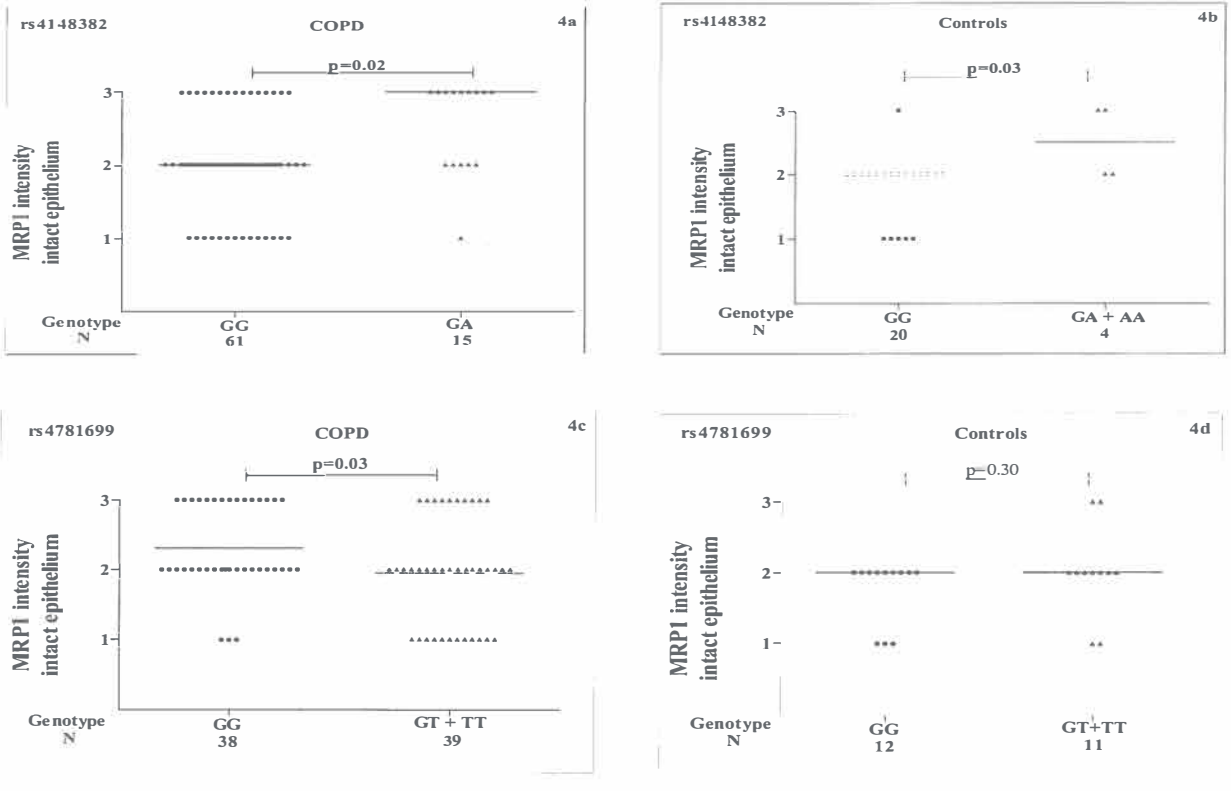
MRP1 protein levels in COPD patients and controls

There were no significant differences in MRP1 protein levels between COPD patients and controls.

Heterozygote (GA) individuals for rs4148382 had a significantly higher MRP1 protein level than wild type (GG) individuals in COPD patients (p=0.026) (Figure 4a) and in the control group minor allele carriers (GA/AA) for rs4148382 had a significantly higher MRP1 protein level than wild type (GG) individuals (p=0.037) (Figure 4b).

Minor allele carriers (GT/TT) for rs4781699 had significantly lower MRP1 protein level than wild type (GG) individuals in COPD patients (p=0.036) (Figure 4c), but there was no significant difference in MRP1 protein level in the control group (Figure 4d).
None of the other 3 SNPs (rs212093, rs504348 and rs35621) associated significantly with MRPl protein levels. Levels of MRP1 were not related to lung function parameters, inflammatory cells in bronchial biopsies or number of packyears.

Figure 4: *MRP1* SNPs and MRP1 protein levels of COPD patients and controls



4a: MRP1 protein levels according to rs4148382 genotype in COPD patients; 4b: MRP1 protein levels according to rs4148382 genotype in controls; 4c: MRP1 protein levels according to rs4781699 genotype in COPD patients; 4d: MRP1 protein levels according to rs4781699 genotype in controls; N= number of individuals

Discussion

This is the first study linking *MRP1* SNPs with the severity of COPD and additionally with the intensity of MRP1 staining in bronchial biopsies. Our results suggest a role of *MRP1* in COPD severity, as indicated by the associations of rs212093 genotypes with a higher level of FEV_1 and less inflammatory cells in bronchial biopsies. Additionally, the SNPs rs504348 and rs4781699 were associated with less airway wall inflammation and rs4148382 with a lower FEV_1 level and increased sputum cell numbers. Moreover, the before mentioned SNPs rs4148382 and rs4781699 were associated with respectively higher and lower levels of MRP1 protein in bronchial biopsies of COPD patients (see summary of the results in Figure 5).

Figure 5: Summary of *MRP1* SNPs` associations for COPD patients

	FEV_1 level	Inflammatory cells		MRP1 protein level
		Bronchial biopsies	Induced sputum	
rs212093	↑	↓	–	–
rs4148382	↓	–	↑	↑
rs504348	–	–	–	–
rs4781699	–	↓	–	↓
rs35621	–	–	–	–

FEV_1 = forced expiratory volume in one second; MRP1 = multidrug resistance-associated protein-1; ↑ = positive association; ↓ = negative association; – = no association.

Since first described in 1992 [4], a fair amount of data on the structure, substrate, function, and regulation of this transporter has been gathered. MRP1 is a member of the human ATP-binding cassette superfamily of transporters which regulates the traffic of molecules across cell membranes. The MRP1 pump confers resistance to several chemotherapeutic agents including vincristine, daunorubicin and methotrexate.[20,21] In addition to protecting cells within the body against drugs, environmental toxins and heavy metals, MRP1 has been suggested to be involved in the cellular oxidative defence system and inflammation [22,23], both being important in COPD development and progression.

We showed that the *MRP1* polymorphism rs212093 was significantly associated with a higher FEV_1 level. In line with this, rs212093 SNP was associated with lower numbers of plasma cells, macrophages, neutrophils and mast cells in bronchial biopsies, cells implicated in COPD previously. Increased numbers of neutrophils have been reported in bronchial biopsies of smokers with airflow limitation, an increase that was associated with a lower FEV_1.[24] Neutrophils and macrophages release proteolytic enzymes and generate oxidants, which cause tissue damage as well as cytokines and chemokines that can potentiate inflammation and trigger an immune response. We previously reported a larger number of B lymphocytes in bronchial biopsies of patients with COPD than in controls without airflow limitation. [25] Furthermore, epithelial cells of smokers with COPD contain higher macrophage and mast cell numbers than smokers without COPD.[26] In a triple knock-out mouse model, we previously demonstrated that the inflammatory response to inhalation of cigarette smoke is reduced when MRP1 is absent.[8] Linking previously reported increased airway wall inflammation in COPD with genetic variants of *MRP1* we found rs212093 to be associated with lower numbers of inflammatory cells in bronchial biopsies, therefore, this SNP might play a protective role in COPD. This SNP located in 3'region is known to be in complete linkage disequilibrium with rs129081 located in the 3' untranslated region [10] and therefore this polymorphism might be involved in the regulation of MRP1 mRNA stability.[11]

One could raise the issue of multiple testing and that we should have adjusted for this in our analyses, but we feel that applying a sequential (classical) Bonferroni correction is not appropriate in the current dataset for a number of reasons.[27] Firstly, our choice for the current study was explicitly driven by our previous findings, suggesting that there might be associations between *MRP1* SNPs and COPD severity. Thus, we explicitly hypothesized on the main outcome variables on forehand. Secondly, a Bonferroni correction would not take into account the potential clustering of outcome variables, which might occur jointly at high or low levels, e.g. a Pearson's correlation coefficient $r = 0.79$ for macrophages and lymphocytes in induced sputum, or are defined as each others ratios.[27] This suggests one might preferentially test a cluster of outcome variables as "one outcome variable" rather than test all variables separately.

It has been shown previously that higher neutrophil percentages in induced sputum correlate with lower FEV_1 levels [28], therefore it is of interest that rs4148382, located in 3'region of *MRP1*, is associated significantly with higher total cells counts and neutrophils in induced sputum and lower FEV_1 level. The association with total cell counts might be driven by the neutrophils which represent 72% of the total cells in induced sputum. The functional consequence of this particular SNP is not known so far and it is not known whether any functional polymorphism is in linkage disequilibrium with it. This polymorphism is located closely to the 5'end of the *MRP6* which maps also on chromosome 16. However, MRP6 mRNA is moderately present in human lung extracts [29] and highly expressed in the liver and kidney [6], which might suggest indeed that the effect of this particular SNP is within *MRP1* and not *MRP6*. How this SNP functionally contributes to COPD severity has to be further unravelled in future studies.

The observed effects in the current study appear to be opposite to previous findings in the same general population as described by Siedlinski et al.[11] In the current study, which extends the previous findings, we observed that rs4148382 associated with a lower FEV_1 level in COPD patients, whereas in the general population from the Doetinchem study rs4148382 associated with a higher FEV_1 level. [11] With respect to these findings it is worth mentioning that the present study was not designed to compare the direction or magnitude of effect estimates between the COPD patients and general population with respect to FEV_1 and genetic factors. The opposite effects are likely due to the fact that we selected a COPD subset of the Doetinchem general population for the current study by matching on the clinical characteristics age, number of packyears and $FEV_1/FVC<70\%$. Although both groups had almost the same number of packyears (median 25^{th} – 75^{th} percentile) (40 (34.1 – 48.7) vs. 41.8 (31.2 -54.7)), the matched COPD subset in the general population had a higher lung function (mean FEV_1 % predicted (SD) =79.7 (13.4)) than our current COPD patients (49.5 (8.8)). This suggests that the COPD subset of subjects from the Doetinchem study who, fulfilled the GOLD criteria of COPD, might be less susceptible to cigarette smoke and COPD development. Therefore, the patients included in the current study with established COPD were probably not comparable with the heavy smokers from the general population based control

cohort (Doetinchem).

Additionally, we have calculated the haplotypes of *MRP1* and assessed the effects of these haplotypes on FEV_1 level and inflammatory cells in bronchial biopsies and induced sputum. We observed that the effects of *MRP1* haplotypes are due to the specific SNP constituting these haplotypes, and therefore didn't add new information. Details on the *MRP1* haplotypes are presented in the data supplement.

Decreased or increased functional MRP1 expression may have a high impact on development and/or progression of lung diseases and protection against air pollution and inhaled toxic compounds such as present in cigarette smoke.[6,7,30] One of our earlier studies showed that the MRP1 intensity in bronchial biopsies of COPD patients was lower compared to healthy individuals.[7] How can we reconcile this with our current findings of MRP1 staining in COPD patients and controls? One option is that this might be due to differences in staining between paraffin and frozen biopsies.[31] More important, it might be due to underlying differences of *MRP1* genotyping distribution in the two populations. It appeared that the previous low intensity of MRP1 staining was driven by wild type individuals [7] and if we would have known this at that time, it might have had a different impact on the interpretation of the results. MRP1 is an essential pump for glutathione (GSH) – conjugates such as the inflammatory mediator leukotriene C4 (LTC4) as well as substrates in the presence of GSH (i.e.glutathione disulphide, GSSG) [32], thereby decreasing intracellular concentrations of toxic compounds. Given the rarity of homozygote mutant (AA) individuals for rs4148382 all the conclusions about this SNP are drawn based on the heterozygote (GA) individuals in COPD patients. It might be that in particular individuals who are heterozygote for rs4148382 SNP can have a locally high MRP1 protein level which therefore might lead to more severe inflammation at that site. Clearly, further research needs to investigate this approach in a larger sample of subjects with or without COPD.

Conclusions

In conclusion, our study is the first to demonstrate that *MRP1* plays a role in COPD severity, given the association of polymorphisms in *MRP1* with airway

wall inflammation, the level of lung function and moreover MRP1 protein levels in subjects with established COPD. This is an important step forward linking *MRP1* polymorphisms with the pathophysiology of COPD.

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Supplementary methods

Population-based control cohort (Doetinchem)

A population based control group was included in the current study to check for differences in genotype distribution between COPD patients and the general population based. We selected 262 subjects as controls from this general population-based control Doetinchem cohort [1] that were comparable with the COPD patients with regard to their clinical characteristics (age between 45 and 75 years, smoking >10 pack years, FEV_1/FVC>70%).

Bronchoscopy and biopsy processing of COPD patients and control group

Fiberoptic bronchoscopy, biopsy processing, immunohistology and analysis were performed as described previously. [2] In brief, as previously described, paraffin embedded biopsies were cut in 4μm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the two morphological best biopsies per patient for analysis (without crushing artefacts, large blood clots, or only epithelial scrapings). [2] Specific antibodies were used against T lymphocytes (CD3 (DAKO, Glostrup, Denmark), CD4 (Novocastra, UK) and CD8 (DAKO, Glostrup, Denmark)), macrophages (CD68 (DAKO, Glostrup, Denmark)), neutrophil elastase (NE (DAKO, Glostrup, Denmark)), mast cell tryptase (AA1 (DAKO, Glostrup, Denmark)) and eosinophils (EG2 (Pharmacia Diagnostics, Uppsala, Sweden)). [2] Bronchoscopy, biopsy collection and processing were performed for the controls in a similar way as for the COPD group and were previously described in detail.[3]

Immunohistochemistry on bronchial biopsies from COPD patients and controls

The immunohistochemical staining was performed on one paraffin section of 4μm per subject with monoclonal antibody MRPr1 for MRP1 purchased from Santa Cruz Biotechnology, Inc. Initially, the tissue slides were deparaffinised with xylene (10 min.) and rehydrated in graded alcohols before staining. Slides were pre-treated with 1mM EDTA buffer in the microwave for 15 minutes. The staining was performed automatically using the Dako Autostainer (DAKO, Copenhagen, Denmark). Pre-incubation with peroxidise blocking reagent (DAKO, Copenhagen, Denmark) was used for blocking endogenous peroxidase. First antibody used

was monoclonal antibody MRPr1 for MRP1 with a dilution of 1:50, the second antibody used was polyclonal antibody GaR^{po} (goat anti-rabbit peroxidase, DAKO, Copenhagen, Denmark) with a dilution of 1:100 and the third antibody used was polyclonal antibody RaG^{po} (rabbit anti-goat peroxidase, DAKO, Copenhagen, Denmark) with a dilution of 1:100. Nova Red (SK4800, Vector, USA) was used as a substrate giving a reddish-brown reaction product. Counterstaining was performed using Mayer's haematoxylin. A negative control was obtained by omission of the primary antibody.

Statistics

Differences in genotypes distribution between COPD patients and population-based controls were tested using Chi-square tests.

***MRP1* haplotypes for COPD patients**

We calculated the haplotypes of *MRP1* and additionally, we investigated the effects of each of the *MRP1* haplotypes on FEV_1 level, inflammatory cells in bronchial biopsies and induced sputum. The most frequent haplotype e.g. 00000, was set as a reference ('wild type haplotype'). For these analyses we used HAPSTAT version 3.0.

Supplementary results

5 *MRP1* SNPs were in Hardy Weinberg Equilibrium (HWE, $p>0.05$) and were not highly correlated with each other ($r^2 <0.8$). (Figure S1)

The clinical characteristics of COPD patients and general population-based controls (Doetinchem) are presented in table S1.

Figure S1: Linkage disequilibrium plot and correlation coefficients (r^2) for 5 *MRP1* polymorphisms genotyped in COPD patients (n=110)

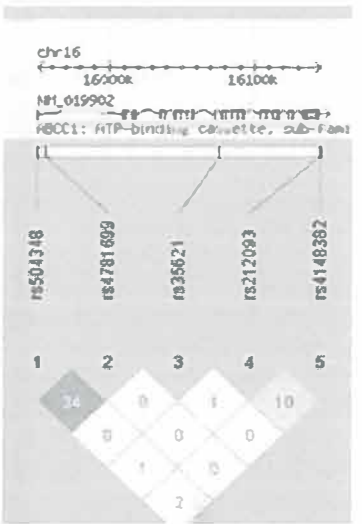

Table S1: Clinical characteristics of COPD patients and general population

	COPD patients (n= 114)	General population (n=262)
Males, n (%)	99 (86.8)	147 (56.1)
Age (years)	61.6 ± 7.7	54.9 ± 6.7
Height (cm)	175.5 ± 7.8	173.3 ± 8.5
Pack-years¶	41.8 (31.2 – 54.7)	23.5 (15.9 – 32.0)
Current smoker, n (%)	72 (63.2)	115 (43.9)
FEV_1/FVC (%)	49.5 ± 8.8	78.6 ± 4.8
FEV_1 (L)	1.8 ± 0.4	3.3 ± 0.7
FEV_1 % pred.*	56 ± 10	105 ± 14

*FEV_1 = forced expiratory volume in one second; FEV_1/FVC = forced expiratory volume in one second/forced vital capacity; * % pred. = percentage of predicted value.*

Table S2: Prevalence of *MRP1* SNPs in COPD patients and general population

SNP		COPD patients n=110 (%)	General population n=262 (%)	p value
rs212093	AA	37 (33.9)	81 (31.4)	0.85
	GA	50 (45.9)	126 (48.8)	
	GG	22 (20.2)	51 (19.8)	
rs4148382	GG	83 (76.1)	213 (82.2)	0.21
	GA	26 (23.9)	44 (17.0)	
	AA	-	2 (0.8)	
rs504348	CC	78 (72.2)	167 (64.2)	0.32
	CG	27 (25.0)	82 (31.6)	
	GG	3 (2.8)	11 (4.2)	
rs4781699	GG	58 (52.7)	128 (49.6)	0.51
	GT	45 (40.9)	104 (40.3)	
	TT	7 (6.4)	26 (10.1)	
rs35621	CC	89 (80.9)	203 (79.3)	0.92
	CT	20 (18.2)	50 (19.5)	
	TT	1 (0.9)	3 (1.2)	

SNP = single nucleotide polymorphism; MRP1 = multidrug resistance-associated protein-1.

We determined the haplotypes of *MRP1* and table S6 shows the frequencies of *MRP1* haplotypes for each of the outcomes e.g. FEV_1 level, inflammatory cells in bronchial biopsies and induced sputum. Compared to the wild type haplotype (00000), none of the other *MRP1* haplotypes had a significant effect on FEV_1 level. The observed effects of *MRP1* haplotypes were in line with our main findings on the effects of *MRP1* SNPs on inflammatory cells in bronchial biopsies and induced sputum: The 01000 haplotype was significantly associated with a higher number of total cells in induced sputum (B (95%CI) =0.59 (0.08 -1.10); p=0.02) as compared to the 00000 haplotype.

Table S3a: *MRP1* SNPs and inflammatory cells in bronchial biopsies of COPD patients

SNP	Model	Ln($CD3^{+}$)	Ln($CD4^{+}$)	Ln($CD8^{+}$)	Ln(plasma cells)
rs212093	a	-0.11 (-0.39-0.17)	-0.14 (-0.48-0.20)	-0.19 (-0.84-0.46)	-0.35 (-0.81-0.11)
	b	-0.27 (-0.61-0.06)	-0.36 (-0.77-0.05)	-0.38 (-1.14-0.38)	**-0.72 (-1.27- -0.18)**
	c	-0.17 (-0.43-0.09)	-0.22 (-0.53-0.10)	-0.26 (-0.89-0.34)	**-0.48 (-0.91- -0.05)**
rs4148382	c	0.16 (-0.13-0.45)	0.27 (-0.07-0.62)	0.43 (-0.22-1.07)	0.07 (-0.41-0.54)
rs504348	a	-0.03 (-0.31-0.25)	0.06 (-0.27-0.39)	0.07 (-0.57-0.70)	-0.03 (-0.31-0.25)
	b	-0.46 (-1.20-0.28)	**-0.98 (-1.87- -0.09)**	-0.30 (-1.98-1.39)	-0.46 (-1.20-0.28)
	c	-0.07 (-0.34-0.20)	-0.04 (-0.36-0.29)	0.03 (-0.58-0.64)	-0.14 (-0.58-0.30)
rs4781699	a	-0.13 (-0.38-0.12)	-0.17 (-0.47-0.13)	-0.45 (-1.01-0.11)	-0.23 (-0.65-0.18)
	b	-0.15 (-0.65-0.35)	-0.27 (-0.88-0.33)	-0.01 (-1.12-0.12)	0.08 (-0.76-0.91)
	c	-0.13 (-0.37-0.11)	-0.18 (-0.47-0.10)	-0.39 (-0.92-0.15)	-0.19 (-0.59-0.21)
rs35621	a	-0.14 (-0.45-0.17)	0.01(-0.37-0.38)	-0.54 (-1.24-0.15)	-0.18 (-0.69-0.34)
	b	-0.14 (-1.40-1.13)	0.06 (-1.48-1.59)	1.18 (-1.65-0.02)	1.19 (-0.92-3.29)
	c	-0.14 (-0.44-0.17)	0.01 (-0.37-0.38)	-0.47 (-1.16-0.22)	-0.13 (-0.64-0.38)

Data are presented as B (95%CI) = regression coefficient (95% confidence interval); ln=natural logarithm; The analyses are adjusted for age, gender and packyears; a: heterozygote versus wild type; b: homozygote mutant versus wild type; c: minor allele vs. wild type; SNPs showing a significant effect are depicted in bold ($p<0.05$).

Table S3b: *MRP1* SNPs and inflammatory cells in bronchial biopsies of COPD patients

SNP	Model	Ln(mast cells)	Ln(macrophages)	Ln(neutrophils)	Ln(eosinophils)
rs212093	a	**-0.25 (-0.47- -0.03)**	-0.39 (-0.78-0.01)	-0.13 (-0.58-0.32)	0.45 (-0.65-1.56)
	b	-0.16 (-0.42-0.10)	**-0.61 (-1.07- -0.15)**	**-0.63 (-1.16- -0.09)**	-0.20 (-1.50-1.10)
	c	**-0.22 (-0.42- -0.02)**	**-0.47 (-0.83- -0.11)**	-0.30 (-0.72-0.12)	0.23 (-0.78-1.24)
rs4148382	c	-0.01 (-0.23-0.22)	0.23 (-0.18-0.63)	0.12 (-0.35-0.58)	0.43 (-0.66-1.51)
rs504348	a	0.06 (-0.15-0.28)	0.03 (-0.36-0.42)	-0.01 (-0.46-0.44)	**-1.09 (-2.15- -0.04)**
	b	0.25 (-0.33-0.84)	-0.18 (-1.23-0.86)	-0.64 (-1.83-0.56)	0.59 (-2.23-3.41)
	c	0.08 (-0.13-0.29)	0.01 (-0.37-0.38)	-0.07 (-0.50-0.37)	-0.94 (-1.96-0.09)
rs4781699	a	-0.02 (-0.21-0.18)	**-0.39 (-0.73- -0.05)**	-0.13 (-0.53-0.27)	-0.45 (-1.41-0.51)
	b	0.09 (-0.31-0.48)	-0.06 (-0.74-0.63)	-0.18 (-0.98-0.63)	-0.34 (-2.26-1.59)
	c	-0.01 (-0.19-0.18)	**-0.34 (-0.67- -0.02)**	-0.14 (-0.52-0.25)	-0.43 (-1.35-0.48)
rs35621	a	0.16 (-0.09-0.40)	-0.10 (-0.53-0.33)	-0.38 (-1.58-0.82)	-0.38 (-1.58-0.82)
	b	0.33 (-0.66-1.32)	1.48 (-0.28-3.24)	0.78 (-4.10-5.65)	0.78 (-4.10-5.65)
	c	0.16 (-0.07-0.40)	-0.04 (-0.47-0.39)	-0.33 (-1.51-0.85)	-0.33 (-1.51-0.85)

Data are presented as B (95%CI) = regression coefficient (95% confidence interval); ln=natural logarithm; The analyses are adjusted for age, gender and packyears; a: heterozygote versus wild type; b: homozygote mutant versus wild type; c: minor allele vs. wild type; SNPs showing a significant effect are depicted in bold ($p<0.05$).

Table S4a: *MRP1* SNPs and inflammatory cells in induced sputum of COPD patients

SNP	Model	Ln (total cells count¶)	Ln (neutrophils)	Ln (macrophages)	Ln (eosinophils)
rs212093	a	-0.09 (-0.58-0.41)	-0.17 (-0.73-0.39)	0.06 (-0.44-0.55)	0.08 (-0.95-1.12)
	b	-0.21 (-0.79-0.37)	-0.23 (-0.89-0.43)	-0.20 (-0.78-0.37)	0.02 (-1.18-1.22)
	c	-0.13 (-0.59-0.33)	-0.19 (-0.71-0.33)	-0.03 (-0.49-0.42)	0.06 (-0.88-1.01)
rs4148382	c	**0.59 (0.11-1.07)**	**0.61 (0.06-1.16)**	0.46 (-0.02-0.94)	0.41 (-0.62-1.43)
rs504348	a	0.03 (-0.45-0.50)	-0.01 (-0.55-0.53)	0.12 (-0.35-0.59)	0.65 (-0.34-1.63)
	b	-1.04 (-2.51-0.44)	-1.15 (-2.82- 0.52)	-1.15 (-2.61-0.31)	0.41 (-2.66-3.47)
	c	-0.05 (-0.51-0.41)	-0.09 (-0.62-0.43)	0.03 (-0.43-0.49)	0.63 (-0.33-1.59)
rs4781699	a	-0.03 (-0.45-0.38)	0.01 (-0.46-0.48)	-0.08(-0.49-0.32)	0.81 (-0.03-1.66)
	b	-0.74 (-1.79-0.31)	0.01 (-1.88-0.51)	-0.10(-2.03-0.04)	0.52 (-1.64-2.67)
	c	-0.09 (-0.49-0.31)	-0.05 (-0.51-0.41)	-0.16 (-0.56-0.24)	0.79 (-0.03-1.61)
rs35621	a	-0.12 (-0.63-0.39)	-0.10 (-0.68-0.48)	-0.01 (-0.52-0.50)	-0.35 (-1.40-71)
	b	0.01 (-2.06-2.07)	0.01 (-2.34-2.35)	0.44 (-1.62-2.50)	1.33 (-2.94-5.59)
	c	-0.11 (-0.61-0.39)	-0.10 (-0.67-0.47)	0.01 (-0.49-0.51)	-0.28 (-1.32-0.76)

Data are presented as B (95%CI) = regression coefficient (95% confidence interval); ln=natural logarithm; The analyses are adjusted for age, gender and packyears; a: heterozygote versus wild type; b: homozygote mutant versus wild type; c: minor allele vs. wild type; ¶ Total cells count refers to total number of non-squamous cells in sputum; SNPs showing a significant effect are depicted in bold ($p<0.05$).

Table S4b: *MRP1* SNPs and inflammatory cells in induced sputum of COPD patients

SNP	Model	Ln (lymphocytes)	Ln (basophils)	Ln (epithelial cells)
rs212093	a	-0.08 (-0.78-0.62)	-0.16 (-0.40-0.09)	0.21 (-0.78-1.19)
	b	-0.37 (-1.19-0.45)	-0.09 (-0.37-0.19)	0.08 (-1.07-1.23)
	c	-0.18 (-0.83-0.46)	-0.13 (-0.36-0.09)	0.16 (-0.74-1.07)
rs4148382	c	0.62 (-0.07-1.32)	0.05 (-0.19-0.29)	-0.53 (-1.50-0.45)
rs504348	a	-0.03 (-0.71-0.65)	-0.10 (-0.33-0.14)	0.24 (-0.71-1.19)
	b	-0.77 (-2.88-1.33)	0.02 (-0.72-0.75)	1.05 (-1.90-4.01
	c	-0.08 (-0.74-0.58)	-0.09 (-0.32-0.14)	0.30 (-0.62-1.22)
rs4781699	a	-0.15 (-0.73-0.44)	-0.06 (-0.26-0.15)	-0.001 (-0.83-0.82)
	b	-0.78 (-2.27-0.72)	-0.05 (-0.57-0.47)	0.55 (-1.55-2.65
	c	-0.20 (-0.77-0.37)	-0.06 (-0.26-0.14)	0.04 (-0.76-0.85)
rs35621	a	-0.37 (-1.08-0.36)	0.13 (-0.17-0.38)	-0.05 -1.07-0.96)
	b	1.04 (-1.87-3.94)	0.02 (-0.99-1.04)	-0.15 (-4.26-3.96)
	c	-0.31 (-1.02-0.40)	0.12 (-0.13-0.37)	-0.06 (-1.05-0.94

Data are presented as B (95%CI) = regression coefficient (95% confidence interval); ln=natural logarithm; The analyses are adjusted for age, gender and packyears; a: heterozygote versus wild type; b: homozygote mutant versus wild type; c: minor allele vs. wild type.

Table S5: *MRP1* SNPs and FEV_1 level of COPD patients

SNP	Model	FEV_1
rs212093	a	0.03 (-0.12 - 0.18)
	b	**0.22 (0.05 - 0.40)**
	c	0.09 (-0.05 - 0.23)
rs4148382	c	**-0.22 (-0.36 - -0.08)**
rs504348	a	0.04 (-0.11 - 0.18)
	b	0.26 (-0.21 - 0.65)
	c	0.06 (-0.08 - 0.20)
rs4781699	a	0.06 (-0.07 - 0.19)
	b	0.09 (-0.17 - 0.35)
	c	0.07 (-0.06 - 0.19)
rs35621	a	0.14 (-0.02 - 0.30)
	b	0.09 (-0.57 - 0.75)
	c	0.14 (-0.02 - 0.30)

Data are presented as B (95%CI) = regression coefficient (95% confidence interval); FEV_1 = forced expiratory volume in one second; The analyses are adjusted for age, gender, height and packyears; General model a: heterozygote versus wild type; General model b: homozygote mutant versus wild type; SNPs showing a significant effect are depicted in bold ($p<0.05$).

Table S6: Frequencies of *MRP1* haplotypes

Haplotypes (threshold > 0.01)		Inflammatory cells	
rs212093 rs4148382 rs504348 rs4781699 rs35621	FEV_1 level	bronchial biopsies	induced sputum
00000	0.2986	0.3087	0.3099
00010	0.0273	0.0271	0.0291
00011	0.0077	0.0075	0.0078
00100	0.0144	0.0140	0.0075
00110	0.0771	0.0770	0.0751
00111	0.0196	0.0194	0.0213
01000	0.0876	0.0848	0.0917
01001	0.0155	0.0157	0.0166
01010	0.0167	0.0147	-
10000	0.2574	0.2555	0.2649
10001	0.0539	0.0530	0.0558
10010	0.0788	0.0756	0.0815
10011	0.0023	0.0031	0.0027
10101	0.0021	0.0022	0.0036
10110	0.0412	0.0417	0.0325

0 = wild type of the corresponding SNP; 1= minor allele carriers of the corresponding SNP

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CHAPTER 3

MRP1 variants, MRP1 protein in bronchial biopsies and lung function decline in COPD

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Abstract

Multidrug resistance-associated protein-1 (MRP1) reduces the oxidative stress generated by smoking, a risk factor for Chronic Obstructive Pulmonary Disease (COPD). We previously showed that MRP1 variants are associated with the level and decline of annual forced expiratory volume in one second (FEV1) in the general population. Moreover, we showed that MRP1 variants are also associated with FEV1 level and inflammatory markers in COPD patients.We investigate in the current study the association of MRP1 protein expression in bronchial biopsies with FEV1 decline in COPD patients using placebo, or inhaled corticosteroids (ICS) with or without long-acting β2-agonists. Additionally we investigate the association of *MRP1* variants with FEV_1 decline.

MRP1 variants (rs212093, rs4148382, rs504348, rs4781699, rs35621) were genotyped in 110 COPD patients. Associations of *MRP1* variants and MRP1 protein expression in bronchial biopsies (obtained at baseline, 6 and 30 months) with FEV_1 decline were analyzed using linear mixed-effect models.

During 30-month ICS treatment, subjects with a moderate staining for MRP1 had less FEV_1 decline than those with a weak staining. In subjects stopping ICS after 6 months followed by 24-month placebo, moderate staining for MRP1 was associated with faster FEV_1 decline than in those with a weak staining. None of the variants was associated with FEV_1 decline.

Our unique study suggests a role of MRP1 protein expression in bronchial biopsies in FEV_1 decline occurring selectively in COPD patients with long-term (30-month) ICS therapy.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is an inflammatory disease characterized by airflow limitation that is not fully reversible, progressive in nature and associated with an abnormal inflammatory response of the lungs to cigarette smoke [1]. Smoking is associated with accelerated lung function decline, which can be tempered by quitting smoking. Smoking generates oxidative stress in the lung, which can be reduced by proteins of the ATP-binding cassette (ABC) superfamily such as multidrug resistance-associated protein 1 (MRP1) [2]. MRP1 (official name ABCC1, ABC subfamily C, member 1) is highly expressed in human lung tissue [2], especially at the basolateral side of bronchial epithelium [3]. MRP1's contribution varies from protecting cells within the body against drugs, environmental toxins and heavy metals, to its involvement in the cellular oxidative defence system and inflammation [4, 5].

In a recent study we showed that the *MRP1* gene is associated with the lung function in two independent general population-based cohorts [6]. In these cohorts five single nucleotide polymorphisms (SNPs) in the *MRP1* gene (rs212093, rs4148382, rs504348, rs4781699 and rs35621) were significantly associated with level of forced expiratory volume in one second (FEV_1) or with annual FEV_1 decline [6]. In a subsequent study in COPD patients we have shown that the *MRP1* gene plays a role in the severity of COPD, since rs212093 and rs4148382 in *MRP1* were associated with less respectively more airway wall inflammation and a higher respectively lower level of lung function [7]. Moreover, rs4148382 was significantly associated with a higher MRP1 protein expression in bronchial biopsies from subjects with established COPD [7]. Due to these novel results with respect to the MRP1 gene and severity of COPD, we aim in the current study to investigate in depth MRP1 protein expression and lung function in the same group of COPD patients.

Thus, consequently, our current study will focus on the associations MRP1 protein expression in airway wall biopsies with the decline of lung function in the same population of COPD patients we previously studied with respect to anti-inflammatory and clinical effects of inhaled corticosteroids (ICS) with or without long-acting β-agonists (LABAs) [8].We investigated whether MRP1 protein expression in

bronchial biopsies obtained at baseline and at 6- and 30- month treatment, are associated with the FEV_1 level at baseline, 6 and 30 months and whether MRP1 protein expression in bronchial biopsies is associated with the course of FEV_1, with and without ICS ± LABA treatment. Additionally, we investigated whether *MRP1* SNPs are associated with the course of FEV_1.

Methods

Study population

We included 114 patients with COPD who participated in a two-center trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study). Patient characteristics and methods have been described in detail previously [9]. In brief, all patients had irreversible airflow limitation, chronic respiratory symptoms [10], did not use a course of oral steroids during the previous 3 months and had no maintenance treatment with inhaled or oral steroids during the previous 6 months. They were current or ex-smokers with a smoking history of ≥10 pack-years, aged between 45 and 75 years without a history of asthma. The study was approved by the medical ethics committees of the University Medical Centers of Leiden and Groningen. All patients gave their written informed consent.

Clinical Characteristics

Reversibility to salbutamol was measured at baseline, 6 and 30 months and FEV_1 post bronchodilator was measured every three months using standardized protocols [11].

Intervention and follow-up procedures

Patients were randomly assigned to receive either: 1) fluticasone propionate, 500µg twice daily, for the first 6 months followed by placebo, twice daily, for 24 months; 2) fluticasone, 500µg twice daily for 30 months; 3) fluticasone, 500µg twice daily and salmeterol, 50µg twice daily, in a single inhaler for 30 months; 4) placebo, twice daily, for 30 months [8].

MRP1 tagging SNPs and genotyping

We selected from all *MRP1* tagging SNPs rs212093, rs4148382, rs35621, rs4781699 and rs504348 based on our previous studies showing a significant associations with FEV_1 level and/or annual FEV_1 decline in the general population [6], as well as with FEV_1 level and inflammatory markers in COPD patients [7]. Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar).

Biopsies and immunohistochemistry on bronchial biopsies

Details on fiberoptic bronchoscopy, biopsy processing and immunohistochemical staining are described in the data supplement. MRP1 protein was scored for staining intensity in intact epithelium of bronchial biopsies with a semiquantitative score: 1=no or weak; 2=moderate; 3=strong staining intensity.

Statistics

Differences between treatment groups in MRP1 staining intensity at baseline, 6 and 30 months were analyzed using Chi-square tests (see additional file 1 for details).
We assessed the association of MRP1 protein expression at baseline, 6 months and 30 months with the FEV_1 levels at those time points using linear mixed-effect models (LME) stratified for treatment group, with adjustment for gender, height and age at baseline.

We used LME models to asses the association of MRP1 protein expression at baseline with FEV_1 decline stratified for treatment groups, separately for the period till 6 months and the time after 6 months because of the change in treatment for one of the groups. We adjusted our analyses for height and age at baseline, gender and the interaction of time with age at baseline and gender.

We assessed the associations of *MRP1* SNPs with FEV_1 decline using LME models (see data supplement for details on the genetic model). Analyses were adjusted for height and age at baseline, gender, treatment and the interactions of time with age at baseline, gender and treatment.
Analyses were performed using SPSS version 16.0 for Windows and values

of $p<0.05$ (tested 2-sided) were considered statistically significant. Linkage Disequilibrium (LD) plots (a threshold of 0.8 for the correlation coefficient (r2) and Hardy Weinberg Equilibrium (HWE) tests were performed with Haploview (version 4.2).

Results

The clinical characteristics of COPD patients are presented in table 1.
All 5 *MRP1* SNPs were in Hardy Weinberg Equilibrium (HWE, $p>0.05$) and were only weakly correlated with each other; the highest r^2 in our population being 0.34 (Figure 1).
Table 2 shows the distribution of MRP1 protein expression within treatment groups.

Table 1: Clinical characteristics of COPD patients

Baseline characteristics, n=114	
Males, n (%)	99 (86.8)
Age (years)	61.6 (7.7)
Height (cm)	175.5 (7.8)
Packyears*	41.8 (31.2 – 54.7)
Current smoker, n (%)	72 (63.2)
FEV_1/FVC (%) ±	50.3 (8.9)
FEV_1 (L) ±	2.03 (0.5)
FEV_1 % predicted ±	62.7 (9.1)
Treatment groups, n=101 † (%)	
Placebo	24 (23.8)
Fluticasone 6 mo, Placebo 24 mo	26 (25.7)
Fluticasone 30 mo	26 (25.7)
Fluticasone/Salmeterol	25 (24.8)

*Data are presented as mean (SD) or * median (25^{th} – 75^{th} percentile); ± postbronchodilator; † number of patients who adhered to therapy (used >70% medication); mo=months; FEV_1 = forced expiratory volume in one second; FEV_1/FVC = FEV_1/forced vital capacity.*

There were no consistent differences between treatment groups in MRP1 staining intensity at baseline, 6 and 30 months. There were no differences in age, lung function and packyears between subjects with at least 1 biopsy with intact epithelium (n=91 (79.8%)) and those without any biopsy (n=23 (20.2%)). There were fewer women with at least 1 biopsy with intact epithelium (n=9 (64.3%)) than men (n=77 (88.5%)).

Figure 1: Linkage disequilibrium plot and correlation coefficients (r^2) for 5 *MRP1* polymorphisms genotyped in COPD patients (n=110)

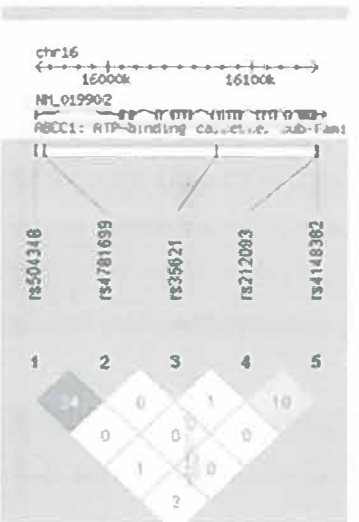

Table 2: Frequencies of MRP1 staining intensity levels within treatment groups

	MRP1 scores baseline				MRP1 scores 6 months				MRP1 scores 30 months			
Treatment group	1	2	3	Nt	1	2	3	Nt	1	2	3	Nt
Placebo, N	3	8	2	13	3	6	4	13	1	9	3	13
Fluticasone 6 months, N	3	8	3	14	3	10	5	18	3	13	2	18
Fluticasone 30 months, N	3	8	4	15	1	12	4	17	3	8	1	12
Fluticasone/Salmeterol, N	8	2	1	11	3	11	1	15	1	12	6	19

MRP1 = multidrug resistance associated-protein 1; MRP1 scores 1= weak staining, 2= moderate staining, 3= strong staining; Nt= total number of subjects per treatment group for each time point.

MRP1 protein expression and FEV_1 level at 0, 6 and 30 months

MRP1 protein expression at 0, 6 and 30 months was not significantly associated with FEV_1 levels at those time points (detailed results are presented in table S1; data supplement).

MRP1 protein expression at baseline and FEV_1 decline (0-6 months and 6-30 months)

Subjects with a moderate or strong intensity of staining for MRP1 protein expression at baseline did not have a slower or faster FEV_1 decline compared to those with weak staining for MRP1 protein expression in any of the treatment groups for the ensuing period of 6-month treatment (Figure 2).

In the group with 30-month ICS treatment, subjects having a moderate intensity of staining at baseline had less FEV_1 decline between 6 and 30 months than those with a weak intensity of staining for MRP1 protein (Figure 2). Conversely, in the treatment group with discontinuation of ICS therapy after 6 months, subjects having a moderate intensity of staining had a faster FEV_1 decline compared to those with a weak intensity of staining for MRP1 protein (Figure 2).

MRP1 protein expression at baseline was not significantly predictive of 30-month FEV_1 decline in the group using placebo or combination of LABAs with ICS (Figure 2).

***MRP1* SNPs and FEV_1 decline (0-6 months and 6-30 months)**

None of the *MRP*1 SNPs was significantly associated with the FEV_1 decline for either the period till 6 months or the time after 6 months (Figure 3).

Additional adjustment for the initial FEV_1 and its interaction with time did not change the size or significance of the estimated effect of the genotypes on FEV_1 decline.

Figure 2: The effect of MRP1 protein level at baseline on FEV_1 decline

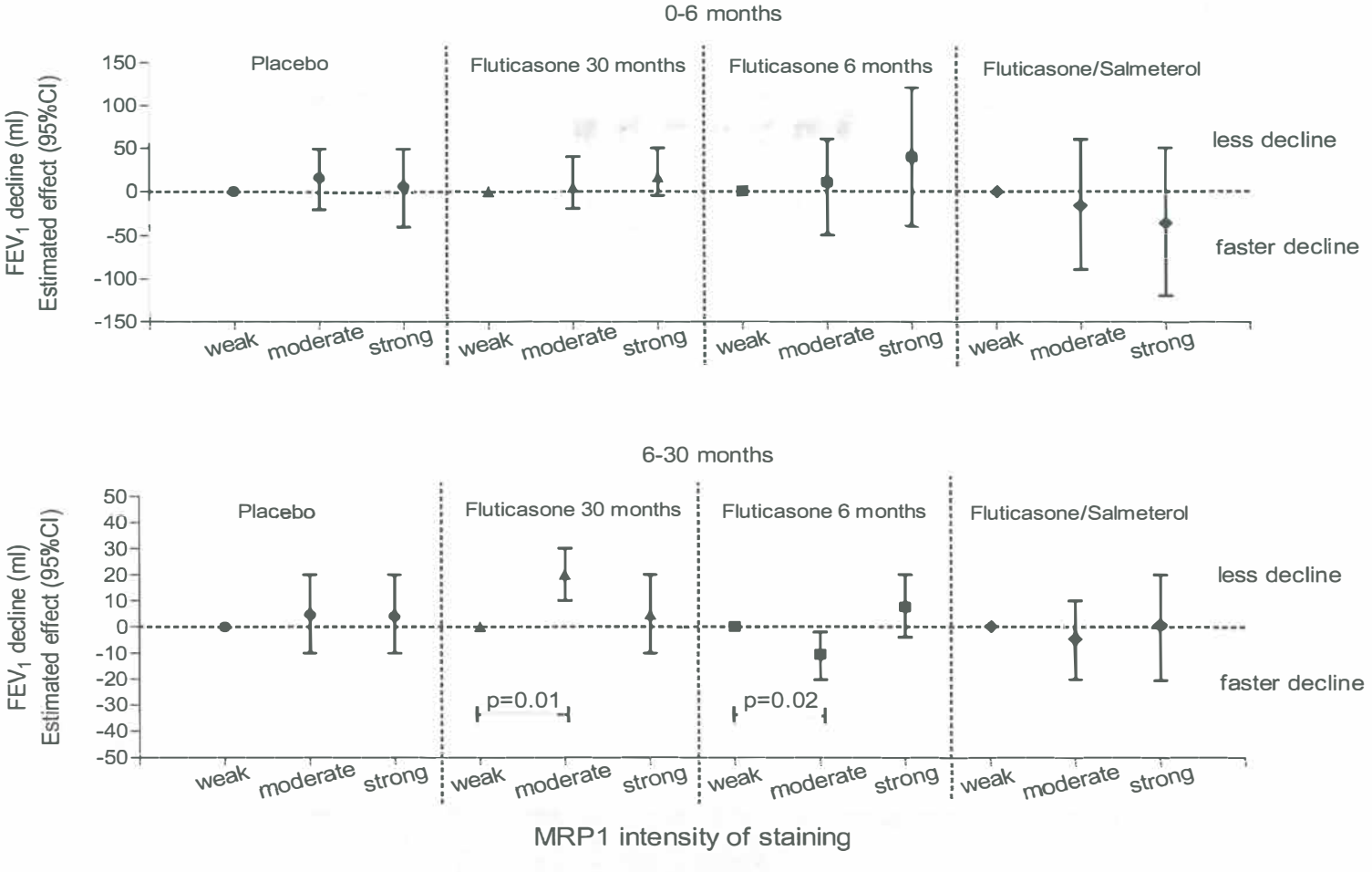

Figure 3: The effect of *MRP1* SNPs on FEV_1 decline (0-6 months, 6-30 months)

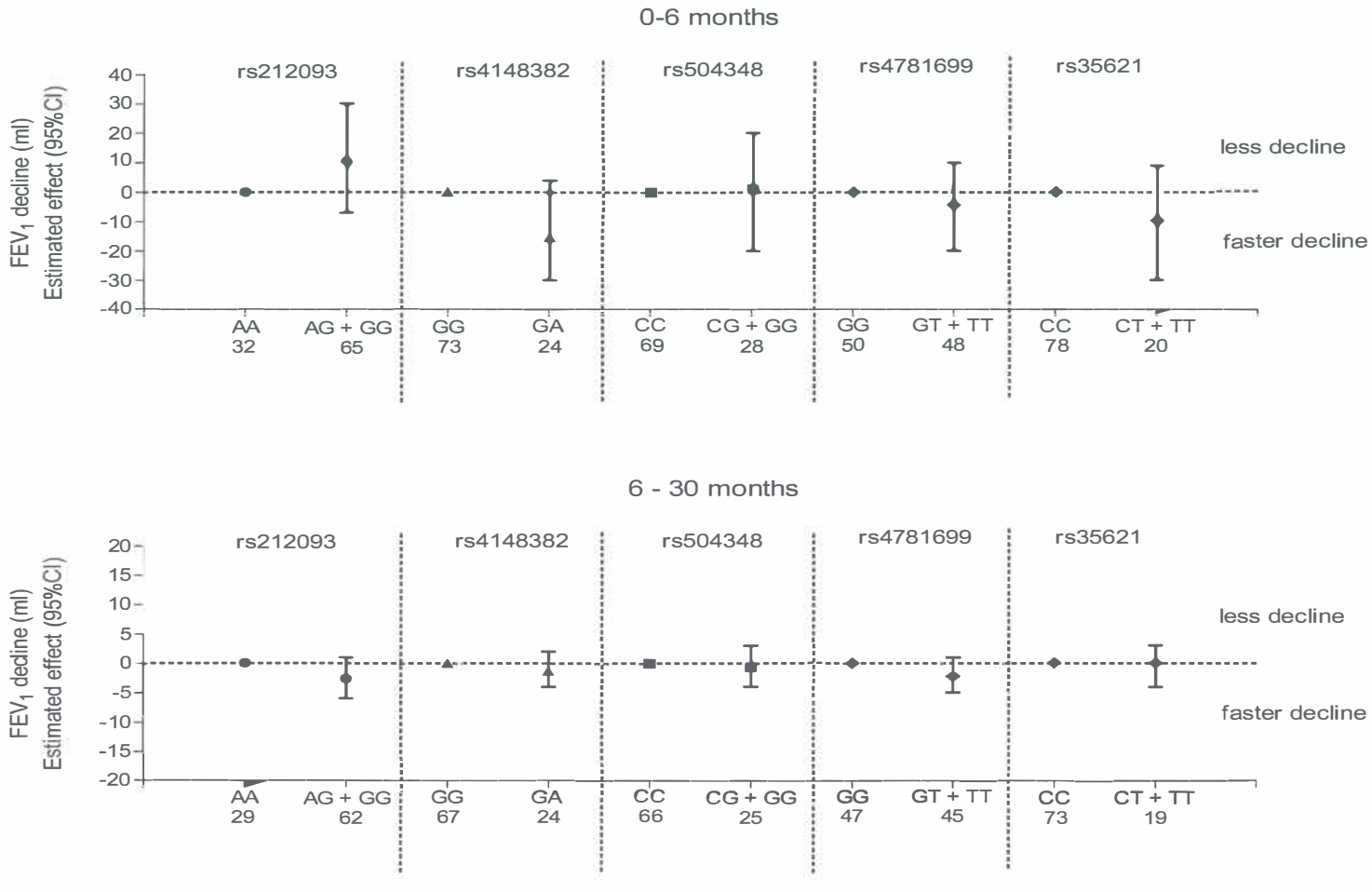

Figure 2: The upper graph is for the first 6 months and the lower graph is for the period from 6 to 30 months; FEV_1 = forced expiratory volume in one second; Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI); The weak staining for MRP1 was set to zero as the reference category; The analyses are adjusted for height, age at baseline, gender and the interaction of time with gender and age at baseline.

Figure 3: The upper graph is for the first 6 months and the lower graph is for the period from 6 to 30 months; FEV_1= forced expiratory volume in one second; Squares represent the regression coefficient (B) and vertical bars the 95% confidence interval (CI); Wild type was set to zero as the reference category; Different numbers for the SNP genotypes are due to missing data on genotype or FEV_1 measurements at every time point; The analyses are adjusted to height and age at baseline, gender, treatment and the interactions of time with age at baseline, gender and treatment.

Discussion

This is the first study showing significant associations of a higher MRP1 protein expression with less accelerated FEV_1 decline in COPD patients using long-term therapy with ICS and a significant association of higher MRP1 protein expression with a faster FEV_1 decline after withdrawal of ICS. These associations were not likely due to the genetic background in *MRP1*, since the SNPs in *MRP1* that were previously associated with the level of FEV_1 [7], in the current study are not significantly associated with FEV_1 decline in the same group of COPD patients. This is in line with our previous findings showing that the same two SNPs are not significantly associated with the decline of lung function in the general population, [6] suggesting that these SNPs may play a role in COPD severity, without any further effects on lung function over time.

Current international guidelines recommend maintenance therapy with LABAs and ICS in moderate to severe COPD with exacerbations [12]. Variable findings have been published regarding the effect of ICS on lung function (decline) and exacerbations in COPD. In one study, fluticasone propionate and salmeterol significantly improved lung function after 2-week treatment and this effect was sustained during 12-month treatment [13]. Although some other studies showed no

effect of fluticasone propionate or budesonide on lung function in COPD patients [14, 15, 16, 17], recent data suggest that disease progression can be modified by ICS [8, 18-20]. Our current study provides further information in that those subjects with a moderate staining for MRP1 level had less lung function decline compared to those with a weak staining in the presence of long-term (30-month) therapy with fluticasone propionate. Importantly, subjects with a moderate intensity of MRP1 staining who stopped fluticasone treatment at 6 months and subsequently used 24-month placebo, had a significantly faster lung function decline compared with those with a weak staining for MRP1. This suggests that continuing therapy with ICS may positively influence the association of MRP1 protein with FEV_1 decline. Furthermore, an in vitro study showed that FP accelerated the conversion of leukotriene C4 (LTC4) to LTD4 and finally leaded to the accelerated degradation of LTC4 to less active LTE4 via LTD4 and this action was observed only after cells were exposed to FP for more than 2 days, suggesting new protein synthesis [21]. The authors speculated that regular use of FP up-regulates the catabolic process of LTC4 to less active LTE4, and therefore contributes to the antiasthma actions [21]. Although the inflammatory processes occurring in asthma and COPD are different, we can speculate that in the current in vivo study, stopping ICS therapy after 6 months could have a negative effect on the action of the MRP1 protein substrate LTC4. Also this is suggestive of an interaction between long term therapy with ICS and MRP1 function. We did not investigate the effect of specific treatment on MRP1 protein expression in bronchial biopsies [22, 23], therefore it is uncertain whether this association of MRP1 protein with lung function decline in the presence of long-term therapy with fluticasone results from the effect of treatment or from MRP1 functionality and/or activity. Further studies, that might include smoking animal models, should also focus on the effect of bronchial epithelium MRP1 protein level in the presence of COPD medication such as fluticasone propionate. Whereas in previous studies in a smoking animal model we studied the association of bronchial MRP1 expression and inflammation [24], it would be of interest in future studies to also focus in COPD/smoking-related animal models on the association of bronchial MRP1 protein and lung function.

A previous study showed that the therapy with salmeterol plus fluticasone

propionate, or with salmeterol and fluticasone propionate separately reduces the rate of decline of FEV_1 in patients with moderate-to-severe COPD [20]. Prior, we showed in the current COPD patients (GLUCOLD) that combination therapy of fluticasone propionate and salmeterol when compared with fluticasone alone has no additional long-lasting effects on inflammatory cells and that it improves FEV_1 level without further influencing FEV_1 decline [8]. These observations may indicate that disease modification may occur in particular sub phenotypes of patients with COPD [8]. Our data suggest that MRP1 protein expression may reflect one of the COPD phenotypes that is sensitive to inhaled corticosteroid therapy. In fact that we did not find a significant association of MRP1 protein expression with 30-month FEV_1 decline in the group using combination of LABA with ICS is likely due to lack of power. Unfortunately, only 2 subjects had analyzable biopsies with a moderate staining for MRP1 protein at baseline in the group randomly selected to use combination of LABAs with ICS.

MRP1 is located in bronchial epithelium, which is the first line of defence against invading microorganisms and inhaled toxic substances like cigarette smoke. Moreover, epithelial cells are the first cells that pulmonary drugs have to come across to reach the underlying tissue and act on it. Thus MRP1 may have a role in development and severity of COPD. We have previously shown that patients with moderate and severe COPD have a lower MRP1 expression in bronchial biopsies than healthy individuals. Furthermore, patients with severe and very severe COPD had again a lower intensity of MRP1 protein expression than those with mild and moderate severe COPD [25]. Thus the spectrum of MRP1 protein expression reaches from higher in healthy subjects to lower in severe disease. Although our previous study [25] is not comparable with our current study due to differences in study design and type of biopsies used for analyses (e.g. frozen vs. paraffin), it is tempting to speculate that the observed association of a higher MRP1 protein expression with a less rapid FEV_1 decline fits with our COPD patients having mild to moderate COPD. This might also explain why we did not observe any significant association of an even higher MRP1 protein expression (coded as strong) with FEV_1 decline (Figure 1). The ultimate effect may, as our data suggest, depend on the medication prescribed, as likely as on doses and time of administration. These findings are of importance since they can contribute to pharmacogenetics of COPD

management in mild to moderate COPD patients, which will result in more accurate and targeted therapy.

We showed previously in an *in vitro* study that cellular concentrations of budesonide affect negatively MRP1 function, likely by inhibiting MRP1 mediated transport that protects against oxidative stress and toxic compounds [22]. How can we reconcile this with our current findings that a higher MRP1 protein expression is associated with less accelerated lung function decline in subjects using 30-month fluticasone propionate therapy? We can speculate on some possible explanations. First, the deposition of drugs may influence its effects. A human *in vivo* study demonstrated that fluticasone propionate concentrations are three times higher in central than peripheral lung tissue [26]. Moreover, the same study showed [26] that fluticasone concentrations in peripheral lung tissue following inhalation of a 1.0 mg dose were higher compared to those reported for budesonide [27]. Thus, there may be dose differences between our previously published *in vitro* study on effects of budesonide and the doses present in the airways and lung tissue with fluticasone *in vivo*.
Second, the action of different inhaled corticosteroids on epithelial tissue may depend on the pharmacokinetic and pharmacodynamic properties of their components. For example, budesonide is less lipophilic than fluticasone and therefore is dissolving freely in airway mucus and is more rapidly absorbed into the airway tissue [28, 29] whereas fluticasone propionate is released slower from the lung lipid compartment with a longer local duration of action [30]. Thus the duration of action of each inhaled steroid may affect the outcome on MRP1 protein association and lung function decline.
Third, it has been previously shown that MRP1 substrates identified by vesicle transport studies compete with each other for transport, even if there are no similarities between the compounds and they are conjugated to different anionic moieties, e.g. LTC4 or $E_2$17βG [31, 32]. This suggests that each substrate is creating an exclusive interaction with MRP1 and it might be that fluticasone propionate and budesonide act also differently in the presence of this protein by being involved in different competing activities with other transported substrates.

We believe that our unique study due to its clear design and including lung function

measurements and biopsy data, has brought new ideas on a possible role of MRP1. This needs further investigation, since is seems possible that MRP1 is differentially influenced by different types of ICS. Our data suggest further exploration of MRP1 in a larger group to establish whether MRP1 could be a candidate marker for individualized ICS prescription in COPD.

Conclusions

In conclusion, this is the first study showing a protective association of a moderate MRP1 protein level in bronchial biopsies with lung function decline in COPD patients using long-term maintenance therapy with fluticasone propionate, when compared with COPD patients having a low intensity of MRP1 protein staining. Conversely, a deleterious association was present when ICS therapy was discontinued after 6-month treatment with fluticasone.

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Supplementary methods

Bronchoscopy and biopsy processing of COPD patients

Fiberoptic bronchoscopy was performed as described previously (Lapperre et al., 2006). In brief, smokers were requested to abstain from smoking on the day of the bronchoscopy. Patients received premedication (400µg salbutamol p.i., 20mg codeine phosphate p.o. and 0.5mg of atropine s.c.) and local anaesthesia with lidocaine (not more than 3mg/kg). Six adequate bronchial biopsies were taken from subsegmental carinae in the right or left lower lobe using the fiberoptic bronchoscope and pairs of cup forceps. Four biopsies were fixed in 4% neutral buffered formalin, processed and embedded in paraffin Two biopsies were directly embedded in Tissue Tek mounting medium, snap frozen in liquid isopentane and finally stored at -80ºC with the necessary security measures to avoid thawing and/or exposure to large changes in temperature. Details on biopsy processing, immunohistology and analysis have been published previously (Lapperre et al., 2006). In brief, four paraffin-embedded biopsies were cut in 4µm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the best morphological biopsy per subject for analysis (without crush artefacts, large blood clots, or only epithelial scrapings).

Immunohistochemistry on bronchial biopsies from COPD patients

Biopsies were obtained at baseline, 6 and 30 months. For each time point, the immunohistochemical staining was performed on one paraffin section of 4µm per subject with monoclonal antibody MRPr1 for MRP1 (Santa Cruz, California, USA). Initially, the tissue slides were deparaffinised with xylene (10 minutes) and rehydrated in graded alcohols before staining. Slides were pre-treated with 1mM EDTA buffer in the microwave for 15 minutes. The staining was performed automatically using the Dako Autostainer (DAKO, Copenhagen, Denmark). Pre-incubation with peroxidise blocking reagent (DAKO, Copenhagen, Denmark) was used for blocking endogenous peroxidase. First antibody used was monoclonal antibody MRPr1 (Santa Cruz Biotechnology, Inc.) for MRP1 with a dilution of 1:50, the second antibody used was polyclonal antibody GaR^{po} (goat anti-rabbit peroxidase, DAKO, Copenhagen, Denmark) with a dilution of 1:100 and the third

antibody used was polyclonal antibody RaG^{po} (rabbit anti-goat peroxidase, DAKO, Copenhagen, Denmark) with a dilution of 1:100. Nova Red (SK4800, Vector, USA) was used as a substrate giving a reddish-brown reaction product. Counterstaining was performed using Mayer's haematoxylin. A negative control was obtained by omission of the primary antibody.

Evaluation of immunohistochemistry on bronchial biopsies

For the current study, some biopsies have been excluded due to limited quality and intact bronchial epithelium (non squamous epithelium) has been selected for analysis based on the microscopic appearance. MRP1 intensity scores for subjects with at least one biopsy with intact epithelium were available from 57 bronchial biopsies at baseline, 68 bronchial biopsies at 6 months and 65 bronchial biopsies at 30 months.

Two observers (S.E.B. and W.T.) performed all evaluations of bronchial biopsies individually, in a blinded manner. Most sections had variable staining for MRP1 in epithelium and parts with the most intense staining were evaluated for scoring.

Statistics

Due to the fact that not all the subjects had a good morphological biopsy to perform immunohistochemical staining or other biopsies had after immunohistochemical staining of poor quality we excluded these subjects for further analyses. To make sure there is no selection bias we analyzed the differences in age, lung function, packyears and gender between subjects with at least 1 biopsy at any time point with intact epithelium and those excluded from the analyses using Mann-Whitney U respectively Chi-square tests.

We coded MRP1 intensity levels of staining (moderate respectively strong) as dummy variables and compared them with the weak staining that was set as reference category.

To asses the association of MRP1 protein expression at baseline with FEV_1 decline we defined FEV_1 decline based on the measurements of FEV_1 level at every 3 months for a total period of 30 months.

In the association of MRP1 protein expression at baseline with FEV_1 decline the

LME model included the main effect of time, MRP1 protein expression at baseline and their interaction with time.

We assessed the associations of *MRP1* SNPs with FEV_1 decline using LME models. Because of the low numbers per genotype we used a dominant genetic model where heterozygote and homozygote variants are pooled and compared to the homozygote wild type. The LME model included the main effect of time, *MRP1* SNPs and their interaction with time.

We performed LME models with a random intercept at the patient level, assuming that data is missing at random.

Supplementary results

Table S1: MRP1 protein expression and FEV1 level at 0, 6 and 30 months

FEV1 level at 0, 6 and 30 months												
	Placebo			Fluticasone/ Salmeterol			Fluticasone 30 months			Fluticasone 6 months		
	B	SE	p	B	SE	p	B	SE	p	B	SE	p
MRP1m	0.11	0.09	0.21	0.04	0.10	0.72	-0.09	0.13	0.48	0.04	0.07	0.60
MRP1s	-0.02	0.10	0.86	0.04	0.11	0.71	-0.10	0.14	0.49	-0.06	0.11	0.58

The data presents the association of MRP1 protein expression at baseline, 6 months and 30 months with the FEV1 levels at those time points using LME stratified for treatment group, with adjustment for gender, height and age at baseline. MRP1m= moderate staining for MRP1 vs. weak staining for MRP1; MRP1s= MRP1 strong staining for MRP1 vs. weak staining for MRP1; B = regression coefficient; SE= standard error; p= p-value.

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CHAPTER 4

Toll-Like Receptor (*TLR2* and *TLR4*) polymorphisms and Chronic Obstructive Pulmonary Disease

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Abstract

Toll-like receptors (TLRs) participate in the defence against bacterial infections that are common in patients with Chronic Obstructive Pulmonary Disease (COPD). We studied all tagging SNPs in *TLR2* and *TLR4* and their associations with the level and change over time of both FEV_1 and sputum inflammatory cells in moderate-to-severe COPD.

Nine *TLR2* SNPs and 17 *TLR4* SNPs were genotyped in 110 COPD patients. Associations of SNPs with lung function and inflammatory cells in induced sputum were analyzed cross-sectionally with linear regression and longitudinally with linear mixed-effect models.

Two SNPs in *TLR2* (rs1898830 and rs11938228) were associated with a lower level of FEV_1 and accelerated decline of FEV_1 and higher number of sputum inflammatory cells. None of the *TLR4* SNPs was associated with FEV_1 level. Eleven out of 17 SNPs were associated with FEV_1 decline, among which rs12377632 and rs10759931 that were additionally associated with higher numbers of sputum inflammatory cells at baseline and with increase over time.

This is the first longitudinal study showing that tagging SNPs in *TLR2* and *TLR4* are associated with the level and decline of lung function as well as with inflammatory cell numbers in induced sputum in COPD patients, suggesting a role in the severity and progression of COPD.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by inflammation and tissue destruction which are partially maintained by the innate immune defence system [1]. The innate immune response in the airways involves the detection of pathogen- or damage-associated molecular patterns by recognition receptors such as Toll-like receptors (TLRs) on cell surfaces [2]. TLRs participate in the defence against viral and bacterial infections, and such infections contribute to disease progression of COPD. TLRs may thus have a role in COPD development and/or progression.
Especially TLR2 and TLR4 have been studied among the TLRs that recognize gram positive [3] and gram negative bacteria. TLR2 and TlR4 are highly expressed on neutrophils and monocytes/macrophages in COPD [4, 5]. The expression of TLR4, but not TLR2, is increased in neutrophils recovered from bronchoalveolar lavage fluid of smokers with COPD and acute respiratory failure [6] and from sputum of patients with stable COPD patients [5].

The potential impact of functional single nucleotide polymorphisms (SNPs) in the *TLR2* and *TLR4* genes on COPD has been previously investigated [7-10]. For instance Asp299Gly in the *TLR4* was shown to be associated with decreased lipopolysaccharide (LPS) signal transduction [11]. Moreover, the prevalence of Asp299Gly SNP (rs498670) in *TLR4* appeared to be lower in COPD patients than controls [8]. One study suggested that the same SNP, rs498670, might not have a major impact on COPD development, since no significant effects of this SNP on lung function were found [9]. Another study focusing on *TLR2* showed that Arg677Trp (no rs designation available) and Arg753Gln (rs5743708) are not associated with either the onset or the course of COPD [7]. So far, other SNPs in *TLR2* and *TLR4* apart from the most extensively studied SNPs mentioned above have not been studied in relation to COPD. Moreover, it is as yet unknown whether the SNPs in *TLR2* and *TLR4* have any effect on lung function decline or changes in the number of inflammatory cells involved in the innate immune response. Therefore, we investigated the association of all tagging SNPs in *TLR2* and *TLR4*

with the level and decline of lung function and with the level and changes in inflammatory cells in induced sputum over time of subjects with established COPD (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; the GLUCOLD study).

Methods

Study population

We included 114 patients with stage II-III COPD (according to the GOLD criteria [12]) who participated in a two-center trial (the GLUCOLD study). Patient characteristics and methods have been described in detail previously [13]. The patients had irreversible airflow limitation and chronic respiratory symptoms [14] and had neither used a course of oral steroids during the previous 3 months, nor maintenance treatment with inhaled or oral steroids during the previous 6 months. They were current or ex-smokers with a smoking history of ≥10 packyears, aged between 45 and 75 years without a history of asthma. The study was approved by the medical ethics committees of the University Medical Centers of Leiden and Groningen. All patients gave their written informed consent.

Clinical characteristics

Lung function and reversibility to salbutamol were measured as described previously [13]. Sputum induction and whole sample processing were performed as described previously [13] according to a validated technique [15]. The patients were in clinically stable condition and had no symptoms or signs of respiratory tract infection for at least two weeks prior to the study and before each visit [13].

Intervention and follow-up procedures

Patients with mild-moderate COPD were randomly assigned to receive either 1) fluticasone propionate, 500μg twice daily, for the first 6 months followed by placebo, twice daily, for 24 months; 2) fluticasone, 500μg twice daily for 30 months; 3) fluticasone, 500μg twice daily and salmeterol, 50μg twice daily, in a single inhaler for 30 months; 4) placebo, twice daily, for 30 months [16].

Selection of the *TLR2* and *TLR4* tagging SNPs and genotyping

We selected the tagging SNPs in *TLR2* and *TLR4* according to HapMap CEU genotype data (release 24) with an r^2 threshold of 0.8 [17] and Minor Allele Frequency (MAF)>1%, resulting in 9 and 17 tagging SNPs respectively. Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar). DNA was available from 110 out of 114 COPD patients [18].

Statistics

Numbers of nonsquamous inflammatory cells in induced sputum were log transformed to achieve normal distribution. We used linear regression analyses to assess the associations of *TLR2* and *TLR4* SNPs with FEV_1 level and with the number of inflammatory cells in induced sputum at baseline. We adjusted our analyses for age, gender, height, packyears and smoking status. Linear mixed-effect (LME) models were used to asses associations of the *TLR2* and *TLR4* SNPs with change in FEV_1 and inflammatory cells in induced sputum from baseline. Analyses were adjusted for age, gender, height, smoking status, the corresponding initial baseline variable (e.g. for FEV_1 decline adjusted for baseline FEV_1), treatment, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time. We performed LME models with a random intercept at the subject's level, assuming that data is missing at random.

To assess the associations of the *TLR2* and *TLR4* SNPs with the outcomes of the present study we used the general genetic model with heterozygote and homozygote variants coded separately as dummy variables and compared them with the homozygote wild type.

Analyses were performed using SPSS version 18.0 for Windows and values of $p<0.05$ (tested 2-sided) were considered statistically significant.

Results

The clinical characteristics of COPD patients are presented in Table 1 and the numbers of inflammatory cells in induced sputum in Table 2.
All SNPs in *TLR2* and *TLR4* were in Hardy Weinberg Equilibrium ($p>0.05$) and were not correlated with each other ($r^2<0.8$). The prevalence of the SNPs in *TLR2* and *TLR4* is presented in Table S1 and Table S2.

Table 1: Clinical characteristics of COPD patients

	COPD patients (n= 114)
Males, n (%)	99 (86.8)
Age (years)	61.6 (7.7)
Height (cm)	175.5 (7.8)
Packyears¶	41.8 (31.2 – 54.7)
Current smoker, n (%)	72 (63.2)
FEV_1/FVC (%)	49.5 (8.8)
FEV_1 (L)	1.8 (0.4)
FEV_1 % pred.*	56 (10)

*Data are presented as mean (standard deviation) or ¶ median (25^{th} – 75^{th} percentile); FEV_1 = Forced Expiratory Volume in one second; FEV_1/IVC = FEV_1/Inspiratory Vital Capacity; * % pred. = percentage of predicted value.*

Table 2: The number of non-squamous inflammatory cells in induced sputum

Induced sputum	Absolute numbers (10^4/ml)	Percentage (%)
Total cell count*	139.7 (77.9 – 311.3)	
Neutrophils	101.6 (46.8 – 228.5)	72.8 (59.9 – 81.7)
Macrophages	31.1 (17.9 – 61.1)	22.1 (14.8 – 33.2)
Eosinophils	1.3 (0.4 – 4.5)	1.1 (0.3 – 2.2)
Lymphocytes	2.2 (1.1 – 6.8)	1.7 (1.2 – 2.3)
Epithelial cells	1.4 (0.6 – 3.4)	1.0 (0.3 – 2.3)

*Data are presented as median (25^{th} – 75^{th} percentile; *Total cell count refers to the number of non-squamous cells in induced sputum*

TLR2 SNPs and FEV_1 level at baseline and FEV_1 decline

Individuals homozygote for rs1898830 and rs11938228 had a significantly lower FEV_1 level at baseline compared with wild-type individuals [B (95%CI= -267 ml (-522.8 – -13.2) and -240.2 ml (-450.3 – 30.1)] (Table 3).

Individuals heterozygote for rs7656411 and rs4696480 had a significantly higher FEV_1 level at baseline compared with wild-type individuals [185 ml (55.1 – 316.2) and 170.2 ml (22.6 – 317.8) respectively] (Table 3).

Individuals heterozygote for rs1898830 and rs11938228 had a significantly accelerated FEV1 decline compared with wild-type individuals [-2.4 ml/yr (-4.4 – -0.4) and -2.2 ml/yr (-4.2 – -0.1) respectively].

Individuals homozygote for rs1898830, rs3804099 and rs7656411 had significantly less FEV1 decline compared with wild-type individuals [5.2 ml/yr (1.4 – 9.0), 3.1 ml/yr (0.2 – 5.6) and 8.6 ml/yr (4.7 – 12.4) respectively] (Table 3). Other SNPs were not significantly associated.

TLR2 SNPs and inflammatory cells in induced sputum: baseline level and changes from baseline

At baseline, individuals heterozygote for rs11938228 had a significantly higher number of sputum neutrophils, macrophages and eosinophils compared with wild-type individuals [0.51 (0.02 – 0.98), 0.48 (0.06 – 0.89) and 0.68 (0.03 – 1.33) respectively] (Figure 1).

Individuals heterozygote for the same SNP had a significant decrease in sputum neutrophil numbers over time compared with the wild-type individuals [-0.03 (-0.05 – -0.01)] (Figure 1).

Individuals heterozygote for rs3804099 had a significant decrease in neutrophil and macrophage numbers over time compared with wild-type individuals [-0.03 (-0.05 – -0.01) and -0.04 (-0.06 – - 0.01) respectively] (Table S3 and Table S4).

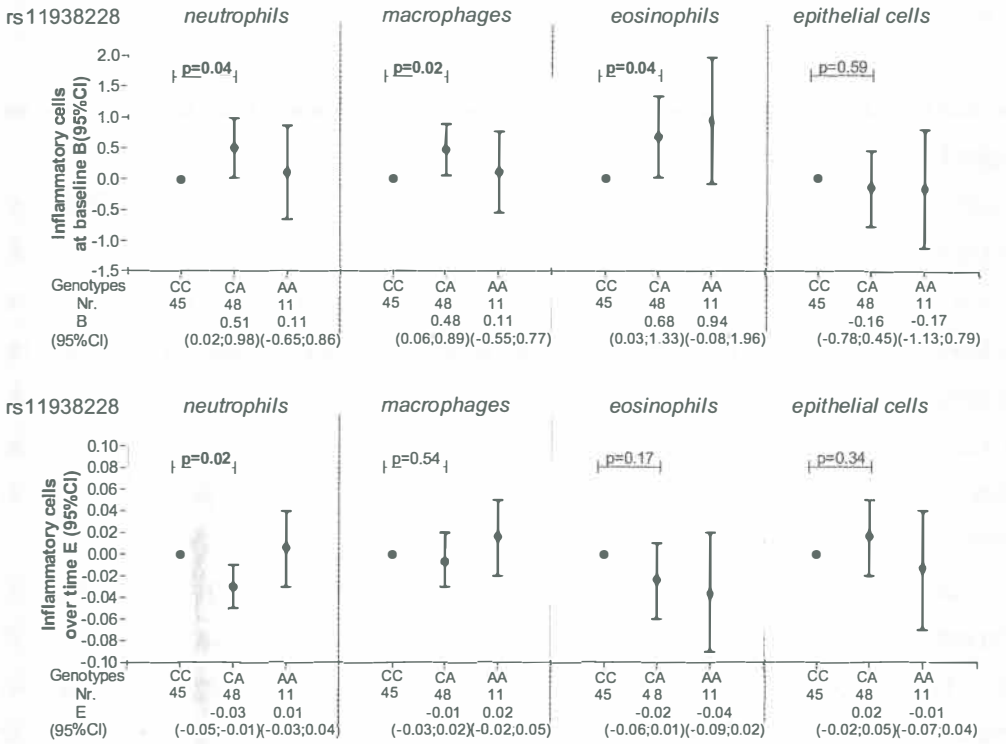
The detailed results of the TLR2 SNPs and inflammatory cells in induced sputum are presented in the data supplement (Table S3, Table S4, Table S5 and Table S6).

Table 3: *TLR2* SNPs and FEV_1 level at baseline and FEV_1 decline

SNP		FEV_1 level (ml) B (95%CI)	p value	FEV_1 decline (ml/yr) E (95%CI)	p value
rs1898830	a	-95.5 (-222.8 - 31.8)	0.140	-2.4 (-4.4 - -0.4)	**0.021**
	b	-267.9 (-522.8 - -13.2)	**0.039**	5.2 (1.4 - 9.0)	**0.008**
rs3804099	a	140.8 (-8.3 - 289.9)	0.064	1.5 (-0.9 - 3.8)	0.219
	b	118.6 (-66.9 - 304.1)	0.208	3.1 (0.2 - 5.9)	**0.036**
rs3804100	a	116.3 (-57.9 - 290.5)	0.188	2.5 (-0.2 - 5.2)	0.073
rs1816702	a	45.8 (-102.7 - 194.2)	0.542	0.1 (-2.3 - 2.4)	0.977
	b	169.9 (-164.5 - 504.4)	0.316	-8.2 (-12.9 - -3.4)	**0.001**
rs11938228	a	-73.0 (-202.4 - 56.5)	0.266	-2.2 (-4.2 - -0.1)	**0.042**
	b	-240.2 (-450.3 - -30.1)	**0.025**	3.1 (-0.2 - 6.3)	0.065
rs7656411	a	185.6 (55.1 - 316.2)	**0.006**	1.9 (-0.2 - 4.0)	0.080
	b	-37.6 (-300.8 - 225.6)	0.778	8.6 (4.7 - 12.4)	**$1.3x10^{-5}$**
rs5743704	a	114.4 (-123.0 - 351.8)	0.341	-2.8 (-6.5 - 0.9)	0.130
rs5743708	a	-72.1 (-268.8 - 124.7)	0.469	-0.1 (-3.1 - 2.8)	0.931
rs4696480	a	170.2 (22.6 - 317.8)	**0.024**	-1.7 (-4.1 - 0.6)	0.143
	b	108.2 (-62.1 - 278.5)	0.210	1.1 (-1.6 - 3.8)	0.413

FEV_1 level adjusted for age, gender, height, pack-year, current smoking; FEV_1 decline adjusted for FEV_1 baseline, age, gender, height, current smoking, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Figure 1: rs11938228 and inflammatory cells in induced sputum



Circles represent the regression coefficient (estimate) and vertical bars the 95% confidence interval (CI); Nr.=number of subjects; Wild type was set as the reference category (CC); At baseline analyses are adjusted for age, gender, height, packyears and smoking status; Over time analyses are adjusted for age, gender, height, smoking status, the corresponding initial baseline variable, treatment, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time.

TLR4 SNPs and FEV_1 level at baseline and FEV_1 decline

None of the TLR4 SNPs was significantly associated with FEV1 level at baseline. Individuals homozygote for rs2737190, rs1927911, rs7846989, rs7037117 and rs10983755 had a significantly accelerated FEV1 decline compared with wild-type individuals [-5.0 ml/yr (-8.7 – -1.3), -5.4 ml/yr (-9.6 – -1.1), -11.0 ml/yr (-20.2 – -1.8), -9.1 ml/yr (-15.1 – -3.2) and -10.7 ml/yr (-19.9 – -1.5) respectively] (Table 4). Individuals heterozygote for rs12377632, rs11536869, rs11536897, rs10759931

and rs11536878 had significantly less FEV1 decline compared with wild-type individuals [2.4 ml/yr (0.1 – 4.7), 6.4 ml/yr (1.1 – 11.7), 7.5 ml/yr (3.8 – 11.3), 2.6 ml/yr (0.2 – 4.9) and 3.5 ml/yr (0.5 – 6.4) respectively] (Table 4).

TLR4 SNPs and inflammatory cells in induced sputum: baseline level and changes from baseline

At baseline individuals heterozygote for rs12377632 had a higher number of sputum neutrophils and eosinophils compared with wild-type individuals [0.50 (0.01 – 0.99) and 1.20 (0.53 – 1.86) respectively] (Figure 2). Individuals homozygote for the same SNP (rs12377632) had a significantly higher number of sputum neutrophils, macrophages and eosinophils at baseline compared with wild-type individuals [1.01 (0.35 – 1.68), 0.85 (0.23 – 1.47) and 1.17 (0.27 – 2.07) respectively] and also a significant increase in numbers over time [0.04 (0.01 – 0.07), 0.06 (0.02 – 0.09) and 0.07 (0.02 – 0.11) respectively] (Figure 2).

Individuals homozygote for rs10759931 had significantly higher sputum neutrophil and macrophage numbers at baseline compared with wild-type individuals [0.88 (0.18 – 1.58) and 0.75 (0.13 – 1.38) respectively] as well as a higher increase in numbers over time [0.05 (0.02 – 0.08) and 0.07 (0.04 – 0.10) respectively] (Figure 3). Individuals heterozygote for the same SNP (rs10759931) had a significantly higher number of eosinophils at baseline and individuals homozygote for the same SNP a significantly higher increase number of eosinophils over time compared with wild-type individuals [0.93 (0.20 – 1.65) and 0.06 (0.02 – 0.11) respectively] (Figure 3).

Individuals carrying rs913930 had significantly lower numbers of macrophages and eosinophils at baseline compared with wild-type individuals [-0.5 (-0.9 – -0.1) and -1.7 (-2.8 – -0.6) in heterozygote and homozygote variants respectively] and a decrease in numbers over time [-0.03 (-0.05 – -0.01) and -0.07 (-0.13 – -0.01) respectively] (Table S8 and Table S9).

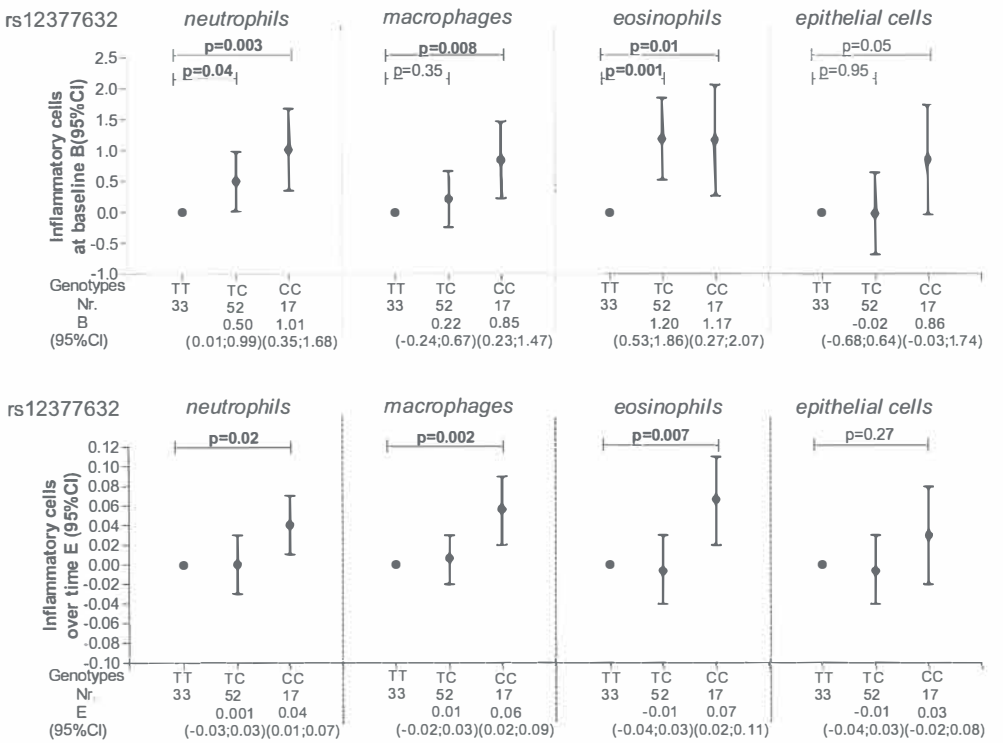
Individuals heterozygote for rs2770150 had a significantly lower number of neutrophils at baseline compared with wild-type individuals [-0.48 (-0.94 – -0.03)] and a larger fall in numbers of neutrophils, macrophages, eosinophils and epithelial cells over time [-0.03 (-0.05; -0.01), -0.02 (-0.04 – -0.001), -0.05 (-0.08 – -0.02) and -0.04 (-0.07 – -0.002) respectively] (Tables S7, S8, S9 and S10).

Table 4: *TLR4* SNPs and FEV_1 level at baseline and FEV_1 decline

SNP		FEV_1 level (ml) B (95%CI)	p value	FEV_1 decline (ml/ yr) E (95%CI)	p value
rs2770150	a	33.3 (-97.8 - 164.3)	0.615	1.7 (-0.3 - 3.7)	0.097
	b	193.2 (-86.3 - 472.6)	0.173	-1.6 (-7.1 - 4.1)	0.587
rs2737190	a	11.3 (-123.1 - 145.5)	0.869	0.8 (-1.3 - 2.7)	0.469
	b	84.6 (-167.3 - 336.5)	0.507	-5.0 (-8.7 - -1.3)	**0.008**
rs10759932	a	-36.9 (-195.6 - 121.8)	0.646	-2.0 (-4.3 - 0.3)	0.084
	b	-19.8 (-503.8 - 463.6)	0.935	-4.6 (-10.4 - 1.3)	0.123
rs1927911	a	56.5 (-77.5 - 190.4)	0.405	-0.2 (-2.2 - 1.9)	0.872
	b	136.6 (-144.8 - 417.9)	0.338	-5.4 (-9.6 - -1.1)	**0.014**
rs4986790	a	-71.9 (-277.5 - 133.8)	0.490	0.8 (-2.4 - 3.9)	0.633
rs11536889	a	-7.7 (-147.2 - 131.9)	0.914	0.8 (-1.4 - 2.9)	0.470
	b	-230.8 (-613.6 - 151.9)	0.234	-1.2 (-6.6 - 4.3)	0.682
rs7856729	a	144.8 (-8.3 - 297.9)	0.063	1.5 (-1.0 - 3.9)	0.244
	b	-6.3 (-467.0 - 454.5)	0.979	-7.3 (-13.8 - -0.8)	**0.028**
rs7846989	a	-168.6 (-348.2 - 11.0)	0.065	-0.9 (-3.5 - 1.7)	0.489
	b	-60.9 (-732.5 - 610.7)	0.858	-11.0 (-20.2 - -1.8)	**0.020**
rs7037117	a	46.8 (-245.6 - 339.1)	0.743	-5.4 (-9.2 - -1.7)	**0.005**
	b	246.7 (-301.1 - 794.4)	0.360	-9.1 (-15.1 - -3.2)	**0.003**
rs10983755	a	-118.2 (-412.6 - 176.3)	0.428	-3.3 (-7.8 - 1.2)	0.151
	b	-43.9 (-712.5 - 624.7)	0.897	-10.7 (-19.9 - -1.5)	**0.023**
rs12377632	a	-7.5 (-150.1 - 135.1)	0.917	2.4 (0.1 - 4.7)	**0.040**
	b	-110.4 (-309.1 - 88.3)	0.273	0.4 (-2.7 - 3.5)	0.793
rs11536857	a	-92.9 (-332.3 - 146.4)	0.443	-0.6 (-4.4 - 3.2)	0.753
	b	-180.2 (-484.4 - 123.9)	0.243	0.2 (-4.1 - 4.5)	0.923
rs11536869	a	-106.1 (-491.7 - 279.5)	0.586	6.4 (1.1 - 11.7)	**0.019**
rs913930	a	-15.6 (-157.6 - 126.3)	0.828	1.4 (-0.7 - 3.5)	0.204
	b	62.9 (-184.3 - 310.1)	0.615	1.8 (-2.4 - 5.9)	0.399
rs11536897	a	110.2 (-115.5 - 335.9)	0.335	7.5 (3.8 - 11.3)	**$9.1x10^{-5}$**
rs10759931	a	-3.3 (-152.9 - 146.4)	0.966	2.6 (0.2 - 4.9)	**0.033**
	b	-94.1 (-292.2 - 104.0)	0.348	-0.9 (-3.9 - 2.1)	0.534
rs11536878	a	143.9 (-28.4 - 316.1)	0.101	3.5 (0.5 - 6.4)	**0.021**
	b	317.1 (-52.2 - 686.4)	0.092	-5.7 (-14.8 - 3.4)	0.217

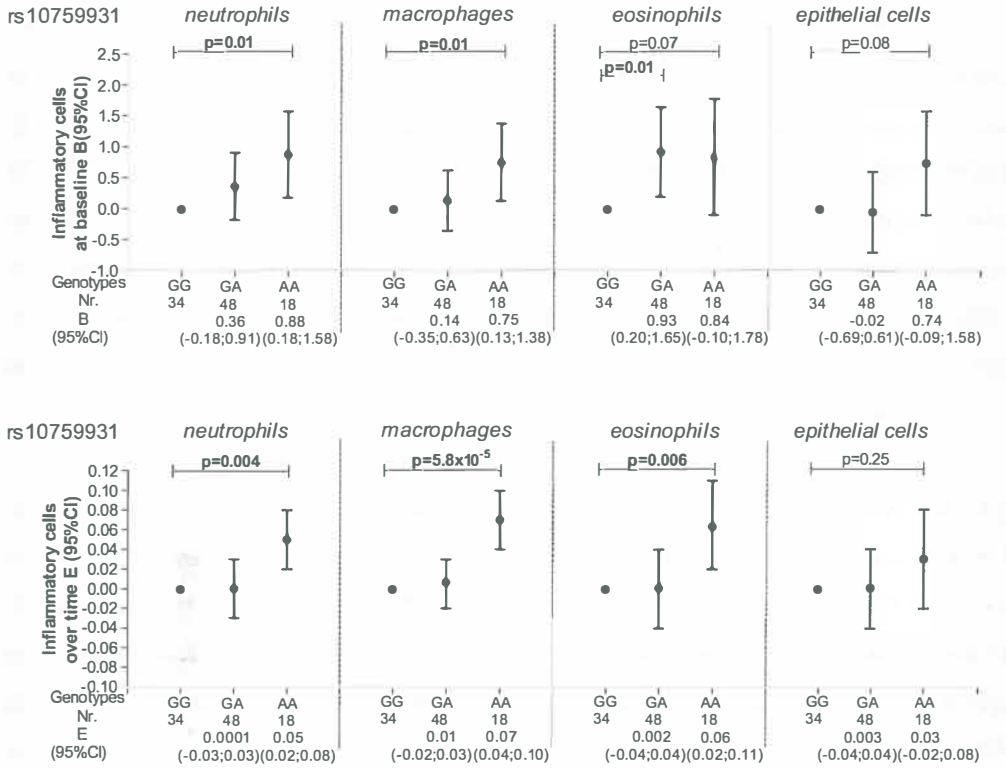
FEV_1 level adjusted for age, gender, height, pack-year, current smoking; FEV_1 decline adjusted for FEV_1 baseline, age, gender, height, current smoking, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Figure 2: rs12377632 and inflammatory cells in induced sputum



Circles represent the regression coefficient (estimate) and vertical bars the 95% confidence interval (CI); Nr.=number of subjects; Wild type was set as the reference category (TT); At baseline analyses are adjusted for age, gender, height, packyears and smoking status; Over time analyses are adjusted for age, gender, height, smoking status, the corresponding initial baseline variable, treatment, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time.

Figure 3: rs10759931 and inflammatory cells in induced sputum



Circles represent the regression coefficient (estimate) and vertical bars the 95% confidence interval (CI); Nr.=number of subjects; Wild type was set as the reference category (GG); At baseline analyses are adjusted for age, gender, height, packyears and smoking status; Over time analyses are adjusted for age, gender, height, smoking status, the corresponding initial baseline variable, treatment, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time.

Discussion

This is the first study investigating comprehensively the genetic contribution of Toll-like receptors to both the severity and the progression of COPD with respect to lung function and inflammation. We investigated 26 tagging SNPs in *TLR2* and *TLR4* and showed consistent associations with the level and decline of FEV_1 as well as with inflammatory cell numbers in induced sputum at baseline and their changes over time.

We found that many SNPs in *TLR2* and *TLR4* were of relevance: 4 out of 9 SNPs in *TLR2* were significantly associated with the level of lung function, 2 with a lower level of FEV_1 (rs1898830 and rs11938228) and 2 with a higher level of FEV_1 (rs7656411 and rs4696480). The SNPs rs1898830, rs3804099 and rs7656411 were significantly associated with less FEV_1 decline and the intronic SNPs rs1898830, rs1816702 and rs11938228 with accelerated FEV_1 decline. The SNP rs11938228 was also associated with an increase in numbers of neutrophils, macrophages and eosinophils in sputum at baseline and with a decrease in numbers of neutrophils over time from baseline, suggesting that these features may either be associated or be influenced by one underlying mechanism. Rs3804099 was significantly associated with a decrease in neutrophils and macrophages over time.

None of the SNPs in *TLR4* was associated with the level of FEV_1, whereas 11 out of 17 SNPs in *TLR4* were significantly associated with accelerated (n=7) or reduced decline of FEV_1 (n=4). Rs12377632 and rs10759931 in *TLR4* were consistently associated with higher numbers of inflammatory cells in induced sputum at baseline and an increase in numbers of inflammatory cells in induced sputum over time from baseline. The other SNPs were associated with either the level or the changes in sputum inflammatory cell numbers from baseline.

Toll-like receptors form a component of the innate immune response which is the first line of defence against invading microorganisms. In humans, 10 functional TLRs have been described [19]. Each TLR expressed in the cellular membrane recognizes molecules such as the lipoproteins of gram-positive bacteria (TLR2) and LPS of gram-negative bacteria (TLR4) [19]. TLRs have been broadly studied in the perspective of microbial and viral infections, inflammation and immune cells [20], but not extensively in COPD. Findings on TLR2 expression on alveolar macrophages, sputum neutrophils and blood monocytes [5,21,22] do suggest a role for this receptor in inflammation that is a characteristic of COPD.
Even less is known on SNPs in *TLR2* and *TLR4* and COPD. Two SNPs in *TLR2* (rs1898830 and rs4696480) are associated with a lower respectively higher level of FEV_1 in the current study and rs1898830 additionally with the decline of FEV_1, suggesting that this SNP might be involved in the progression of the disease as well.

This is compatible with observations in asthma where the same SNPs have been shown to modify the effect of $PM_{2.5}$ exposure on the prevalence of asthma from birth up to 8 years of age [23]. Rs1898830, located in intron 1, was previously associated with TLR2-mediated cellular activation [24]. The authors suggested that this effect might be caused by the effect of the rs13150331 on transcriptional activities of the *TLR2* gene promoter, a SNP in the 5'-flanking region that is in strong LD with rs1898830 [24]. The observed associations in the current study may also be due to the effects of other SNPs in *TLR2* in LD with the intronic SNPs rs1898830 and rs4696480. However, there is accumulating evidence that mutations in the splice, donor and acceptor sites or enhancer, intron and promoter elements may all be important in genetic expression and regulation [25]. Therefore, functional assays are of interest to elucidate the molecular mechanisms underlying these associations.

We confirmed previous observations that there is no significant association of rs5743708 (Arg753Gln), a SNP shown to affect transmembrane signalling of *TLR2* [26], with progression of COPD [7]. Furthermore there was no association with numbers of inflammatory cells in induced sputum, suggesting that this specific SNP may have no impact on COPD.
Interestingly, rs11938228, an intronic SNP in *TLR2* that has not been previously studied, was associated with a lower level of FEV_1 and increased numbers of inflammatory cells in induced sputum at baseline, suggesting that this SNP negatively affects the severity of COPD.

A reduced *TLR4* gene expression was found in nasal epithelium of smokers and severe COPD patients [27]. Other studies showed that the expression of *TLR4* mRNA is inhibited by LPS in a mouse macrophage cell line [28], and stimulated in human neutrophils and monocytes [29]. This apparent discrepancy may reflect the differences in cell type and/or differentiation stages [20]. These findings are of interest given our observation that different SNPs in *TLR4* are associated either with the number of neutrophils or the number of macrophages (Table S8 and Table S9). Two SNPs in *TLR4* namely rs12377632 and rs10759931 were consistently associated with lung function and inflammation. However, the results from baseline and longitudinal analyses for heterozygotes and homozygotes separately require

careful interpretation. For instance both heterozygotes and homozygotes for rs12377632 had a non-significantly lower level of lung function, but a significantly higher number of inflammatory cells in induced sputum, suggesting that this particular SNP may be involved in inflammatory processes representing the first line of defence as reflected in sputum. Additionally, heterozygote individuals for rs12377632 had less lung function decline, while homozygote individuals for the same SNP had a significantly higher number of inflammatory cells over time. This may signify that disease modification can be achieved for particular genotypes in COPD and that changes in induced sputum may represent an activation of the first line of defence yet this is not interrelated with changes in lung function.

Rs10759931 in *TLR4* may have functional consequences on TLR4 expression or signalling activity given its location in the promoter region. This may influence exclusively innate immunity and inflammation, which in turn may affect COPD severity and progression. Therefore, its association with less accelerated FEV_1 decline and increase in numbers of macrophages over time is intriguing since one generally would anticipate that an increase in inflammatory burden associates with accelerated lung function decline. It is thus difficult to reconcile these two observations. This may be due to other genetic effects interacting with this particular SNP and/or differential effects of this SNP on many other underlying mechanisms of changes in lung function, like extracellular matrix turnover or effects on oxidative stress responses. Moreover, gene expression profiling combined with genetics should elucidate whether this SNP indeed is an eQTL (expression quantitative trait locus). Since rs10759931 could be a GATA2 binding site [23], future studies should unravel how the studied SNPs functionally contribute to COPD severity and progression.

Only 2 SNPs in *TLR4* have been previously investigated with respect to COPD; Asp299Gly (rs498670) and Thr399Ile (rs498671) [8-10]. Rs498670 appeared not to be present among patients with COPD who had never smoked [8] and there were no homozygote variants for rs498670 in smokers from the general population (>10 packyears and >40 years of age) [8], comparable to our study. We also confirm the previous findings that the presence of the *TLR4* rs498670 did not have any

significant impact on lung function level [8] and extend this observation by showing that it was also not significantly associated with FEV_1 decline. This indicates that rs498670 has no impact on the severity of COPD at a population level as well as in patients with established COPD.

We here show that rs2770150 was consistently associated with lower numbers of inflammatory cells in induced sputum in COPD although the effect size was small. This promoter SNP might thus positively influence inflammation in COPD patients. Since signalling through TLR2 and TLR4 by hyaluronan may be important in the maintenance of epithelial integrity in the lung after inflammatory insults and in repair [30], it could be that *TLR2* and *TLR4* SNPs are also exhibiting effects in the inflammatory processes in COPD in order to down regulate detrimental signals. More studies are clearly needed to validate these findings and to understand the mechanism by which the *TLR2* and *TLR4* polymorphisms affect the pathological role of TLRs in the signalling pathways involved in COPD, in particular taking into account the effects on level of expression of these receptors on different cell types. For instance there is an increased expression of TLR4 and TLR9 on lung $CD8^+$ T cells [31].

It has been speculated that TLRs could delay FEV_1 decline and thus serve as a therapy target for COPD patients [31,32]. Of reference to our study, COPD patients have reduced TLR4 expression in epithelial cells and corticosteroids dose dependently reduced TLR4 mRNA in an epithelial cell line [27] and increased TLR2 expression [33]. Our patients used inhaled corticosteroids in the randomized GLUCOLD study [16]. Therefore, we adjusted for the period with a change in treatment and its interaction with treatment to avoid any interference with the treatment response. Due to the relatively low number of participants in the study and hence low power, a formal study on gene-treatment interaction was not feasible. However, even with the low numbers of individuals, we were able to find consistent associations of SNPs in *TLR2* and *TLR4* with level and decline of lung function and number of inflammatory cells in induced sputum. Although effects on lung function decline are small, they are consistent for different SNPs. Given the consistency of our results, it is of importance that future studies with a larger sample size of COPD patients confirmed the clinical significance of our findings.

In the current dataset we did not apply a multiple testing correction (i.e. Bonferroni) given the clustering of outcome variables, which might occur jointly at high or low levels account (e.g. a Pearson's correlation coefficient r = 0.79 for macrophages and lymphocytes in induced sputum) or their definition as each others ratios [16].

In summary, previous studies focused on functional SNPs only and studied COPD development exclusively, whereas our is the first study with longitudinal data showing that tagging SNPs in the *TLR2* and *TLR4* genes are associated with the level and decline of lung function as well as with (changes in) numbers of inflammatory cells in induced sputum. These different associations provide insights for future investigations on how these polymorphisms may produce different signatures of genes' activation and how they could eventually contribute to pharmacogenetics in COPD management, which will result in more accurate and targeted therapy.

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Supplementary methods

Clinical characteristics

Sputum induction and processing were performed as described previously [1] according to a validated technique [2]. After inhaling 200 μg salbutamol, patients inhaled hypertonic sodium chloride aerosols (4.5% weight/volume) during three periods of 5 min. Differential cell counts were expressed as a percentage of nucleated cells, excluding squamous cells. A sputum sample was considered adequate when the percentage of squamous cells was <80%.

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Supplementary tables

Table S1: Prevalence of the *TLR2* SNPs

SNP	Genotypes	N (%)	Location	AA position
rs1898830	AA AG GG	46 (43.0) 54 (50.5) 7 (6.5)	Intron 1	-
rs3804099	TT TC CC	27 (25.7) 56 (53.3) 22 (21.0)	Exon 3	Asn199Asn
rs3804100	TT CT CC	93 (85.3) 16 (14.7) 0 (0)	Exon 3	Ser450Ser
rs1816702	CC CT TT	79 (71.8) 27 (24.5) 4 (3.6)	Intron 2	-
rs11938228	CC CA AA	45 (43.3) 48 (46.2) 11 (10.6)	Intron 2	-
rs7656411	TT TG GG	64 (59.3) 38 (35.2) 6 (5.6)	3'region	-
rs5743704	CC CA AA	98 (92.5) 8 (7.5) 0 (0)	Exon 3	His631Pro
rs5743708	GG GA AA	95 (88.0) 13 (12.0) 0 (0)	Exon 3	Gln753Arg
rs4696480	TT TA AA	29 (26.6) 54 (49.5) 26 (23.9)	Intron 1	-

Table S2: Prevalence of the *TLR4* SNPs

SNP	Genotypes	N (%)	Location	AA position
rs2770150	TT TC CC	55 (50.9) 47 (43.5) 6 (5.6)	promotor	-
rs2737190	AA AG GG	50 (47.2) 47 (44.3) 9 (8.5)	promotor	-
rs10759932	TT TC CC	79 (75.2) 23 (21.9) 3 (2.9)	promotor	-

rs1927911	CC CT TT	61 (57.0) 39 (36.4) 7 (6.5)	Intron 1	-
rs4986790	AA AG GG	93 (88.6) 12 (11.4) 0 (0)	Exon 3	Asp299Gly
rs11536889	GG GC CC	75 (68.8) 31 (28.4) 3 (2.8)	3'region	-
rs7856729	GG GT TT	80 (76.2) 23 (21.9) 2 (1.9)	3'region	-
rs7846989	TT TC CC	89 (82.4) 18 (16.7) 1 (0.9)	3'region	-
rs7037117	AA AG GG	19 (63.3) 9 (30.0) 2 (6.7)	3'region	-
rs10983755	GG GA AA	101(93.5) 6 (5.6) 1 (0.9)	promotor	-
rs12377632	TT TC CC	33 (32.4) 52 (51.0) 17 (16.7)	Intron 2	-
rs11536857	CC CT TT	96 (88.1) 8 (7.3) 5 (4.6)	promotor	-
rs11536869	AA AG GG	104 (97.2) 3 (2.8) 0 (0)	Intron 1	-
rs913930	TT TC CC	45 (42.5) 52 (49.1) 9 (8.5)	3'region	-
rs11536897	GG GA AA	99 (91.7) 9 (8.3) 0 (0)	3'region	-
rs10759931	GG GA AA	34 (34.0) 48 (48.0) 18 (18.0)	promotor	-
rs11536878	CC CA AA	87 (82.1) 16 (15.1) 3 (2.8)	Intron 2	-

Table S3: *TLR2* SNPs and neutrophils in induced sputum

SNP		(ln)neutrophils baseline B (95%CI)	p value	(ln) neutrophils change E (95%CI)	p value
rs1898830	a	0.4 (-0.1 - 0.8)	0.114	-0.02 (-0.1 - $3.0x10^{-5}$)	*0.050*
	b	-0.2 (-1.1 - 0.7)	0.622	-0.01 (-0.03 -0.05)	0.746
rs3804099	a	0.2 (-0.3 - 0.7)	0.446	-0.03 (-0.05 - -0.01)	**0.031**
	b	-0.01 (-0.7 - 0.7)	0.972	-0.01 (-0.04 - 0.02)	0.641
rs3804100	a	0.1 (-0.6 - 0.7)	0.915	0.001 (-0.03 - 0.03)	0.967
rs1816702	a	-0.3 (-0.8 - 0.3)	0.332	0.01 (-0.01 - 0.04)	0.313
	b	-1.2 (-2.5 - 0.2)	0.081	0.02 (-0.04 - 0.1)	0.481
rs11938228	a	0.5 (0.1 - 1.0)	**0.041**	-0.03 (-0.05 - -0.01)	**0.021**
	b	0.1 (-0.7 - 0.9)	0.777	0.01 (-0.03 - 0.04)	0.842
rs7656411	a	0.1 (-0.5 - 0.5)	0.921	-0.02 (-0.04 - 0.01)	0.098
	b	-0.3 (-1.3 - 0.6)	0.507	-0.002 (-0.04 - 0.04)	0.944
rs5743704	a	-0.7 (-1.5 - 0.1)	0.085	0.04 (-0.0001 - 0.07)	0.051
rs5743708	a	-0.2 (-0.9 - 0.5)	0.540	-0.01 (-0.04 - 0.02)	0.567
rs4696480	a	0.4 (-0.2 - 0.9)	0.177	-0.01 (-0.03 - 0.02)	0.518
	b	0.2 (-0.5 - 0.8)	0.631	0.001 (-0.03 - 0.03)	0.985

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for neutrophils at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S4: *TLR2* SNPs and macrophages in induced sputum

SNP		(ln)macrophages baseline B (95%CI)	p value	(ln)macrophages change E (95%CI)	p value
rs1898830	a	0.4 (-0.1 - 0.8)	0.083	-0.002 (-0.03 - 0.02)	0.907
	b	-0.5 (-1.3 - 0.3)	0.236	0.04 (-0.01 - 0.08)	0.087
rs3804099	a	0.3 (-0.2 - 0.8)	0.178	-0.04 (-0.06 - - 0.01)	**0.006**
	b	0.4 (-0.6 - 0.6)	0.886	-0.02 (-0.05 - 0.01)	0.226
rs3804100	a	-0.1 (-0.7 - 0.5)	0.811	-0.03 (-0.06 - 0.01)	0.083
rs1816702	a	-0.3 (-0.7 - 0.2)	0.289	0.02 (-0.01 - 0.04)	0.212
	b	-0.5 (-1.6 - 0.8)	0.464	0.03 (-0.03 - 0.08)	0.289
rs11938228	a	0.5 (0.1 - 0.9)	**0.027**	-0.01 (-0.03 - 0.02)	0.546
	b	0.1 (-0.6 - 0.8)	0.748	0.02 (-0.02 - 0.05)	0.408
rs7656411	a	0.1 (-0.3 - 0.6)	0.577	-0.02 (-0.05 - 0.01)	0.066
	b	0.1 (-0.9 - 0.7)	0.793	-0.03 (-0.07 - 0.02)	0.267
rs5743704	a	-0.8 (-1.5 - -0.1)	**0.027**	0.02 (-0.03 - 0.06)	0.453
rs5743708	a	-0.2 (-0.9 - 0.4)	0.496	0.01 (-0.02 - 0.04)	0.527
rs4696480	a	0.1 (-0.4 - 0.6)	0.604	-0.01 (-0.04 - 0.01)	0.302
	b	-0.1 (-0.6 - 0.5)	0.802	-0.03 (-0.05 - 0.01)	0.096

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for macrophages at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S5: *TLR2* SNPs and eosinophils in induced sputum

SNP		(ln)eosinophils baseline B (95%CI)	p value	(ln)eosinophils change E (95%CI)	p value
rs1898830	a	0.5 (-0.2 - 1.1)	0.134	-0.02 (-0.05 - 0.02)	0.273
	b	0.5 (-0.7 - 1.7)	0.399	-0.03 (-0.09 - 0.04)	0.433
rs3804099	a	-0.5 (-1.2 - 0.2)	0.167	-0.01 (-0.04 - 0.03)	0.721
	b	-1.1 (-1.91 - -0.2)	**0.015**	-0.01 (-0.04 - 0.05)	0.790
rs3804100	c	-0.4 (-1.3 - 0.5)	0.604	0.01 (-0.04 - 0.06)	0.664
rs1816702	a	-0.1 (-0.8 - 0.6)	0.735	0.03 (-0.01 - 0.06)	0.118
	b	-1.1 (-2.9 - 0.8)	0.251	0.01 (-0.07 - 0.09)	0.812
rs11938228	a	0.7 (0.03 - 1.3)	**0.043**	-0.02 (-0.06 - 0.01)	0.179
	b	0.9 (-0.1 - 1.9)	0.071	-0.04 (-0.09 - 0.02)	0.199
rs7656411	a	-0.3 (-0.9 - 0.4)	0.483	-0.002 (-0.04 - 0.03)	0.922
	b	-1.6 (-2.9 - 0.4)	**0.012**	-0.01 (-0.06 - 0.07)	0.820
rs5743704	a	-1.0 (-2.1 - 0.1)	0.070	0.04 (-0.02 - 0.09)	0.141
rs5743708	a	0.1 (-0.9 - 1.02)	0.879	-0.02 (-0.06 - 0.03)	0.421
rs4696480	a	-0.1 (-0.8 - 0.7)	0.872	0.03 (-0.01 - 0.06)	0.168
	b	-0.5 (-1.4 - 0.3)	0.210	0.01 (-0.04 - 0.05)	0.745

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for eosinophils at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S6: *TLR2* SNPs and epithelial cells in induced sputum

SNP		(ln) epithelial cells baseline B (95%CI)	p value	(ln) epithelial cells change E (95%CI)	p value
rs1898830	a	-0.3 (-0.9 - 0.3)	0.313	0.01 (-0.03 - 0.04)	0.632
	b	-0.4 (-1.5 - 0.8)	0.523	-0.01 (-0.07 - 0.06)	0.837
rs3804099	a	0.4 (-0.3 - 1.02)	0.249	0.01 (-0.03 - 0.05)	0.749
	b	1.02 (0.2 - 1.8)	**0.013**	0.01 (-0.04 - 0.06)	0.622
rs3804100	a	0.3 (-0.5 - 1.1)	0.462	-0.01 (-0.06 - 0.04)	0.689
rs1816702	a	0.3 (-0.4 - 0.9)	0.441	0.03 (-0.02 - 0.06)	0.222
	b	-0.5 (-2.2 - 1.2)	0.536	-0.01 (-0.09 - 0.07)	0.814
rs11938228	a	-0.2 (-0.8 - 0.5)	0.599	0.02 (-0.02 - 0.05)	0.342
	b	-0.2 (-1.1 - 0.8)	0.727	-0.01 (-0.07 - 0.04)	0.671
rs7656411	a	0.2 (-0.4 - 0.8)	0.559	0.01 (-0.03 - 0.04)	0.841
	b	0.5 (-0.7 - 1.6)	0.455	-0.02 (-0.09 - 0.05)	0.587
rs5743704	a	-0.5 (-1.6 - 0.5)	0.298	-0.01 (-0.07 - 0.06)	0.911
rs5743708	a	-0.4 (-1.2 - 0.5)	0.376	0.02 (-0.04 - 0.06)	0.583
rs4696480	a	0.1 (-0.6 - 0.7)	0.900	0.03 (-0.02 - 0.06)	0.209
	b	0.1 (-0.6 - 0.9)	0.751	-0.01 (-0.05 - 0.03)	0.642

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for epithelial cells at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S7: *TLR4* SNPs and neutrophils in induced sputum

SNP		(ln) neutrophils baseline B (95%CI)	p value	(ln) neutrophils change E (95%CI)	p value
rs2770150	a	-0.5 (-0.9 - -0.03)	**0.039**	-0.03 (-0.05 - -0.01)	**0.022**
	b	0.1 (-0.9 - 1.1)	0.806	-0.02 (-0.08 - 0.04)	0.482
rs2737190	a	-0.3 (-0.8 - 0.02)	0.237	-0.01 (-0.03 - 0.02)	0.784
	b	-0.8 (-1.6 - 0.02)	0.058	0.01 (-0.03 - 0.05)	0.571
rs10759932	a	-0.4 (-0.9 - 0.2)	0.176	-0.01 (-0.03 - 0.02)	0.513
	b	-1.9 (-3.20 - -0.6)	**0.006**	0.02 (-0.04 - 0.08)	0.524
rs1927911	a	0.01 (-0.5 - 0.5)	0.969	0.001 (-0.02 - 0.02)	0.980
	b	-0.5 (-1.4 - 0.5)	0.336	0.02 (-0.03 - 0.06)	0.523
rs4986790	a	-0.4 (-1.1 - 0.4)	0.338	-0.03 (-0.06 - 0.01)	0.098
rs11536889	a	0.1 (-0.4 - 0.6)	0.623	-0.01 (-0.03 - 0.01)	0.386
	b	1.3 (-0.1 - 2.6)	0.061	0.05 (-0.01 - 0.10)	0.098
rs7856729	a	-0.1 (-0.6 - 0.5)	0.879	0.01 (-0.02 - 0.04)	0.552
	b	0.5 (-1.1 - 2.1)	0.523	0.01 (-0.06 - 0.07)	0.858
rs7846989	a	-0.3 (-0.9 - 0.4)	0.463	-0.03 (-0.05 - 0.01)	0.087
	b	-0.8 (-3.1 - 1.6)	0.504	-0.08 (-0.17 - 0.01)	0.066
rs7037117	a	0.2 (-0.8 - 1.1)	0.750	-0.02 (-0.07 - 0.03)	0.427
	b	0.3 (-1.5 - 1.9)	0.763	-0.09 (-0.16 - -0.01)	**0.025**
rs10983755	a	-0.7 (-1.7 - 0.4)	0.188	0.02 (-0.03 - 0.07)	0.427
	b	-0.9 (-3.1 - 1.4)	0.456	-0.08 (-0.17 - 0.02)	0.097
rs12377632	a	0.5 (0.01 - 1.0)	**0.045**	0.002 (-0.03 - 0.03)	0.988
	b	1.0 (0.4 - 1.7)	**0.003**	0.04 (0.01 - 0.07)	**0.023**
rs11536857	a	-0.7 (-1.6 - 0.2)	0.120	-0.06 (-0.10 - -0.01)	**0.013**
	b	-0.6 (-1.7 - 0.5)	0.258	0.02 (-0.03 - 0.06)	0.475
rs11536869	a	-0.5 (-1.9 - 0.8)	0.430	-0.02 (-0.03 - 0.07)	0.470
rs913930	a	-0.4 (-0.9 - 1.1)	0.128	-0.01 (-0.03 - 0.01)	0.426
	b	-0.8 (-1.6 - 0.1)	0.074	-0.01 (-0.06 - 0.04)	0.665
rs11536897	a	0.2 (-0.7 - 1.1)	0.657	-0.01 (-0.05 - 0.03)	0.676
rs10759931	a	0.4 (-0.2 - 0.9)	0.187	7.5x10-5 (-0.03-0.03)	0.995
	b	0.9 (0.2 - 1.6)	**0.014**	0.05 (0.02 - 0.08)	**0.004**
rs11536878	a	0.5 (-0.2 - 1.1)	0.127	0.001 (-0.03 - 0.03)	0.961
	b	-0.1 (-1.4 - 1.2)	0.880	0.08 (-0.01 - 0.16)	0.089

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for neutrophils at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S8: *TLR4* SNPs and macrophages in induced sputum

SNP		(ln)macrophages baseline B (95%CI)	p value	(ln)macrophages change E (95%CI)	p value
rs2770150	a	-0.3 (-0.7 - 0.2)	0.234	-0.02 (-0.04- -0.001)	**0.042**
	b	0.02 (-0.9 - 0.9)	0.962	-0.04 (-0.10 - 0.03)	0.273
rs2737190	a	-0.3 (-0.7 - 0.2)	0.283	-0.01 (-0.03 - 0.02)	0.623
	b	-0.6 (-1.4 - 0.1)	0.096	-0.001 (-0.04 - 0.04)	0.970
rs10759932	a	-0.4 (-0.9 - 0.2)	0.184	-6.0x10-5 (-0.03 -0.03)	0.996
	b	-1.2 (-2.4 - 0.1)	0.058	-0.01 (-0.07 - 0.05)	0.797
rs1927911	a	-0.2 (-0.6 - 0.3)	0.369	0.002 (-0.02 - 0.03)	0.863
	b	-0.7 (-1.5 - 0.2)	0.118	0.01 (-0.03 - 0.06)	0.608
rs4986790	a	0.1 (-0.6 - 0.7)	0.825	-0.04 (-0.07 - -0.003)	**0.034**
rs11536889	a	0.5 (0.02 - 0.9)	**0.043**	-0.02 (-0.04 - 0.01)	0.206
	b	1.2 (0.1 - 2.4)	**0.036**	0.04 (-0.02 - 0.09)	0.232
rs7856729	a	-0.2 (-0.7 - 0.3)	0.411	0.001 (-0.03 - 0.03)	0.941
	b	0.3 (-1.1 - 1.8)	0.653	0.04 (-0.03 - 0.10)	0.300
rs7846989	a	0.1 (-0.6 - 0.6)	0.916	-0.03 (-0.05 - 0.01)	0.090
	b	-0.2 (-2.3 - 1.9)	0.864	-0.08 (-0.17 - 0.02)	0.094
rs7037117	a	-0.2 (-1.0 - 0.6)	0.574	0.01 (-0.04 - 0.05)	0.783
	b	0.5 (-1.0 - 1.9)	0.535	-0.05 (-0.12 - 0.02)	0.164
rs10983755	a	-0.4 (-1.3 - 0.6)	0.428	0.02 (-0.03 - 0.08)	0.373
	b	-0.2 (-2.3 - 1.9)	0.846	-0.07 (-0.16 - 0.03)	0.138
rs12377632	a	0.2 (-0.3 - 0.7)	0.354	0.01 (-0.02 - 0.03)	0.667
	b	0.9 (0.2 - 1.5)	**0.008**	0.06 (0.02 - 0.09)	**0.002**
rs11536857	a	-0.5 (-1.3 - 0.4)	0.259	-0.07 (-0.11 - -0.03)	**0.001**
	b	0.02 (-0.9 - 1.0)	0.964	0.01 (-0.04 - 0.05)	0.737
rs11536869	a	-0.7 (-1.9 - 0.5)	0.236	0.04 (-0.02 - 0.10)	0.153
rs913930	a	-0.5 (-0.9 - -0.1)	**0.037**	-0.03 (-0.05 - -0.01)	**0.031**
	b	-0.4 (-1.2 - 0.3)	0.269	-0.01 (-0.05 - 0.04)	0.692
rs11536897	c	0.3 (-0.6 - 1.1)	0.537	-0.03 (-0.07 - 0.02)	0.227
rs10759931	a	0.2 (-0.4 - 0.6)	0.567	0.01 (-0.02 - 0.03)	0.700
	b	0.8 (0.1 - 1.4)	**0.019**	0.07 (0.04 - 0.10)	**5.9x10-5**
rs11536878	a	0.2 (-0.4 - 0.8)	0.441	-0.001 (-0.03 - 0.03)	0.982
	b	-0.8 (-1.9 - 0.3)	0.156	0.09 (-0.002 - 0.18)	0.056

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for macrophages at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S9: *TLR4* SNPs and eosinophils in induced sputum

SNP		(ln)eosinophils baseline B (95%CI)	p value	(ln)eosinophils change E (95%CI)	p value
rs2770150	a	-0.5 (-1.1 - 0.2)	0.129	-0.05 (-0.08 - -0.02)	**0.003**
	b	-1.0 (-2.3 - 0.3)	0.112	-0.07 (-0.16 - 0.02)	0.097
rs2737190	a	-0.1 (-0.7 - 0.6)	0.828	-0.01 (-0.04 - 0.03)	0.653
	b	-0.6 (-1.7 - 0.6)	0.321	0.05 (-0.01 - 0.11)	0.084
rs10759932	a	-0.2 (-1.0 - 0.6)	0.570	-0.02 (-0.06 - 0.02)	0.219
	b	-0.6 (-2.5 - 1.3)	0.532	0.09 (0.01 - 0.17)	**0.035**
rs1927911	a	-0.3 (-0.9 - 0.4)	0.436	-0.01 (-0.04 - 0.03)	0.841
	b	-0.1 (-1.3 - 1.2)	0.954	0.09 (0.02 - 0.15)	**0.013**
rs4986790	a	0.1 (-0.9 - 1.0)	0.940	-0.04 (-0.08 - 0.02)	0.158
rs11536889	a	0.5 (-1.02 - 1.2)	0.143	0.01 (-0.03 - 0.04)	0.852
	b	0.1 (-1.7 - 1.9)	0.919	0.10 (0.02 - 0.18)	**0.012**
rs7856729	a	-0.2 (-1.0 - 0.5)	0.546	0.03 (-0.01 - 0.07)	0.149
	b	1.3 (-0.9 - 3.4)	0.236	0.08 (-0.05 - 0.19)	0.220
rs7846989	a	0.1 (-0.8 - 1.0)	0.825	-0.03 (-0.07 - 0.02)	0.204
	b	-0.9 (-4.0 - 2.3)	0.573	-0.03 (-0.17 - 0.11)	0.719
rs7037117	a	0.2 (-1.2 - 1.7)	0.756	-0.05 (-0.12 - 0.03)	0.196
	b	0.8 (-1.9 - 3.4)	0.562	-0.07 (-0.22 - 0.08)	0.339
rs10983755	a	-0.1 (-1.5 - 1.3)	0.876	0.03 (-0.05 - 0.10)	0.448
	b	-1.0 (-4.1 – 2.1)	0.536	-0.02 (-0.15 - 0.11)	0.760
rs12377632	a	1.2 (0.5 - 1.9)	**0.001**	-0.01 (-0.04 - 0.03)	0.815
	b	1.2 (0.3 - 2.1)	**0.011**	0.07 (0.02 - 0.11)	**0.007**
rs11536857	a	-1.4 (-2.6 - -0.2)	**0.020**	-0.7 (-0.13 - 0.001)	0.053
	b	0.3 (-1.2 - 1.7)	0.738	-0.01 (-0.08 - 0.05)	0.679
rs11536869	a	-0.2 (-2.0 - 1.6)	0.831	0.01 (-0.08 - 0.08)	0.935
rs913930	a	-0.5 (-1.1 - 0.1)	0.105	-0.04 (-0.07 - -0.01)	**0.023**
	b	-1.7 (-2.8 - -0.6)	**0.003**	-0.07 (-0.13 - -0.01)	**0.037**
rs11536897	a	0.5 (-0.7 - 1.7)	0.427	0.02 (-0.05 - 0.08)	0.578
rs10759931	a	0.9 (0.2 - 1.7)	**0.013**	0.002 (-0.04 - 0.04)	0.903
	b	0.9 (-0.1 - 1.8)	0.079	0.06 (0.02 - 0.11)	**0.006**
rs11536878	a	-1.3x10-5 (-0.9- 0.9)	1.000	0.03 (-0.02 - 0.08)	0.168
	b	0.9 (-0.9 - 2.7)	0.308	0.08 (-0.05 - 0.21)	0.231

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for eosinophils at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table S10: *TLR4* SNPs and epithelial cells in induced sputum

SNP		(ln) epithelial cells baseline B (95%CI)	p value	(ln) epithelial cells change E (95%CI)	p value
rs2770150	a	0.1 (-0.5 - 0.7)	0.772	-0.04 (-0.07- -0.002)	**0.035**
	b	-0.6 (-1.9 - 0.6)	0.310	-0.09 (-0.19 - 0.01)	0.060
rs2737190	a	-0.6 (-1.2 - 0.1)	0.068	0.02 (-0.02 - 0.05)	0.375
	b	-0.2 (-1.3 - 0.8)	0.660	0.04 (-0.02 - 0.10)	0.192
rs10759932	a	-0.6 (-1.3 - 0.2)	0.108	0.02 (-0.02 - 0.06)	0.279
	b	0.1 (-1.7 - 1.7)	0.964	0.04 (-0.05 - 0.13)	0.373
rs1927911	a	-0.7 (-1.3 - 0.1)	**0.022**	0.04 (0.01 - 0.08)	**0.030**
	b	-0.3 (-1.4 - 0.8)	0.591	0.04 (-0.03 - 0.11)	0.235
rs4986790	a	0.5 (-0.5 - 1.4)	0.299	-0.05 (-0.11 - 0.01)	0.072
rs11536889	a	0.3 (-0.4 - 0.9)	0.443	-0.03 (-0.06 - 0.01)	0.166
	b	0.6 (-1.1 - 2.2)	0.488	0.02 (-0.07 - 0.10)	0.692
rs7856729	a	-0.5 (-1.2 - 0.2)	0.147	0.04 (0.001 - 0.09)	**0.048**
	b	01. (-1.9 - 2.1)	0.922	0.01 (-0.09 -0.11)	0.812
rs7846989	a	0.7 (-0.1 - 1.5)	0.081	-0.03 (-0.07 - 0.02)	0.252
	b	1.3 (-1.5 - 4.1)	0.364	0.03 (-0.12 - 0.17)	0.724
rs7037117	a	-0.6 (-1.5 - 0.3)	0.173	0.03 (-0.03 - 0.09)	0.270
	b	0.5 (-1.2 - 2.2)	0.551	-0.01(-0.09 - 0.09)	0.955
rs10983755	a	-0.9 (-2.2 - 0.4)	0.166	-0.02 (-0.10 - 0.06)	0.656
	b	1.1 (-1.8 - 3.9)	0.456	0.04 (-0.11 - 0.18)	0.647
rs12377632	a	-0.02 (-0.7 - 0.7)	0.952	-0.01 (-0.04 - 0.03)	0.792
	b	0.9 (-0.1 - 1.8)	0.059	0.03 (-0.02 - 0.08)	0.278
rs11536857	a	0.1 (-1.0 - 1.2)	0.875	0.01 (-0.06 - 0.08)	0.872
	b	0.4 (-1.0 - 1.7)	0.579	-0.05 (-0.11 - 0.02)	0.190
rs11536869	a	0.01 (-1.7 - 1.7)	0.991	0.01 (-0.08 - 0.09)	0.916
rs913930	a	-0.4 (-1.0 - 0.3)	0.228	-0.02 (-0.06 - 0.01)	0.198
	b	-0.5 (-1.6 - 0.6)	0.358	-0.002 (-0.07 - 0.07)	0.967
rs11536897	a	-0.02 (-1.2 - 1.1)	0.966	0.03 (-0.04 - 0.09)	0.409
rs10759931	a	-0.1 (-0.7 - 0.6)	0.894	0.002 (-0.04 - 0.04)	0.906
	b	0.8 (-0.1 - 1.6)	0.084	0.03 (-0.02 - 0.08)	0.257
rs11536878	a	-0.4 (-1.2 - 0.5)	0.376	0.04 (-0.02 - 0.08)	0.175
	b	-0.7 (-2.4 - 0.9)	0.389	0.03 (-0.12 - 0.17)	0.737

Baseline analysis are adjusted for age, gender, pack-year, current smoking; Change analysis are adjusted for epithelial cells at baseline, age at baseline, gender, current smoking at baseline, treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time; a= heterozygotes vs. wild-type;b= homozygote variant vs. wild-type.

CHAPTER 5

HHIP associates with higher FEV_1 in smokers of the general population, and protects against accelerated FEV_1 decline in COPD patients

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Abstract

GWA studies have identified variants near the *Hedgehog-interacting protein* (*HHIP)* gene to be associated with COPD and lung function.

We investigated both the level of lung function and lung function decline using LME models in subjects from a general population-based cohort Doetinchem (n=1,152), and in subjects with established COPD (n=110, GLUCOLD). We used linear regression for the associations of rs1032295 and rs13147758 with level of forced expiratory volume in one second (FEV_1) in the total cohorts and stratified according to smoking status.

In the general population, rs13147758 was associated with a higher level of FEV_1 in smokers [B (95%CI)=163.4ml (14.6; 312.1)], but not with FEV_1 decline. In COPD, this SNP was associated with less FEV_1 decline, an effect that was larger in smokers [4.4ml/yr (1.6; 7.2)].

This study shows protective effects in smokers and COPD patients. The level of lung function was higher in smokers having the rs13147758 SNP. Of interest, variants in *HHIP* were significantly associated with less lung function decline in COPD, both in the total population and in smokers.

Introduction

Cigarette smoking is the leading cause in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD). However, since not all smokers develop COPD, other factors play an important role which may indicate different individual susceptibility to COPD [1]. Accumulating evidence suggests that an interaction between the "host" factors such as genetics, lung growth, airway responsiveness, gender and race, and the "environmental" factors such as air pollution, smoking, socio-economic status, bacterial and viral infections and diet, may influence the development of COPD [1]. In addition there are suggestions that genes originally linked to lung developmental processes could be implicated in the development of COPD, as indicated by microarray profiling and genome wide association (GWA) studies in human subjects and animal models [2].

One of the genes involved in lung developmental processes is *Hedgehog-interacting protein (HHIP)*, which was discovered by screening a mouse cDNA expression library for proteins that bind to Sonic hedgehog (SHH) [3]. A study showed that a loss in *HHIP* function in *Hip1*- null mice leads to recessive postnatal lethality due to respiratory distress and hypoplastic lungs [4]. Therefore, HHIP is an important regulator of the Hedgehog pathway and has been implicated in development and repair. Moreover, airway branching is inhibited in the lungs of *HHIP*-null mice due to increased SHH activity and almost complete suppression of fibroblast growth factor 10 (Fgf10) expression in the developing lung [4]. Thus, lower levels of HHIP lead to reduced levels of Fgf10, lung hypoplasia and insufficient lung growth [4, 5]. An increasing number of studies stress the importance of early life events in the development of obstructive lung diseases such as COPD, and *HHIP* might be a developmental gene involved in COPD, a disease associated with structural defects in small airways and inappropriate lung growth [6].

Therefore, it is of interest that GWA studies identified that single nucleotide polymorphisms (SNPs) located on chromosome 4 in an intergenic region near the *HHIP* gene are associated with COPD and lung function level. [7;, 8] Furthermore, in a meta-analysis of GWAs results, 27 SNPs in the *HHIP* region reached genome-wide significance for their association with FEV_1/FVC [9].

Previous studies have focused on the association of the SNPs in the *HHIP* region with level of lung function (FEV_1 and FEV_1/FVC) in the general population. However, these studies can provide information only about the cross-sectional association of the gene with lung function level, not about the natural occurring decline in lung function. We had the unique opportunity to additionally investigate the association of these SNPs with annual lung function decline in subjects from the general population-based cohort Doetinchem.

Since COPD patients already have an impaired lung function we expect that the *HHIP* gene acts differently in the presence of the disease. We hypothesize that there is a relation between the impaired lung function and this developmental gene involved in the airway branching morphogenesis. Therefore, we investigated whether these SNPs are also associated with small airway function and lung function level and decline after COPD has developed. Furthermore, since previous studies suggested a protective effect of the gene in the presence of smoking, we investigated the above mentioned associations also by stratifying our data according to smoking status.

Methods

Doetinchem cohort study

Subjects from the Doetinchem cohort study [10], a prospective part of the larger MORGEN study [11] were included in the present study. A random sub-sample of 1152 subjects was selected from the total cohort with spirometry tests and DNA available (n=3224) as described elsewhere [12].

In the Doetinchem cohort study subjects were tested for pre-bronchodilator lung function (FEV_1 and FVC) three times with 5-year intervals (1994-2007) [12] according to the European Respiratory Society (ERS) guidelines. [13] In the random sample 100%, 100% and 70, 4% subjects participated in the first, second and third survey respectively.

GLUCOLD

A total of 114 COPD patients who participated in a two-center trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study) were included in the study. Patient characteristics and methods have been

described in detail previously [14]. In brief, inclusion criteria were: current or ex-smokers with a smoking history of ≥10 pack-years, aged between 45 and 75 years without a history of asthma. They had irreversible airflow limitation, chronic respiratory symptoms and did not use a course of oral steroids during the previous 3 months and had no maintenance treatment with inhaled or oral steroids during the previous 6 months. The study was approved by the medical ethics committees of the University Medical Centers of Leiden and Groningen. All patients gave their written informed consent.

Reversibility to salbutamol was measured at baseline, 6 and 30 months and FEV_1 and IVC post bronchodilator were measured every three months using standardized protocols [15]. We used the measurements at baseline, 6 and 30 months for instantaneous expiratory flows ($FEF_{50-75\%}$), total lung capacity (TLC) and residual volume (RV) that were measured as previously described [14]. Reference values for all lung function measurements were obtained from Quanjer and colleagues [13].

Intervention and follow-up procedures

Patients were randomly assigned to receive either: 1) fluticasone propionate, 500μg twice daily, for the first 6 months followed by placebo, twice daily, for 24 months; 2) fluticasone, 500μg twice daily for 30 months; 3) fluticasone, 500μg twice daily and salmeterol, 50μg twice daily, in a single inhaler for 30 months; 4) placebo, twice daily, for 30 months [16].

Selection of the HHIP SNPs and genotyping

From the previous *HHIP* SNPs identified in the GWAs we selected 2 SNPs (rs1032295 and 13147758) that were not highly correlated with each other (r2=0.74). The correlation was assessed using Haploview (version 4.2) [17]. Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar). DNA was available from 110 out of 114 COPD patients [18], but for one subject the genotyping failed. Data was available for 109 COPD patients and for 1152 subjects from the general population.

Statistics

We used linear regression to assess the association of the *HHIP* SNPs and FEV_1,

FEV_1/IVC, $FEF_{50-75\%}$, RV and RV/TLC ratio at baseline in GLUCOLD and FEV_1, FEV_1/FVC level in Doetinchem. We adjusted our analyses for age, gender and height.

We used linear mixed-effect (LME) models to asses the association of the *HHIP* SNPs with FEV_1 and FEV_1/IVC decline, small airway function ($FEF_{50-75\%}$) and air trapping (RV and RV/TLC ratio) over time in GLUCOLD, and FEV_1 and FEV_1/FVC decline in Doetinchem. Analyses were adjusted for age, gender, height, smoking status, the corresponding initial baseline variable (i.e. for FEV_1 decline adjusted for FEV_1 initial) and the interaction of all variables with time. In the GLUCOLD study we additionally adjusted our analyses for treatment, the period when there is a change in treatment and its interaction with treatment and their interaction with time. We performed LME models with a random intercept at the subject's level, assuming that data is missing at random.

To asses the associations of the *HHIP* SNPs with the outcomes in the present study we used the general genetic model with heterozygote and homozygote variants coded separately as dummy variables and compared to the homozygote wild type. Analyses were performed using SPSS version 16.0 for Windows and p-values $p<0.05$ (tested 2-sided) were considered statistically significant.

Results

The clinical characteristics of subjects from the general population (Doetinchem) and COPD patients (GLUCOLD) are presented in tables 1 and S1 (see supplementary material).

The SNPs rs1032295 and rs13147758 in the *HHIP* region genotyped in our populations were in Hardy Weinberg Equilibrium (HWE, $p>0.05$) and were not correlated with each other, r^2 being 0.54 in COPD patients and 0.55 in the general population-based cohort. Table S2 (supplementary material) shows the prevalence of rs1032295 and rs13147758 in our study populations.

1. Doetinchem general population based-cohort

The SNPs in the *HHIP* region were not significantly associated with the level of FEV_1 or FEV_1/FVC at the first survey. The results are presented in the data supplement (Table S3; supplementary material). When stratified according to smoking status, currently smoking individuals homozygote for rs13147758 had a significantly higher level of FEV_1 (B (regression coefficient) (95%CI) =163.4 ml (14.6; 312.1)) compared to wild-type individuals (Table 2).

Both SNPs were not significantly associated with FEV_1 decline in the total population or after stratification according to smoking status (Table S4; supplementary material). No significant association was found with FEV_1/FVC decline in the total population, but ex-smokers individuals heterozygote for rs1032295 had significantly less FEV_1/FVC decline (E (estimate) (95%CI) = 0.2 (0.1; 0.4) compared to wild-type (Table S5; supplementary material).

Table 1: Clinical characteristics at baseline

	Doetinchem, n=1152	GLUCOLD, n=114
Duration of follow-up, years	5	2.5
Number of surveys, median	3	6
Males, n (%)	541 (47.0)	99 (86.8)
Age, years	45.3 (9.9)	61.6 (7.7)
Height, cm	172.6 (9.1)	175.5 (7.8)
Pack-years*	5.5 (0 – 16)	41.8 (31.2 – 54.7)
Current smoker, n (%)	351 (30.5)	72 (63.2)
Ex-smoker, n (%)	446 (38.7)	42 (36.8)
Never smoker, n (%)	355 (30.8)	-
FEV_1, L	3.6 (0.8)	2.03 (0.5)
FEV_1/IVC (%)	77.4 (7.2)**	48.2 (8.5)
FEV_1 % predicted	106.3 (14.1)	63.0 (8.7)

*Data are presented as mean (SD) or *median (25th-75th percentile) for the subjects with available SNPs genotypes. **FEV_1/FVC; FEV_1= forced expiratory volume in one second; FVC=forced vital*

capacity; IVC =inspiratory vital capacity. In GLUCOLD the measurements for lung function are post bronchodilator.

2. COPD patients (GLUCOLD)

SNPs in the *HHIP* region were not significantly associated with any of the baseline lung function parameters (FEV_1, FEV_1/IVC, $FEF_{50-75\%}$, RV and RV/TLC). The detailed results of these analyses are presented in the data supplement (Tables S3 and S8; supplementary material). Additional adjustment for smoking status did not change these results.

Rs1032295 was not significantly associated with annual FEV_1 decline in COPD patients in the total population or stratified according to smoking status (Table S6; supplementary material). Individuals heterozygote for rs13147758 had significantly less FEV_1 decline per year (E (95%CI) = 2.7 ml/yr (0.7; 4.8)) compared to wild-type individuals (Table S6; supplementary material). In smokers, individuals heterozygote for rs13147758 had significantly less FEV_1 decline per year (4.4 ml (1.6; 7.2)) compared to wild-type individuals (Figure 1). In the genetic dominant model (where heterozygote and homozygote variants are pooled together), minor allele carriers for rs13147758 had significantly less FEV_1 decline per year (2.9 ml (0.3; 5.8)) compared to wild-type individuals as well.

Both rs1032295 and rs13147758 were significantly associated with less FEV_1/IVC decline (0.06 (0.01; 0.11) and 0.09 (0.04; 0.13) respectively) (Table S7; supplementary material).This association was seen especially in ex-smokers where individuals heterozygote for rs1032295 had less FEV_1/IVC decline (0.10 (0.02; 0.17)) while smokers homozygote for the same SNP had an accelerated FEV_1/IVC decline (-0.12 (-0.22;-0.01)) compared to wild-type individuals (Table S7; supplementary material).

Neither of the 2 SNPs in the *HHIP* region was significantly associated with annual decline in $FEF_{50-75\%}$, RV and RV/TLC (Table S8; supplementary material).

Table 2: The association of SNPs in the *HHIP* region and FEV_1 at baseline stratified according to smoking status in the general population and COPD patients

SNP		FEV_1 (ml) level, B (95%CI)	p	FEV_1 (ml) level, B (95%CI)	p
		Doetinchem current smokers		**GLUCOLD current smokers**	
rs1032295	a	-9.7 (-116.1 - 96.6)	0.858	52.8 (-83.5 - 189.1)	0.442
	b	125.7 (-27.6 - 279.1)	0.108	187.2 (-27.6 - 402.1)	0.087
rs13147758	a	11.2 (-95.4 - 117.7)	0.837	58.5 (-79.88 - 196.9)	0.401
	b	163.4 (14.6 - 312.1)	**0.031**	7.1 (-201.1 - 215.2)	0.946
		Doetinchem ex-smokers		**GLUCOLD ex-smokers**	
rs1032295	a	-36.5 (-130.2 - 57.2)	0.445	33.1 (-192.7- 258.9)	0.768
	b	-102.9 (-235.2 - 29.6)	0.128	-143.5 (-431.2 -144.2)	0.318
rs13147758	a	-7.2 (-100.9 - 86.5)	0.880	0.4 (-231.9 - 232.6)	0.997
	b	-58.3 (-186.8 - 70.2)	0.373	54.4 (-237.2 - 345.9)	0.707
		Doetinchem never smokers			
rs1032295	a	36.2 (-66.8 - 139.2)	0.490		
	b	53.6 (-94.1 - 201.2)	0.476		
rs13147758	a	-10.4 (-116.1 - 95.2)	0.846		
	b	51.4 (-90.47 - 193.24)	0.477		

HHIP =hedgehog interacting protein; B (95%CI) = regression coefficient (95% confidence interval); FEV_1= forced expiratory volume in one second; Analyses are adjusted for gender, age and height. The significant results are depicted in bold with p-value >0.05; a=heterozygote vs. wild-type; b=homozygote vs. wild-type.

Discussion

In the current study we found that the intergenic SNP rs13147758 near the *HHIP* gene is significantly associated with a higher level of FEV_1, but not with FEV_1 decline

in smokers from the general population rs13147758. Of interest, in COPD patients, we observed that rs13147758 was significantly associated with less accelerated decline in lung function (FEV_1 and FEV_1/IVC), an effect that was enhanced in smokers with small effect sizes. The second SNP we investigated, rs1032295, was significantly associated with changes in FEV_1/IVC in COPD patients, and effects were modest here as well.

SNPs in the *HHIP* region have been identified in GWA studies to be associated with COPD, level of lung function and height in the general population [7, 8, 19]. Moreover, some of these SNPs reached genome-wide significance for association with the level of FEV_1/FVC and FEV_1 in a meta-analysis of the latest GWAs results [9]. In table 3 we present an overview of the associations of rs1032295 and rs13147758 with lung function measurements in studies of the general population, including the current study and table 4 shows the results of the meta-analyses of the p-values from the general populations presented in table 3. Rs13147758 shows a consistent pattern of association with lung function level among all studies. Our study had a similar direction of effect, though the association did not reach significance probably due to a smaller sample size (Table 3). A striking observation in our study is that individuals homozygote for rs13147758 have a higher level of FEV_1 (50.5 ml (-29.9; 130.9)) compared to wild-type, an effect that is even more pronounced in smokers (163.4 ml (14.6; 312.1)). This is in line with a previous study showing that the effect size increases and p-values decrease after restricting analyses to ever smokers [8]. A former study on the *HHIP* gene found rs13118928, which is highly correlated with rs13147758 (r^2=0.96, in HapMap CEU reference sample), to be associated with a lower risk of COPD [20]. Moreover, the protective effect of having one or more variant alleles was particularly present in subjects having > 36 packyears smoking (OR=0.8, p-value=0.007) [20]. Thus, it seems that particularly smokers in the general population benefit in lung function when being homozygote for rs13147758.

To our knowledge this is the first study showing that rs1032295 and 13147758 were significantly associated with less FEV_1/IVC decline in patients with established COPD.

Moreover, rs13147758 was significantly associated with less FEV_1 decline, a positive association that was enhanced in smokers only, suggesting that this SNP has a protective effect in the presence of smoking. In line with this, rs13147758 was significantly associated with a lower FEV_1/IVC ratio and FEV_1 decline in COPD patients with > 41.8 packyears (median of population) (Table S9; supplementary material). Interestingly, we observed in COPD patients significant positive associations with lung function decline for heterozygotes, but not homozygotes for rs13147758, which might be due to low numbers of homozygotes, thus, lack of power. Our results were consistent across our outcome variables since we found the same pattern of associations with FEV_1/IVC in the total COPD population and in smokers. Given the relatively small effect size of these SNPs and the putative clinical relevance, replication of these findings is needed. Hedgehog signalling is activated in human bronchial epithelial cells exposed to cigarette smoke [21]. The *HHIP* gene encodes the Hedgehog-interacting protein functioning as a Hedgehog inhibitor [3], thus rs13147758 might positively influence *HHIP* promoter activity upon smoke exposure, alone or through other causal variants. Future studies should focus on the functionality of the rs1032295 and rs13147758 SNPs and investigate whether they have regulatory effects on *HHIP* function.

There is strong evidence that an impaired airway function can start *in utero* [22] and tracks from birth towards early childhood [23] and from childhood to adulthood [24]. Evidently, genetic factors may play a modifying role during different phases of life, affecting the predisposition to COPD. A deficit in airway branching [4] or smooth muscle cell differentiation [25] in combination with exposure to cigarette smoke later in life might lead to development of COPD. We here observe in smokers from our general population that the *HHIP* gene is associated with a higher level of FEV_1 only, which may be the ultimate effect of lung development and smoking in relation to the *HHIP* gene alterations.

It is well known that remodelling can occur in COPD as a response to injury and inflammation, changes that might influence the maintenance of normal lung tissue function [26]. This inflammatory response is followed by a repair process, which if successful, results in recovery of tissue architecture and function [27].

Table 3: Summary of the association of SNPs in the *HHIP* region and lung function measurements

HHIP SNPs	Sample size	FEV_1 level	FEV_1 decline	FEV_1/FVC level	FEV_1/FVC decline	Presence of COPD	Cohort/Study
rs1032295	1,152	- (0.84)	- (0.91)	- (0.92)	+ (0.15)	- (0.84)	Doetinchem, current study
	17,500	+ (0.26)	n.i.	n.i.	n.i.	n.i.	British 1958 birth cohort (a)
	20,890	- ($4.37x10^{-2}$)	n.i.	- (**$6.28x10^{-9}$**)	n.i.	n.i.	CHARGE GWAs (b)
	16,178	- (**$5.01x10^{-7}$**)	n.i.	- (**$1.15x10^{-7}$**)	n.i.	n.i.	SpiroMeta replication (c)
	37,068	- ($7.95x10^{-7}$)	n.i.	- (**$4.37x10^{-15}$**)	n.i.	n.i.	CHARGE+SpiroMeta (d)
rs13147758	1,152	+ (0.21)	+ (0.42)	+ (0.63)	+ (0.93)	- (0.96)	Doetinchem, all current study
	351	+ (**0.03**)	- (0.89)	+ (0.50)	+ (0.88)	- (0.46)	*within smokers, current study*
	17,500	+ (0.12)	n.i.	n.i.	n.i.	n.i.	British 1958 birth cohort (a)
	20,890	+ (**$5.30x10^{-2}$**)	n.i.	+ (**$4.90x10^{-11}$**)	n.i.	n.i.	CHARGE GWAs (b) #
	16,178	+ (**$1.43x10^{-10}$**)	n.i.	+ (**$1.09x10^{-10}$**)	n.i.	n.i.	SpiroMeta replication (c)
	37,068	+ (**$7.95x10^{-7}$**)	n.i.	+ (**$3.21x10^{-20}$**)	n.i.	n.i.	CHARGE+SpiroMeta (d)
	7,691	+ (0.03)^	n.i.	+ (**$2.3x10^{-8}$**)^	n.i.	n.i.	Framingham Heart Study (e)
	4,141	+ (0.09)^		+ ($8.1x10^{-5}$)^			*within smokers*
	823	+ (0.004)^	n.i.	+ (0.0002)^	n.i.	n.i.	Family Heart Study (e)
	485	+ (0.0001)^		+ ($2.2x10^{-6}$)^			*within smokers*
	1,609	+ (0.058)	n.i.	+ (**$1.9x10^{-4}$**)	n.i.	n.i.	ECLIPSE (f) ¶
	742	n.i.	n.i.	n.i.	n.i.	- (**$1.0x10^{-4}$**)	Rotterdam Study (g) ¶
	277					- (**0.007**)	*especially for >36 pack-year*
	4,395	i	n.i.	n.i.	n.i.	- ($1.67x10^{-7}$)	GWAs COPD (h)
	4,320	n.i.	n.i.	n.i.	n.i.	- (**$9.24x10^{-7}$**)	GWAs COPD (i) ¶

*The table shows the direction of the association with the corresponding p-value between brackets; FEV_1= forced expiratory volume in one second; FVC=forced vital capacity; * FEV_1/IVC (FEV_1/inspiratory vital capacity) was used; + = positive association (i.e. higher FEV_1 level or less rapid FEV_1 decline); - = negative association (i.e. lower FEV_1 level or faster FEV_1 decline); n.i. = not investigated; i. = One association without a mentioned direction; ^= data is presented for % predicted; # =rs13147758 in Linkage Disequilibrium (LD) with rs1980057 (r^2=1); ¶ = rs13147758*

in LD with rs13118928 (r^2=0.96);The significant results are depicted in bold with p-value >0.05 or any threshold for the specific study ($x10^{-4}$, $x10^{-7}$or $x10^{-8}$); (a) = (Power and Elliott, 2006); (b) = (Hancock, 2010; Psaty, 2009); (c) = (Hancock, 2010; Repapi, 2010); (d) = (Hancock, 2010); (e) = (Wilk, 2009); (f) = (Pillai, 2010); (g) = (van Durme, 2010); (h) = (Pillai, 2009); (i) = (Cho, 2010).

Table 4: Meta-analyses of the p-values from the general populations presented in table 3

SNP	lung function parameter	p-value meta-analyses
rs1032295	FEV_1 level	$2.18x10^{-10}$
	FEV_1/FVC level	$1.59x10^{-25}$
rs13147758	FEV_1 level	$3.74x10^{-16}$
	FEV_1/FVC level	$5.69x10^{-46}$

FEV_1= forced expiratory volume in one second; FVC=forced vital capacity

While in subjects from the current general population-based cohort we did not see a significant association with FEV_1 decline, in subjects with established COPD, the *HHIP* gene was associated with less accelerated FEV_1 decline in smokers suggesting that this gene might be involved in a balance between repair and cigarette induced lung injury. It is therefore likely that an understanding of the process by which lung development occurs could be connected to redevelop normal lung structure where damage has already occurred [28].

Since the *HHIP* gene is a developmental gene involved in branching morphogenesis, we investigated its association with changes in small airways function. However, rs1032295 and rs13147758 were not associated with levels or changes of small airways function in COPD, as defined by the $FEF_{50-75\%}$ measurements and air trapping or hyperinflation (RV and RV/TLC). This may be a true finding, or reflect lack of power to detect associations, since we had only 3 data points for the air trapping parameters.

All together our findings are of importance since the hedgehog pathway is involved in airway branching during lung development. It would be interesting to study this gene in the context of lung growth and its interaction with environmental as well as personal smoke exposure, since we observed positive associations with the level and decline of FEV_1 especially in smokers. This specific association should be

assessed in cohorts followed from birth up to the plateau phase in lung development. Continuing elucidation of the mechanisms of lung development may identify novel therapeutic targets in the quest to prevent and treat COPD.

In conclusion, this study corroborates the findings of previous studies and extends these by showing that this developmental gene may play an important role in protection to accelerated lung function loss in COPD.

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Supplementary Results

Table S1: Clinical characteristics at baseline for small airways parameters in participants of GLUCOLD

GLUCOLD n=114	
FEF_{50}, L/s	0.9 (0.4)
FEF_{75}, L/s)	0.3 (0.1)
RV, L	3.6 (0.9)
TLC, L	7.4 (1.3)
RV/TLC, %	48.2 (8.0)

FEF_{50} = forced expiratory flow at 50% of the FVC (forced vital capacity); FEF_{75}= forced expiratory flow at 75% of the FVC; RV =residual volume; TLC =total lung capacity.

Table S2: Prevalence of the SNPs in the *HHIP* region

		Doetinchem	GLUCOLD	p-value
rs1032295	TT	417 (36.6)	48 (44.0)	0.290
	TG	559 (49.0)	46 (42.2)	
	GG	164 (14.4)	15 (13.8)	
rs13147758	AA	392 (34.5)	39 (35.8)	0.930
	GA	563 (49.6)	54 (49.5)	
	GG	181 (15.9)	16 (14.7)	

Table S3: The association of SNPs in the *HHIP* region with FEV_1 and FEV_1/IVC at baseline in Doetinchem and GLUCOLD.

		Doetinchem		GLUCOLD	
SNP		**FEV_1 (ml) level, B (95%CI)**	**p-value**	**FEV_1 (ml) level B (95%CI)**	**p-value**
rs1032295	a	-5.73 (-64.08-52.63)	0.337	56.06 (-59.26-171.37)	0.337
	b	15.69 (-67.46 - 98.85)	0.711	31.29 (-135.95 - 198.54)	0.711
rs13147758	a	-6.68 (-61.59 - 56.25)	0.326	57.95 (-58.50 - 174.40)	0.326
	b	50.49 (-29.96 - 130.94)	0.821	18.91 (-146.61 - 184.43)	0.821
SNP		**FEV_1/FVC (%) level, B (95%CI)**	**p-value**	**FEV_1/IVC (%) level, B (95%CI)**	**p-value**
rs1032295	a	0.04 (-0.79 - 0.88)	0.482	1.18 (-2.13 - 4.48)	0.482
	b	0.88 (-0.31 - 2.07)	0.631	1.17 (-3.63 - 5.96)	0.631
rs13147758	a	0.04 (-0.81 - 0.88)	0.597	0.90 (-2.47 - 4.27)	0.597
	b	0.51 (-0.65 - 1.66)	0.627	-1.18 (-5.96 - 3.61)	0.627

HHIP =hedgehog interacting protein; B (95%CI) = regression coefficient (95% confidence interval); FEV_1= forced expiratory volume in one second; FVC=forced vital capacity; IVC =inspiratory vital capacity. Analyses are adjusted for gender, age, height and smoking dummy variables. In GLUCOLD additional adjustment for smoking status did not change the size or significance of the effect.

a=heterozygote vs wild type; b=homozygote vs. wild type.

Table S4: The association of SNPs in the *HHIP* region and FEV_1 decline in Doetinchem

Doetinchem total population*			
SNP		**FEV_1 (ml) decline E (95%CI)**	**p-value**
rs1032295	a	-0.31 (-5.78 - 5.17)	0.912
	b	0.21 (-7.62 - 8.04)	0.958
rs13147758	a	3.84 (-1.74- 9.42)	0.177
	b	3.13 (-4.49 - 10.75)	0.421
Doetinchem current smokers			
SNP		**FEV_1 (ml) decline, E (95%CI)**	**p-value**
rs1032295	a	-5.37 (-15.90 - 5.16)	0.317
	b	-5.99 (-21.16 - 9.18)	0.438
rs13147758	a	4.27 (-6.48- 15.00)	0.436
	b	-1.05 (-16.00- 13.92)	0.891
Doetinchem ex-smokers			
SNP		**FEV_1 (ml) decline, E (95%CI)**	**p-value**
rs1032295	a	5.24 (-3.58 - 14.06)	0.244
	b	6.85 (-5.59 - 19.29)	0.280
rs13147758	a	4.82 (-4.11 - 13.75)	0.290
	b	5.51 (-6.66 - 17.68)	0.375
Doetinchem never smokers			
SNP		**FEV_1 (ml) decline, E (95%CI)**	**p-value**
rs1032295	a	-1.34 (-10.58 - 7.91)	0.777
	b	-2.69 (-16.07 - 10.70)	0.694
rs13147758	a	2.15 (-7.34 - 11.64)	0.657
	b	3.21 (-9.64 - 16.06)	0.624

*HHIP =hedgehog interacting protein, E (95%CI) = estimate (95% confidence interval), FEV_1= forced expiratory volume in one second, Analyses are adjusted for time, initial FEV_1, gender, age baseline, height, *smoking dummy variables at baseline and the interaction of all variables with time. a=heterozygote vs wild type; b=homozygote vs. wild type.*

Table S5: The association of SNPs in the *HHIP* region and FEV_1/FVC decline in Doetinchem.

Doetinchem total population*			
SNP		**FEV_1/FVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.08 (-0.03 - 0.16)	0.152
	b	-0.02 (-0.15 - 0.12)	0.836
rs13147758	a	0.09 (-0.01 - 0.19)	0.062
	b	0.01 (-0.13 - 0.14)	0.932
Doetinchem current smokers			
SNP		**FEV_1/FVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.02 (-0.17 - 0.20)	0.889
	b	-0.12 (-0.39 - 0.15)	0.395
rs13147758	a	0.12 (-0.07- 0.31)	0.206
	b	0.02 (-0.24 - 0.28)	0.881
Doetinchem ex-smokers			
SNP		**FEV_1/FVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.21 (0.07 - 0.36)	**0.004**
	b	0.10 (-0.11 - 0.30)	0.361
rs13147758	a	0.11 (-0.04 -0.26)	0.138
	b	-0.03 (-0.24 - 0.17)	0.748
Doetinchem never smokers			
SNP		**FEV_1/FVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	-0.02 (-0.17 - 0.14)	0.821
	b	-0.05 (-0.28 - 0.17)	0.656
rs13147758	a	0.04 (-0.12 - 0.19)	0.660
	b	0.01 (-0.21 - 0.22)	0.974

*HHIP =hedgehog interacting protein, E (95%CI) = estimate (95% confidence interval), FEV_1/FVC=forced expiratory volume in one second/forced vital capacity, Analyses are adjusted for time, initial FEV_1/FVC, gender, age baseline, height, *smoking dummy variables at baseline and the interaction of all variables with time. a=heterozygote vs wild type; b=homozygote vs. wild type.*

Table S6: The association of SNPs in the *HHIP* region and FEV_1 decline stratified according to smoking status in GLUCOLD

GLUCOLD (all patients)			
SNP		**FEV_1 (ml/yr) decline, E (95%CI)**	**p-value**
rs1032295	a	0.53 (-1.52- 2.59)	0.610
	b	-2.27 (-5.32- 0.78)	0.144
rs13147758	a	2.70 (0.65 - 4.76)	**0.010**
	b	-1.65 (-4.66- 1.36)	0.282
GLUCOLD smokers			
SNP		**FEV_1 (ml/yr) decline, E (95%CI)**	**p-value**
rs1032295	a	1.27 (-1.60 - 4.14)	0.384
	b	-2.78 (-7.22 - 1.67)	0.220
rs13147758	a	4.39 (1.55 - 7.22)	**0.003**
	b	-3.04 (-7.34 - 1.27)	0.166
GLUCOLD ex-smokers			
SNP		**FEV_1 (ml/yr) decline, E (95%CI)**	**p-value**
rs1032295	a	0.23 (-3.00 - 3.47)	0.886
	b	-1.88 (-6.08 - 2.32)	0.378
rs13147758	a	0.43 (-2.90 - 3.75)	0.801
	b	0.06 (-4.17 - 4.29)	0.978

FEV_1 = forced expiratory volume in one second; E (95%CI) =estimate and the 95% confidence interval; The analyses are adjusted for time, initial FEV_1, treatment dummy variables, gender, age baseline, height, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time; The significant results are depicted in bold with p-value >0.05. a=heterozygote vs wild type; b=homozygote vs. wild type.

Table S7: The association of SNPs in the *HHIP* region and FEV_1/IVC (%) decline stratified according to smoking status in GLUCOLD

GLUCOLD (all patients)			
SNP		**FEV_1/IVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.06 (0.01 - 0.11)	**0.021**
	b	-0.03 (-0.10 - 0.05)	0.459
rs13147758	a	0.09 (0.04 - 0.13)	**0.001**
	b	-0.03 (-0.10 - 0.05)	0.475
GLUCOLD smokers			
SNP		**FEV_1/IVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.03 (-0.04 - 0.09)	0.453
	b	-0.12 (-0.22 - -0.01)	**0.035**
rs13147758	a	0.07 (-0.01 - 0.14)	*0.054*
	b	-0.10 (-0.19 - 0.01)	*0.071*
GLUCOLD ex-smokers			
SNP		**FEV_1/IVC (%) decline, E (95%CI)**	**p-value**
rs1032295	a	0.10 (0.02 - 0.17)	**0.011**
	b	0.07 (-0.03 - 0.16)	0.175
rs13147758	a	0.05 (-0.03 - 0.13)	0.200
	b	0.06 (-4.17 - 4.29)	0.423

FEV_1/IVC= forced expiratory volume in one second/inspiratory vital capacity; E (95%CI) =estimate and the 95% confidence interval; The analyses are adjusted for time, initial FEV_1/IVC, treatment dummy variables, gender, age baseline, height, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time; The significant results are depicted in bold with p-value >0.05. a=heterozygote vs wild type; b=homozygote vs. wild type.

Table S8: The association of SNPs in the *HHIP* region and $FEF_{50-75\%}$, RV and RV/TLC at baseline

SNP		FEF_{50} (ml/s) B (95%CI)	p-value
rs1032295	a	6.49 (-119.36 - 132.33)	0.337
	b	-24.93 (-217.71 - 167.86)	0.711
rs13147758	a	-16.38 (-143.05 - 110.30)	0.798
	b	-135.65 (-318.18 - 46.89)	0.143
SNP		**FEF_{75} (ml/s) B (95%CI)**	**p-value**
rs1032295	a	26.17 (-15.57 - 67.91)	0.216
	b	-21.50 (-92.14 - 49.13)	0.546
rs13147758	a	-0.49 (-43.11 - 42.13)	0.982
	b	-31.39 (-99.87 - 37.10)	0.365
SNP		**RV (ml) B (95%CI)**	**p-value**
rs1032295	a	-274.06 (-629.31 - 81.20)	0.129
	b	-289.90 (-832.87 - 253.07)	0.292
rs13147758	a	-211.97 (-577.45 - 153.52)	0.252
	b	-158.80 (-683.18 - 365.58)	0.549
SNP		**RV/TLC (%) B (95%CI)**	**p-value**
rs1032295	a	-2.26 (-5.29 - 0.78)	0.143
	b	-1.01 (-5.64 - 3.63)	0.667
rs13147758	a	-1.59 (-4.70 - 1.53)	0.314
	b	-1.92 (-6.39 - 2.55)	0.396

HHIP =hedgehog interacting protein; B (95%CI) = regression coefficient (95% confidence interval); FEF_{50} = forced expiratory flow at 50% of the FVC (forced vital capacity); FEF_{75}= forced expiratory flow at 75% of the FVC; RV =residual volume; TLC =total lung capacity. Analyses are adjusted for gender, age, height and smoking status. a=heterozygote vs wild type; b=homozygote vs. wild type.

Table S9: The association of SNPs in the HHIP region with FEV_1 and FEV_1/IVC decline for COPD patients with more than 41.8 pack-years

		GLUCOLD	
SNP		**FEV_1 decline E (95%CI)**	**p-value**
rs1032295	a	1.45 (-1.34 - 4.23)	0.307
	b	-3.85 (-8.85 - 1.14)	0.130
rs13147758	a	2.91 (0.07- 5.75)	**0.045**
		-2.94 (-6.96 - 1.08)	0.151
SNP		**FEV_1/IVC decline, E (95%CI)**	**p-value**
rs1032295	a	0.06 (-0.01 - 0.13)	0.072
	b	-0.07 (-0.19 - 0.05)	0.242
rs13147758	a	0.08 (0.01 - 0.15)	**0.026**
	b	-0.07 (-0.17- 0.03)	0.179

FEV_1 = forced expiratory volume in one second; FEV_1/IVC=FEV_1/inspiratory vital capacity; E (95%CI) =estimate and the 95% confidence interval; The analyses are adjusted for time, initial FEV_1 or FEV_1/IVC, treatment dummy variables, gender, age baseline, smoking status baseline, height, the period when there is a change in treatment and its interaction with treatment and the interaction of all variables with time; The significant results are depicted in bold with p-value >0.05. a=heterozygote vs wild type; b=homozygote vs. wild type.

CHAPTER 6

Nicotinic acetylcholine receptor variants are related to smoking habits, but not directly to COPD

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Abstract

Genome-wide association studies identified single nucleotide polymorphisms (SNPs) in the *nicotinic acetylcholine receptors (nAChRs)* cluster as a risk factor for nicotine dependency and COPD. We investigated whether SNPs in the *nAChR* cluster are associated with smoking habits and lung function decline, and if these potential associations are independent of each other.

The SNPs rs569207, rs1051730 and rs8034191 in the *nAChR* cluster were analyzed in the Vlagtwedde-Vlaardingen cohort (n=1,390) that was followed for 25 years. We used GEE and LME models to analyze the associations of the SNPs with quitting or restarting smoking and with the annual FEV_1 decline respectively.

Individuals homozygote (CC) for rs569207 were more likely to quit smoking (OR (95%CI) =1.58 (1.05 - 2.38)) compared to wild-type (TT) individuals. Individuals homozygote (TT) for rs1051730 were less likely to quit smoking (0.64 (0.42; 0.97)) compared to wild-type (CC) individuals. None of the SNPs was significantly associated with the annual FEV_1 decline in smokers and ex-smokers.

We show that SNPs in the *nAChR* region are associated with smoking habits such as quitting smoking, but have no significant effect on the annual FEV_1 decline in smokers and ex-smokers, suggesting a potential role of these SNPs in COPD development via smoking habits rather than via direct effects on lung function.

Introduction

Cigarette smoking is a major concern influencing public health worldwide. It is the main risk factor for Chronic Obstructive Pulmonary Disease (COPD), but the risk to develop airway obstruction varies between smokers due to differences in genetic susceptibility.

Over time, studies in animal models [1, 2], candidate gene or family and twin studies [3, 4] have tackled the underlying mechanisms and genetic background influencing nicotine addiction and smoking habits [5]. Recent reviews highlighted the important role of genetic factors in the inter-individual variation to initiate, maintain or to quit smoking and their effects on lung function [6, 7].

The genetic contribution to the variation in smoking behaviour recognizes two classes of genes: genes influencing the response to nicotine, like nicotinic acetylcholine receptors (*nAChR)* and nicotine metabolism (*CYP2D6*), and genes predisposing to addictive behaviour due to their effects on key neurotransmitter pathways like dopamine (*DRD1)* and serotonin (*TPH*) [8].

Nicotinic acetylcholine receptor (nAChR) is a controversial gene with respect to its association with COPD and smoking addition. Single nucleotide polymorphisms (SNPs) rs1051730 and rs8034191 in the *nAChR* cluster have been identified in cross-sectional genome-wide association (GWA) studies as a risk for COPD [9]. Furthermore, studies showed cross-sectional associations of the SNPs in the *nAChR* cluster with nicotine dependency based on the reported number of cigarettes per day [10] or the Fagerstrom Test of Nicotine Dependence (FTND), a test reliably predicting smoking cessation and correlating with biochemical measurements of nicotine dependency [11]. Other studies have found a cross-sectional association of the same variants with the level of lung function and COPD [12].

Since smoking is a risk factor for COPD itself, it is not clear from these cross-sectional studies whether these SNPs in the *nAChR* cluster are directly and independently a risk for COPD development or whether they are associated with COPD through their association with nicotine dependency and smoking habits [10, 11, 13].

We had the unique opportunity to study longitudinally the association of the *nAChR*

variants with changes in smoking habits and lung function in the population-based Vlagtwedde-Vlaardingen cohort.

Methods

Ethics Statement

The study protocol was approved by the local university medical hospital ethics committee, University Medical Center Groningen, University of Groningen, The Netherlands and all patients gave their written informed consent. In 1984, the Committee on Human Subjects in Research of the University of Groningen reviewed the study and affirmed the safety of the protocol and study design (http://www.ccmo-online.nl/main.asp?pid=14&sid=16&ssid=33&inid=16).

Study population

The Vlagtwedde-Vlaardingen cohort (n=1390) has been previously described in detail [14, 15]. The cohort was prospectively followed for 25 years with lung function measurements every 3 years using a water-sealed spirometer (Lode Intruments, the Netherlands). Lung function was determined by measurement of the Forced Expiratory Volume in 1 second (FEV_1). The median number of surveys during the follow-up was 7. Current, ex-smokers and never smokers definition is based on validated questionnaires. A current smoker has been defined as having smoked in the previous 12 months and an ex-smoker as not having smoked in the previous 12 months.

Selection and genotyping of the SNPs in the *nAChR* cluster

We selected three SNPs rs1051730, rs8034191 and rs569207 in the *nAChR* cluster based on previous findings [9, 11-13, 16, 17]. The SNPs belong to the linkage-disequilibrium block containing *nAChR* genes, but do not represent the overall CHRNA5-A3-B4 group. Rs1051730 is a synonymous mutation and rs569207 an intronic SNP belonging to the *nAChR* subunit genes, *CHRNA3* and *CHRNA5* respectively, and the third SNP, rs8034191, is located near *nAChR* subunit genes on 15q25 on *LOC123688*. Genotyping was performed by K-Bioscience (UK) using their patent-protected competitive allele specific PCR system (KASPar) (http://

www.kbioscience.co.uk/index.html).

Statistics

Data on smoking habits and lung function collected during the surveys from 1965 to 1990 have been used for analyses. To study the changes in smoking habits such as quitting smoking within smokers and restarting smoking within ex-smokers, we compared the information of two successive surveys. The paired observations had a minimum interval of 3 years and every subject who had smoked at any time contributed to the analyses with a maximum of 7 paired observations. Analyses of the paired observations have been performed separately for smokers and ex-smokers: To investigate quitting smoking we selected all paired observations in which the subject smoked at the first observation of 2 successive surveys. The smoking habit (i.e. smoking or ex-smoking) at the nearest follow-up survey (i.e. the second observation of two successive surveys) was used as dependent variable in our analyses. To investigate restarting smoking we selected all paired observations in which the subject was an ex-smoker at the first of 2 successive surveys. Again, the smoking habit (i.e. ex-smoking or restarted smoking) at the nearest follow-up survey was the dependent variable in our analyses.

1. SNPs in the *nAChR* cluster and changes in smoking habits in smokers and ex-smokers

Generalized estimating equations (GEE) were used to investigate in smokers and ex-smokers separately the associations of the SNPs in the *nAChR* cluster with quitting and restarting smoking, respectively. This method takes into account the dependence of multiple measurements within one subject and adjusts for the fact the intervals between the observations are not constant and the number per subject was variable. In the GEE model, an OR > 1 should be interpreted as an increased chance to stop or restart smoking and an OR < 1 should be interpreted as a decreased chance to stop or restart smoking. In the GEE model, the smoking habit was the dependent variable (categorical). We adjusted our analyses for gender and the time between two successive surveys.

2. SNPs in the *nAChR* cluster and the annual FEV_1 decline in smokers and ex-smokers

Linear mixed-effects models (LME) were used in smokers and ex-smokers separately to investigate the associations of the SNPs in the *nAChR* cluster with the annual FEV_1 decline (defined as the difference in FEV_1 between the 2nd and 1st observation of two successive surveys divided by the time in years between these surveys). In the LME model, the annual FEV_1 decline was the dependent variable (continuous). We adjusted our analyses for gender, the time between two successive surveys and age and height at the first of two successive surveys. Quitting and restarting smoking were also used in the analyses as independent variables for smokers respectively ex-smokers. In the LME model, a negative regression coefficient should be interpreted as an excess FEV_1 decline per year and a positive regression coefficient as less FEV_1 decline per year as compared to the wildtype. In the LME model effect estimates are considered significant if the confidence interval does not include 0. Since E, estimated from the LME model, can be interpreted as a regression coefficient, we indicated it as a B throughout the paper to improve consistency of the presented results and ease of interpretation.

3. Changes in smoking habits and the annual FEV_1 decline in smokers and ex-smokers

LME models were used to investigate the associations of quitting and restarting smoking with the annual FEV_1 decline separately in smokers and ex-smokers. The analyses were adjusted for gender, age and height at the first of two successive surveys and the time between two successive surveys.

4. SNPs in the *nAChR* cluster and cigarettes smoked per day, packyears and COPD at the last survey

We used linear regression to assess the associations of the SNPs and number of cigarettes smoked per day at the last survey in current smokers. The associations of the SNPs with packyears were performed in ever smokers (current smokers and ex-smokers together). We adjusted for age and gender. We used chi-square tests to assess the differences in the prevalence of the SNPs between subjects with ($FEV_1/FVC < 70\%$) and without ($FEV_1/FVC > 70\%$) COPD. In the linear regression model, a negative regression coefficient should be interpreted as a lower number of

cigarettes per day and less packyears, and a positive regression coefficient should be interpreted as a higher number of cigarettes per day and higher number of packyears.

To asses the associations of the SNPs in the *nAChR* cluster with changes in smoking habits, with the annual FEV_1 decline in smokers and ex-smokers, and with the daily number of cigarettes and packyears at the last survey we used the general genetic model where heterozygote and homozygote variants were coded separately as dummy variables and compared to the homozygote wild type.

All analyses were performed using SPSS version 16.0 for Windows and values of $p<0.05$ (tested 2-sided) were considered statistically significant. Linkage Disequilibrium (LD) plots (a threshold of 0.8 for the correlation coefficient (r^2) and Hardy Weinberg Equilibrium (HWE) tests were performed with Haploview (version 4.2) [18].

LME models were used with a random intercept at the subject level, assuming data are missing at random. We included only FEV_1 measurements from the age of 30 years onwards because it is assumed that the maximal lung function is reached before that age and the FEV_1 is considered to be either in the plateau or decline phase [19].

Results

The clinical characteristics of the Vlagtwedde-Vlaardingen cohort are presented in table 1.
The 3 SNPs in the *nAChR* cluster rs1051730, rs8034191 and rs569207 were all in Hardy Weinberg Equilibrium ($p>0.05$) and their prevalence was relatively high (9% to 57%; Table S1).

1. SNPs in the *nAChR* cluster and changes in smoking habits in smokers and ex-smokers

In smokers, individuals homozygote variant (CC) for rs569207 were more likely to

quit smoking (OR (odds ratio) (95%CI (confidence interval)) =1.58 (1.05 - 2.38)) compared to wild type (TT) individuals (Figure 1, upper graph).

Individuals homozygote variant (TT) for rs1051730 were more likely to continue smoking (OR (95%CI) =0.64 (0.42 - 0.97)) compared to wild type (CC) individuals (Figure 1, upper graph). The rs8034191 SNP was not associated with quitting smoking. None of the SNPs were associated with restarting smoking (Figure 1, lower graph). Heterozygotes showed no significant associations compared to wild-types. Detailed data on these associations are presented in the supplementary material (Table S2).

Table 1: Clinical characteristics of the Vlagtwedde-Vlaardingen cohort at the last survey

Vlagtwedde-Vlaardingen cohort (n=1,390)	
Duration of follow-up (years)	21 (16-22)
Median nr. of visits	7 (5-8)
Males, number (%)	714 (51.4)
Age (years)*	52 (43-60)
Height (cm)	170.3 (9.3)
Never smokers, number (%)	445 (32.0)
Ex-smokers, number (%)	456 (32.8)
Current smokers, number (%)	489 (35.2)
Packyears *	8.9 (0-24)
FEV_1 (L)	2.8 (0.7)
VC (L)	3.8 (0.9)
FEV_1/VC (%)	73.8 (8.9)
FEV_1 (% predicted)	92.6 (15.4)
FEV_1 change (ml/year)	-20.8 (22.9)
No COPD, number (%)	993 (73.8)
COPD, GOLD stage I, number (%) #	185 (13.8)
COPD, GOLD stage II, number (%) #	146 (10.9)
COPD, GOLD stage III, number (%) #	21 (1.6)

*Data are presented as mean (standard deviation) or * median (range); FEV_1 = forced expiratory volume in one second; VC = vital capacity; COPD = Chronic Obstructive Pulmonary Disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease; GOLD stage I = mild COPD; GOLD stage II = moderate COPD; GOLD stage III = severe COPD; # According to ATS/ERS standards[20]*

Figure 1: SNPs in the *nAChR* cluster and OR (95%CI) for quitting smoking in subjects who smoke (upper graph), and OR (95%CI) for restarting to smoke in ex-smokers (lower graph)

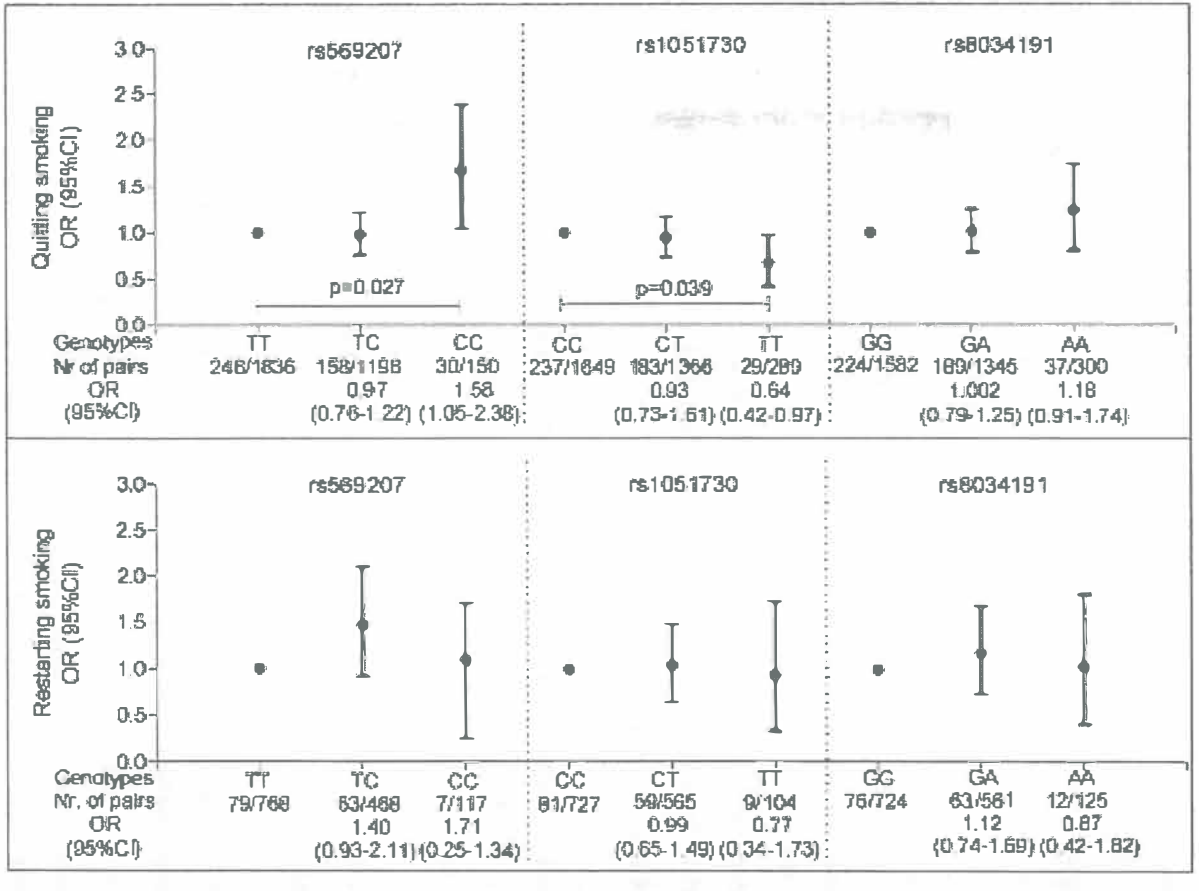


Figure 1: Nr. of pairs= number of paired observations in which the subject stopped respectively restarted smoking/ total number of paired observations included in the analysis; Circles represent the odd ratio (OR) and vertical bars represent 95% confidence interval (CI); Wild type was set to one as the reference category; Different total number of paired observations for the SNP genotypes are due to missing data on genotype or smoking habits. The analyses are adjusted for gender and the time between 2 successive surveys. rs569207 TT=wild-type, TC=heterozygote, CC=homozygote variant rs1051730 CC= wild-type CT=heterozygote TT=homozygote variant rs8034191 GG= wild-type, GA= heterozygote, AA=homozygote variant.

2. SNPs in the *nAChR* cluster and annual FEV_1 decline in smokers and ex-smokers

The numbers of available FEV_1-pairs in smokers and ex-smokers are presented in the supplementary material (Table S3).

None of the SNPs was significantly associated with the annual FEV_1 decline in smokers or ex-smokers, with or without correction for quitting and restarting smoking, respectively (Table 2). Also, there was no association between the SNPs and the annual FEV_1 decline in never smokers (Table 2). Additionally we investigated the associations of smoking status with the decline in annual FEV_1 using an LME model, independent of the genotypes. There was a significant difference in annual FEV_1 decline between smokers and never smokers and between smokers and ex-smokers independent of the genotypes. Detailed results are presented in the data supplement Table S4.

3. Smoking habits and annual FEV_1 decline in smokers and ex-smokers

Subjects who quitted smoking had (B (regression coefficient) (95%CI) = 20.3 ml (9.49 - 31.15)) significantly less FEV_1 decline per year than subjects who continued smoking. Ex-smokers who restarted smoking had a faster FEV_1 decline (B (95%CI) = -14.7 ml (-31.5 - 2.1)) per year than ex-smokers who did not restart smoking, although this did not reach significance.

4. SNPs in the *nAChR* cluster and cigarettes smoked per day, packyears, and COPD at the last survey

In current smokers none of the SNPs was significantly associated with the number of cigarettes smoked per day at the last survey (Table 3).

In current and ex-smokers, only individuals homozygote variant (CC) for rs569207

had a lower number of packyears (B (95%CI) = -5.9 (-11.5 - -0.3)) at the last survey as compared to wild-type (TT) individuals (Table 3).

Table 2: SNPs in the *nAChR* cluster and annual FEV_1 decline (ml/year) in smokers, ex-smokers and never smokers

SNPs		annual FEV_1 decline (smokers)[1]			annual FEV_1 decline (ex-smokers)[2]			annual FEV_1 decline (never smokers)[3]		
		B	95%CI	p	B	95%CI	p	B	95%CI	p
rs569207	a	0.3	-7.6 – 8.3	0.939	4.1	-6.4 – 14.5	0.451	2.6	-5.5 - 10.6	0.531
	b	13.1	-5.7 – 31.9	0.172	0.9	-15.9 – 17.8	0.916	2.9	-13.7 - 19.7	0.727
rs1051730	a	0.1	-7.8 – 8.1	0.973	-2.4	-12.3 – 7.6	0.643	-5.3	-13.1 - 2.6	0.189
	b	5.4	-8.2 – 18.9	0.437	3.9	-14.3 – 22.2	0.674	-0.7	-15.1 - 13.6	0.925
rs8034191	a	-1.1	-9.2 – 7.1	0.919	-0.3	-10.2 – 9.8	0.964	-3.6	-11.4 - 4.3	0.370
	b	3.9	-9.6 – 17.4	0.405	5.4	-11.5 – 22.3	0.531	-4.8	-18.6 - 9.1	0.499

B= regression coefficient from the linear mixed-effect model, adjusted for 1=quitting smoking, 2=restarting smoking, 3=never smoker, gender, height and age at the first of two successive surveys and time between two successive surveys. "Smokers" refer to those paired observations in which the subject was a smoker at the first of two successive surveys and quitted smoking or continued smoking at the nearest follow-up survey. "Ex-smokers" refer to those paired observations in which the subject was an ex-smoker at the first of two successive surveys and continued being an ex-smoker or restarted smoking at the nearest follow-up survey. "Never smoker" refer to those paired observations in which the subject was a never smoker at the first of two successive surveys and continued being a never smoker at the nearest follow-up survey. a= heterozygotes vs. wild-type; b= homozygote variant vs. wild-type.

Table 3: SNPs in the *nAChR* cluster with cigarettes smoked per day in current smokers, and packyears in ever smokers at the last survey

SNP		cigarettes/day in current smokers*			packyears in ever smokers**		
		B	95%CI	p-value	B	95%CI	p-value
rs569207	a	-1.5	-3.3 - 0.4	0.111	-1.5	-4.1 - 1.0	0.238
	b	-2.8	-7.6 - 2.0	0.252	-5.9	-11.5 - -0.3	**0.039**
rs1051730	a	1.1	-0.6 - 2.9	0.207	0.1	-2.4 - 2.6	0.943
	b	-1.5	-4.6 - 1.6	0.338	0.3	-4.1 - 4.7	0.901
rs8034191	a	0.2	-1.6 - 2.0	0.819	-0.7	-3. - 1.9	0.600
	b	-1.2	-4.3 - 1.9	0.469	-0.6	-4.8 - 3.7	0.800

*B=regression coefficient derived from linear regression model, adjusted for age and gender; * only current smokers at the last survey; **ever smokers are current smokers and ex-smokers together; a= heterozygote vs. wild-type; b= homozygote variant vs. wild-type.*

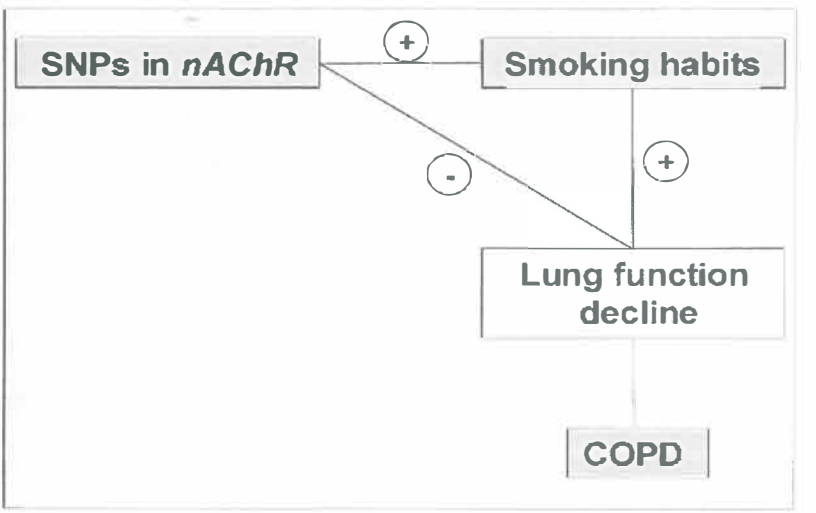
Discussion

The nAChRs are highly expressed in the nervous system and their binding to nicotine activates physiological and pharmacological responses to tobacco smoking [11]. Variants in the *nAChR* cluster have been associated with nicotine dependency and smoking status [10, 11, 21]. So far, studies showed cross-sectional associations of the *nAChR* variants with nicotine dependency and the level of lung function and COPD [10-12, 22-25]. Since smoking is a risk factor for COPD itself, cross-sectional studies can not elucidate whether the effect of the *nAChR* variants determine COPD development directly or indirectly via smoking addiction.
Our longitudinal study shows that rs1051730 and rs569207 in the *nAChR* are associated with an increased and decreased ability to quit smoking, respectively, but the SNPs are not associated with the annual FEV_1 decline. This suggests that these SNPs may be involved in COPD development via smoking habits rather their effect on (accelerated) lung function decline (Figure 2).

First, we observed in smokers that individuals homozygote variant (CC) for rs569207 were more likely to quit smoking compared to wild type (TT) individuals.

This is compatible with a previous study showing that the haplotype for rs569207 confers protective effects on nicotine addiction compared with haplotypes for other SNPs within the same Linkage Disequilibrium (LD) block [13]. We additionally found that individuals homozygote variant (TT) for rs1051730 were less likely to quit smoking compared to wild type (CC) individuals, a finding that is in line with a previous study [10]. Although r^2 between rs569207 and rs1051730 was only 0.11 in our study population (Figure S1), we observed that individuals having the homozygote variant for rs569207 are also the ones who have the wild type for rs1051730 (and thus are less likely to quit smoking), strengthening our findings of a protective effect of the variant in rs569207 for smoking habits within the *nAChR* cluster. Rs1051730 and rs8034191 were highly correlated (r^2=0.91), yet rs8034191 was not associated with quitting smoking among smokers, suggesting that other factors at the subject's level might explain the significant association of rs1051730 with the inability to quit smoking. Moreover, although rs8034191 does not encode an actual receptor protein, we have selected this SNP for analyses with smoking habits since it has been identified in a previous GWA study as a risk for COPD [9].

Figure 2: Summary of the observed associations in the current study



SNPs = single nucleotide polymorphisms; COPD = Chronic Obstructive Pulmonary Disease; nAChR = nicotinic acetylcholine receptor; + = association; - = no association.

Second, none of the *nAChR* SNPs was significantly associated with annual FEV_1 decline in smokers or ex-smokers and neither with annual FEV_1 decline in never smokers. Additionally, none of the SNPs was significantly associated with the level of FEV_1 among smokers, ex-smokers and never smokers (data not shown). Pillai et al performed a GWA study in four independent populations and demonstrated that the 15q/25 region is associated with COPD defined by airway obstruction [9]. The authors suggested that the rs1051730 (*CHRNA3*) or rs8034191 (*LOC123688*) are causal variants for COPD [26]. In line with this, rs1051730 and rs8034191 were found to be associated with lower levels of FEV_1 in the British 1958 birth cohort. However, the latter two studies looked into cross-sectional level of FEV_1, and thus they cannot disentangle whether the SNPs act via nicotine dependency, or have independent effects. Our longitudinal data strongly suggest that these SNPs are not associated with lung function decline since we found no effect of the SNPs on FEV_1 decline, and additionally that this lack of effect was independent of smoking.

Third, we observed that smokers who quit smoking had less annual FEV_1 decline compared with subjects who continued smoking. Our findings are consistent with the results from previous studies demonstrating in a smoking cessation program that quitters had less FEV_1 decline compared with persistent smokers with COPD [27].

Results of two independent cohorts showed that rs1051730 is additionally associated with emphysema, a feature of COPD, but not with nicotine addiction when defined as the number of packyears smoked [12]. We also found that neither rs1051730 nor rs8034191 were associated with the number of packyears smoked at the last survey suggesting that the number of packyears does not necessarily reflect addiction. Interestingly, in current and ex-smokers homozygote variant (CC) for rs569207 had a lower number of packyears at the last survey than wild type (TT) individuals. This may reflect that carriers of this variant are able to quit smoking more easily, highlighting the consistency of our results.

Our longitudinal results draw the attention to rs569207 in the CHRNA5 gene. This SNP showed a protective effect in relation to smoking habits in the general

population, without an effect on annual FEV_1 decline. This clearly suggests that rs569207 has a protective effect on COPD development via smoking habits exclusively and not via lung function decline (Figure 2). This increased likelihood of quitting smoking was previously seen in individuals carrying *CYP2A6* variants [28]. *CYP2A6*, the gene that influences the response to nicotine besides *nAChRs*, is responsible for the metabolic inactivation of nicotine to cotinine [29]. It is tempting to speculate that as for *CYP2A6* variants, individuals carrying rs569207 do not build up a tolerance to nicotine which is thought to play a critical role in the development and maintenance of nicotine dependency. However, this seems to be unlikely since rs569207 is located in intron 1 and might not have effects other than the regulation of the gene/protein expression. So, from the current study we can not draw firm conclusions on the biological effects of the SNPs in *nAChR* cluster and future functional studies should focus on this in detail. Moreover, pre-clinical studies have demonstrated that bupropion is a non-competitive antagonist at the *nAChRs* subunits [30-32] and thus it is plausible that genetic variation in *CHRNA5* influences bupropion's efficacy for treatment of tobacco dependence [32]. It has been shown that rs871058, an intronic SNP in *CHRNA5,* appears to have pharmacogenetic relevance [32]. This intronic SNP rs871058 is highly correlated with rs16969968 in *CHRNA5*. The latest emerged as the strongest risk variant in a nicotine dependence association study of over 3,000 SNPs in over 300 candidate genes [21, 32]. Therefore, it might be that the intronic SNP, rs569207, from the current study is in strong linkage disequilibrium with other SNPs that are able to alter the subunit properties or even have a pharmacological effect, since there is a high degree of linkage disequilibrium across *CHRNA5* [32]. Based on our data we can not state that our observations are the results of monogenic effects. Furthermore, pharmacogenetic variation can significantly alter susceptibility to, and response to treatment for, drug dependence [33]. In a recent study it has been hypothesized that slower nicotine metabolism, as measured by the nicotine metabolite ratio, would decrease the influence of genotypes for rs16969968, rs1051730 in *CHRNA5* and rs578776 in *CHRNA3* [34]. The authors found, in a sample of 1030 treatment seekers, significant independent and additive associations of nicotine metabolite ratio , rs16969968, and rs1051730 with cigarettes per day, but the interactions of the nicotine metabolite ratio with genotype on cigarettes per day were not significant

[34]. A recent randomized clinical trial of nicotine replacement therapy showed that fast nicotine metabolizers are less likely to succeed at quitting smoking, as compared to slow nicotine metabolizers, when offered free nicotine patches [35].

This is the first longitudinal study suggesting that SNPs in the *nAChR* cluster potentially have a causal role in COPD via smoking habits. We hypothesize therefore that these variants are related to the onset of COPD via their association with smoking habits, and they are not independently related to COPD development.

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Supplementary Results

Figure S1: Linkage disequilibrium plot and correlation coefficients (r^2) for 3 polymorphisms in the *nAChR* cluster genotyped in Vlagtwedde-Vlaardingen cohort (n=1,390)

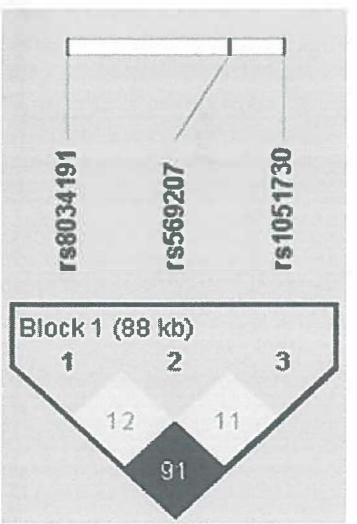

Table S1: Prevalence of the *nAChR* SNPs in Vlagtwedde – Vlaardingen

SNPs	Gene	Genotypes	N=1390 (%)
rs569207	CHRNA5	TT	735 (56.8)
		TC	487 (37.6)
		CC	72 (5.6)
rs1051730	CHRNA3	CC	685 (51.5)
		CT	537 (40.4)
		TT	108 (8.1)
rs8034191	LOC123688	GG	666 (50.3)
		GA	540 (40.8)
		AA	117 (8.9)

rs569207 TT=wild-type, TC=heterozygote, CC=homozygote variant rs1051730 CC= wild-type CT=heterozygote TT=homozygote variant rs8034191 GG= wild-type, GA= heterozygote, AA=homozygote variant

Table S2: The effect of the *nAChR* SNPs on smoking habits in smokers (pairs=3393) and ex-smokers (pairs=1468)

		quit smoking			restart smoking		
SNPs		OR	95%CI	p-value	OR	95%CI	p-value
rs569207	a	0.97	0.76 - 1.22	0.782	1.40	0.93 – 2.09	0.105
	b	1.58	1.05 – 2.38	**0.027**	0.58	0.25 – 1.33	0.200
rs1051730	a	0.93	0.73 – 1.61	0.520	0.99	0.65 – 1.48	0.960
	b	0.64	0.42 – 0.97	**0.039**	0.77	0.34 – 1.73	0.529
rs8034191	a	1.004	0.79 – 1.27	0.972	1.12	0.74 – 1.69	0.572
	b	1.180	0.81 - 1.75	0.375	0.88	0.42 – 1.82	0.740

Table S3: Annual FEV_1 declines in smokers and ex-smokers

		786 smokers with 3276 annual FEV_1 declines		467 ex-smokers with 1426 annual FEV_1 declines	
SNP	Genotype	Nr. subjects	Nr. FEV_1 declines	Nr. subjects	Nr. FEV_1 declines
rs569207	TT	418	1767	242	743
	TC	275	1171	155	455
	CC	39	140	32	114
rs1051730	CC	390	1586	232	709
	CT	312	1316	183	545
	TT	60	286	33	101
rs8034191	GG	375	1521	225	707
	GA	309	1298	183	541
	AA	65	296	40	121

Table S4: Differences in annual FEV1 decline according to smoking status

Differences in annual FEV_1 decline			
	B	95%CI	p-value
Smokers vs. never smokers	-6.4	-12.5 – -0.3	**0.039**
Ex-smokers vs. never smokers	1.5	-5.3 – 8.4	0.662
Smokers vs. ex-smokers	-7.9	-13.8 – -2.0	**0.009**

B=regression coefficient; LME model adjusted for gender, height and age at the first of two successive surveys and time between two successive surveys. The results showing a significant association are depicted in bold.

CHAPTER 7

Identifying gene pathways involved in COPD

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Submmited

Abstract

Single genes are not contributing on their own standing to Chronic Obstructive Pulmonary Disease (COPD) and therefore identification of genes interacting in pathways is necessary. We used 2 different methods to assess which genes that are linked to COPD belong to the same pathway or cluster together.

First, we explored 26 candidate genes that previously were shown to be significantly associated with lung function and COPD in the general population-based cohort Vlagtwedde-Vlaardingen, using DAVID (Database for Annotation, Visualization and Integration Discovery). Secondly, we investigated how single nucleotide polymorphisms (SNPs) in these candidate genes were categorized in relation to COPD using factor analysis.

We identified 5 functional gene classification groups from which the combination of *MMP1, MMP2, MMP9, MMP12* and *ADAM33* ranked the highest based on their biological significance. Factor analysis showed that 10 factors explained 62.5% of the total variance in our data set: 1)*ADAM33*, 2)*ADAM33*, 3)*TGF-β1*, 4)*MRP1*, 5) *SOD2*, 6)*SFTPD* and *SFTPA2*, 7)*MRP1*, 8)*KEAP,* and *TGF-β1*, 9)*GCLM, GCLC* and *GSTT1* and 10)*NFE2L, GSTM1* and *SOD3*.

Our study indicates that using 2 different data mining methods may help to better understanding of how genes and their corresponding SNPs function together in the pathogenesis of COPD.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of death worldwide. Studies in the general population have investigated several candidate genes for COPD in the past [1-7]. These candidate genes are involved in well known mechanisms of COPD i.e. lung tissue destruction and repair, protease-antiprotease and oxidant-antioxidant imbalance. Since genes do not function on their own standing, gene pathway identification could be useful for understanding the mechanisms of developing a complex disease like COPD. We previously investigated 26 genes (*MRP1, ADAM33, MMP1, MMP2, MMP9, MMP12, TIMP1, GSTP1, GSTM1, GSTT1, SFTPA1, SFTPA2, SFTPB, SFTPD, HMOX1, TGFβ, DCN, GCLC, GCLM, SOD2, SOD3, NFE2L2, KEAP1, SIRT1, CHRNA3* and *CHRNA5*) in our general population-based Vlagtwedde-Vlaardingen cohort, based on their putative function in the development of COPD, i.e. oxidative stress, protease-antiprotease balance and surfactant as well as the *CHRNA* genes related to nicotine addiction and COPD [1-8]. The aim of the current study was to assess which of these genes that are linked to COPD belong to the same pathway, and which SNPs group together. To this aim we used two different methods: 1) DAVID (the Database for Annotation, Visualization and Integration Discovery) [9] in order to identify associations of specific pathways with different biological terms (e.g. "lung function"), and 2) factor analysis that assessed how the SNPs in our candidate genes are categorized together in order to find a pattern of relationship among them.

Methods

1. Genes functional classification and pathway analysis

We set out to identify significant pathways from a set of genes that are involved in biological conditions with the use of a software package Database for Annotation, Visualization and Integration Discovery (DAVID) version 6.7, which is able to extract biological features/terms associated with large gene lists [10]. DAVID is using a novel method to identify related genes by measuring the similarity of their global annotation profiles based on the hypothesis that if two genes have similar

annotation profiles, they should be functionally related [11].

We included in our analyses 26 candidate genes (*MRP1, ADAM33, MMP1, MMP2, MMP9, MMP12, TIMP1, GSTP1, GSTM1, GSTT1, SFTPA1, SFTPA2, SFTPB, SFTPD, HMOX1, TGFβ, DCN, GCLC, GCLM, SOD2, SOD3, NFE2L2, KEAP1, SIRT1, CHRNA3* and *CHRNA5*) that had been previously investigated with respect to lung function and COPD development in the general population-based Vlagtwedde-Vlaardingen cohort [1-8]. Since DAVID was designed to collect gene identifiers and well-known publicly available annotation categories [10] we used the default parameters for our analyses, with some exceptions. In the category of associated diseases we selected only the terms involved in the "genetic association disease" that fitted to the purpose of the current study, i.e. lung function. The enriched terms within the "genetic association disease" category are presented in the descending order of their p-values. The enrichment p-values are calculated based on EASE Score [12] (modified Fisher Exact p-value). In the DAVID analysis, the genes are clustered based on their enrichment score (ES). The ES is the geometric mean of p-values from the enriched annotation terms associating with one or more of the gene group members and its algorithm has been described in details previously [11]. According to the DAVID website an ES of 1 is equivalent to a p-value of 0.1 and an ES of 1.3 is equivalent to a p-value of 0.05 (http://david.abcc.ncifcrf.gov/forum/cgi-bin/ikonboard.cgi?act=ST&f=8&t=163&st=#entry1). For the pathway analysis in DAVID we used the default parameters involving 2 pathways namely KEGG and BIOCARTA.

2. Factor analysis

Study population

We included a total of 1,390 subjects of the Vlagtwedde-Vlaardingen cohort that has been previously described in detail [13, 14]. The cohort was prospectively followed for 25 years with lung function measurements every 3 years using a water-sealed spirometer (Lode Instruments, the Netherlands). The median number of surveys during the follow up was 7 (range 2-8).

SNP selection

From 78 SNPs genotyped previously in 26 genes in Vlagtwedde-Vlaardingen, we selected 26 SNPs for our analysis. The selection was based on previously reported findings showing the significant associations of these SNPs with the level or decline of lung function in Vlagtwedde-Vlaardingen [1-8]. We excluded SNPs that were highly correlated with other SNPs from our list of 78 SNPs ($r^2>0.8$) or that were out of Hardy-Weinberg Equilibrium ($p<0.05$).

Statistics for factor analysis

26 SNPs were included in the factor analysis, a data reduction technique that consists of clustering of variables into independent subgroups of variables called "factors" and then simplifying the factor structure by Varimax rotation [15]. We evaluated the sampling adequacy based on correlation using Kaiser-Meyer-Olkin (KMO) a measure that indicates that data are likely to cluster well since correlations between pairs of variables can be explained by the other variables. The coefficients, either positive or negative, that link the parameters to factors are called "factor loadings" and are the correlation coefficient between the parameters and the factors. We have chosen the number of factors as small as possible (10), but large enough to account for most of the variation within the data (>50%) [15]. We used the Varimax rotation, which offers the possibility to simplify the structure with the optimal loadings in such a way that each variable would have a high loading on one factor, whereas its loadings on other factors would be low.

For the SNPs analyses we used SPSS version 18.0.3 for Windows.

The Linkage Disequilibrium pattern was plotted with Haploview version 4.2 [16].

Results

The characteristics of the Vlagtwedde-Vlaardingen study are presented in data supplement table S1 (supplementary material).

Genes functional classification and pathway analysis

Table 1 shows the genes included in the pathway analysis using DAVID. Based on the functional annotation chart from the "genetic association diseases" category there are 167 enriched terms associated with our gene list. Since our focus was on lung function, we selected the 6 terms (Table 2) out of 167 enriched terms observed, that fitted to lung function. The selected enriched functional terms were represented by 1) "lung function" ($1.7x10^{-14}$), 2) "chronic obstructive pulmonary disease/COPD" ($9.7x10^{-11}$), 3) "chronic obstructive pulmonary disease" ($1.2x10^{-7}$), 4) "emphysema" ($9.7x10^{-7}$), 5) "cystic fibrosis"; "chronic bronchopulmonary diseases"; "chronic obstructive pulmonary disease/COPD" ($2.4x10^{-2}$) and 6) "chronic obstructive pulmonary disease/COPD emphysema" ($2.4x10^{-2}$) (Table 2).

The major gene families were classified in 5 groups in the gene functional classification analyses (Table 3), based on the enrichment scores that are able to order their relative importance of the gene groups. A higher enrichment score for a group indicates that the group members are involved in more important ("enriched") functions [11]. Notably, the enrichment scores provide a guideline and should not be used as decision maker. The first group with the highest enrichment scores (ES=4.23, i.e. p-value<0.05) included 5 genes: *MMP1, MMP2, MMP9, MMP12* and *ADAM33*. The second group (ES = 3.87) included the surfactant genes, the third group (ES = 2.43) the glutathione genes and the fourth and fifth groups (ES = 1.19 and 0.90 respectively, i.e. 0.05<p-value<0.10) included glutamate (*GCLs*) and cholinergic receptor genes (*CHRNA3* and *CHRNA5*).

Systematically mapping of reduced lung function and COPD [1-7] to their biological terms using DAVID [10] led to identification of 5 functional groups having similar annotation profiles, and being functionally related, i.e. 1) *metalloproteinases*, including *ADAM33*, 2) *surfactants*, 3) *glutathione transferases*, 4) *glutamate-cysteine ligases* and 5) *nicotinic cholinergic receptor; alpha* genes.

The KEGG pathway revealed 5 overrepresented pathways, in which "glutathione metabolism" showed the lowest p-value ($5.5x10^{-6}$), while only 3 overrepresented pathways were in the BIOCARTA pathway from which "Inhibition of Matrix Metalloproteinases" showed the lowest p-value ($1.9x10^{-3}$) (supplementary material Table S2).

Table 1: Genes included in the analysis using DAVID

Nr	Gene symbol	Gene name	Mechanism/ Process
1	*ADAM33*	*a disintegrin and metalloprotease domain 33*	Metalloprotease
2	*MMP1*	*matrix metalloprotease 1*	
3	*MMP2*	*matrix metalloprotease 2*	
4	*MMP9*	*matrix metalloprotease 9*	
5	*MMP12*	*matrix metalloprotease 12*	
6	*TIMP1*	*tissue inhibitor of matrix metalloprotease-1*	Inhibitor MMP
7	*TGFβ1*	*transforming growth factor, beta 1*	Extracellular Matrix (ECM)
8	*DCN*	*decorin*	
9	*SFTPA1*	*surfactant protein A1B; surfactant protein A1*	Surfactant
10	*SFTPA2*	*surfactant protein A2; surfactant protein A2B*	
11	*SFTPB*	*surfactant protein B*	
12	*SFTPD*	*surfactant protein D*	
13	*GSTP1*	*glutathione S-transferase pi 1*	Oxidative stress
14	*GSTM1*	*glutathione S-transferase mu 1*	
15	*GSTT1*	*glutathione S-transferase theta 1*	
16	*MRP1/ ABCC1*	*ATP-binding cassette, sub-family C (CFTR/ MRP), member 1*	
17	*SOD3*	*superoxide dismutase 3, extracellular*	
18	*SOD2*	*superoxide dismutase 2, mitochondrial*	
19	*HMOX1*	*heme oxygenase (decycling) 1*	
20	*GCLC*	*glutamate-cysteine ligase, catalytic subunit*	
21	*GCLM*	*glutamate-cysteine ligase, modifier subunit*	
22	*SIRT1*	*sirtuin 1*	
23	*NFE2L2*	*nuclear factor (erythroid-derived 2)-like 2*	
24	*KEAP1*	*kelch-like ECH-associated protein 1*	
25	*CHRNA3*	*cholinergic receptor, nicotinic, alpha 3*	Nicotine addiction
26	*CHRNA5*	*cholinergic receptor, nicotinic, alpha 5*	

Nr=number

Table 2: Functional Annotation Summary according to genetic association disease (selected)

Annotation/ Term	Genes	%	p-value	Benjamini
lung function	*ADAM33 GSTM1 GSTP1 GSTT1 HMOX1 MMP1 MMP12 MMP9 SOD2 SOD3*	38.5	$1.7x10^{-14}$	$8.3x10^{-12}$
chronic obstructive pulmonary disease/ COPD	*GCLC GCLM GSTM1 GSTP1 HMOX1 MMP1 MMP12 MMP9 SFTPB TGFβ1*	38.5	$9.7x10^{-11}$	$2.4x10^{-8}$
emphysema	*GSTM1 GSTP1 GSTT1 HMOX1 MMP9*	19.2	$9.7x10^{-7}$	$4.7x10^{-5}$
cystic fibrosis; chronic bronchopulmonary diseases; chronic obstructive pulmonary disease/COPD	*GSTM1 GSTP1*	7.7	$2.4x10^{-2}$	$1.6x10^{-1}$
chronic obstructive pulmonary disease/ COPD emphysema	*GSTP1 MMP1*	7.7	$2.4x10^{-2}$	$1.6x10^{-1}$

% = percentage representing how many genes are overrepresented by a specific term in the total number of gene; P-value = modified Fisher Test p-value; Benjamini = the false discovery rate controlling the multiple testing procedure.

Table 3: Gene functional classification

Group	Enrichment Score	Genes	Term records
1	4.23	*MMP2, MMP12, MMP9, MMP1, ADAM33*	188 (short sequence motif: cysteine switch; extracellular matrix; protease; zinc ion binding; macrophage differentiation; peptidase activity)
2	3.87	*SFTPB, SFTPD, SFTPA1, SFTPA2*	146 (lung; gaseous exchange; lung development; cell motility; pulmonary surfactant; surface film; respiratory distress syndrome)
3	2.43	*GSTT1, GSTM1, GSTP1*	45 (glutathione metabolism; drug metabolism; transferase; metabolism of xenobiotics by cytochrome P450)
4	1.19	*GCLM, GCLC*	138 (regulation of tube size; response to oxidative stress; circulatory system process; glutathione metabolic process)
5	0.90	*CHRNA3, CHRNA5*	146 (behavioural response to nicotine; neurotransmitter receptor activity; ion transport; response to organic substance)

Enrichment Score = ranks the biological significance of genes groups based on all enriched annotation terms. Term records = reports the major enriched annotation terms associated with the group.

Factor analysis

Table S3 (supplementary material) shows the SNPs with the corresponding genes that were previously found to be significantly associated with level or decline of lung function and/or COPD development in the Vlagtwedde-Vlaardingen cohort. We included 351 subjects with an FEV1/FVC <70%. In figure 1 we highlight all 26 SNPs included in the factor analysis that were not highly correlated with each other. Factor analysis showed that 10 factors explained 62.5 % of the total variance in our data set. The correlations and the variance explained by each factor are presented in table 4. We identified the following clustering of genes: 1) *ADAM33* (2 SNPs), 2) *ADAM33* (4 SNPs), 3) *TGF-β1* (2 SNPs), 4) *MRP1* (2 SNPs), 5) *SOD2* (2 SNPs), 6) *SFTPD* and *SFTPA2* (2 SNPs), 7) *MRP1* (3 SNPs), 8) *KEAP,* and *TGF-β1* (2 SNPs), 9) *GCLM, GCLC* and *GSTT1* (2 SNPs and a null allele) and 10) *NFE2L, GSTM1* and *SOD3* (2 SNPs and a null allele).

Figure 1: Linkage disequilibrium plot for all the SNPs genotyped previously in Vlagtwedde-Vlaardingen

Highlighted SNPs are used in the factor analysis. GSTM1 null allele and GSTT1 null allele are not displayed in the LD plot.

Table 4: Varimax rotated factor loading matrix from the factor analysis

SNPs	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10
rs3918396_ADAM33	**0.921**	0.005	-0.039	-0.043	-0.031	-0.011	0.012	-0.027	-0.047	0.038
rs17548907_ADAM33	**0.918**	0.105	-0.018	-0.040	-0.042	-0.070	-0.076	-0.030	0.038	-0.038
rs2280091_ADAM33	-0.188	**0.832**	0.021	-0.011	0.132	0.057	0.076	0.038	-0.127	0.011
rs17548913_ADAM33	0.426	**0.758**	0.016	-0.064	0.021	0.009	-0.058	-0.031	0.127	-0.040
rs528557_ADAM33	0.579	**0.741**	0.011	-0.019	0.039	-0.003	-0.061	-0.033	-0.048	-0.031
rs597980_ADAM33	0.454	**-0.513**	0.002	-0.026	0.020	0.150	-0.129	0.078	-0.207	-0.042
rs1982073_TGFβ1	0.011	0.033	**0.913**	-0.093	0.015	-0.059	0.066	-0.043	-0.015	-0.044
rs1800469_TGFβ1	-0.061	0.006	**0.900**	-0.006	0.004	-0.019	0.081	0.036	-0.060	-0.003
rs4781699_MRP1	-0.075	0.018	-0.026	**0.877**	0.048	-0.085	0.0005	-0.038	-0.066	-0.048
rs504348_MRP1	-0.022	-0.059	-0.075	**0.872**	0.086	-0.053	-0.011	0.026	-0.003	0.050
rs2842958_SOD2	-0.045	0.071	-0.024	0.091	**0.844**	0.019	-0.016	-0.016	0.072	-0.049
rs4880_SOD2	0.003	0.061	0.062	0.045	**0.825**	-0.035	0.024	0.044	-0.053	0.090
rs721917_ SFTPD	-0.067	0.001	-0.018	-0.107	-0.025	**0.805**	-0.018	-0.009	-0.088	-0.011
rs17886395_SFTPA2	0.027	0.010	-0.061	-0.023	0.005	**0.766**	0.113	-0.106	0.091	-0.016
rs4148382_MRP1	0.029	0.011	-0.057	-0.027	0.049	-0.040	**-0.713**	-0.245	-0.109	-0.012
rs212093_MRP1	-0.090	0.084	0.046	-0.072	0.020	-0.010	**0.639**	-0.055	-0.043	-0.075
rs35621_MRP1	0.044	-0.063	0.119	0.220	-0.010	0.176	**0.414**	-0.161	-0.263	0.101
rs11085735_KEAP1	-0.076	-0.061	0.053	0.046	-0.021	-0.026	0.016	**0.715**	-0.033	-0.164
rs6957_TGFβ1	-0.001	0.071	-0.139	-0.141	-0.018	-0.184	0.037	**0.534**	-0.114	0.220
rs41303970_GCLM	-0.100	0.058	-0.067	-0.053	-0.059	0.021	-0.051	-0.041	**0.641**	0.011
rs17883901_GCLC	0.164	-0.067	0.097	0.171	0.046	0.233	-0.069	0.431	**0.457**	0.199
GSTT1 null allele	0.054	-0.142	-0.013	-0.105	0.242	-0.101	0.326	-0.171	**0.425**	0.012
rs2364723_NFE2L2	-0.083	-0.012	-0.110	-0.038	0.081	0.078	0.189	0.011	-0.279	**0.633**
GSTM1 null allele	-0.015	0.085	-0.157	-0.061	-0.055	-0.040	0.257	-0.077	-0.148	**-0.523**
rs1799895_SOD3	0.056	0.067	-0.067	-0.009	-0.099	-0.186	0.012	-0.062	0.289	**0.522**
rs243865_MMP2	0.048	-0.029	0.096	0.006	-0.308	-0.032	0.102	0.297	0.162	-0.331
Initial Eigen value	2.89	2.09	1.95	1.71	1.55	1.41	1.28	1.18	1.11	1.10
Variance explained 62.5%	9.7	8.3	6.8	6.5	6.2	5.6	5.3	4.7	4.7	4.7

Bold values represent the highest loadings of the variable. The threshold was set to 0.4.

Discussion

Over the past 10 years, biologically plausible genetic candidates have been identified for both the level and decline of lung function and COPD development in the general population [1-7, 17]. It has become clear that complex diseases such as COPD are not determined by a single gene, but by gene pathways based on shared biological features. Therefore we applied an approach different from candidate genes to identify potential gene-networks underlying COPD and representing the biological processes behind the disease, using DAVID [11]. Interestingly, the first function group identified by DAVID is represented by genes involved in the metalloproteinase processes. Matrix metalloproteinases (MMP) and a disintegrin and metalloproteinases (ADAM) are members of the metzincins superfamily of zinc-based proteinases [18]. These proteinases are considered to play a central role as regulators of the tissue microenvironment under physiological conditions during development and tissue remodelling and they can contribute to tissue destruction under pathological conditions [18].

The second functional gene group indicated as important in our gene input list is represented by the surfactant genes known to play a role in host defence and control of inflammation. Therefore it is not surprising that alterations in surfactants like *SFTPA1* and *SFTPD* have been associated with the progression of COPD [2] while *SFTPB* is involved in COPD exacerbations [19].

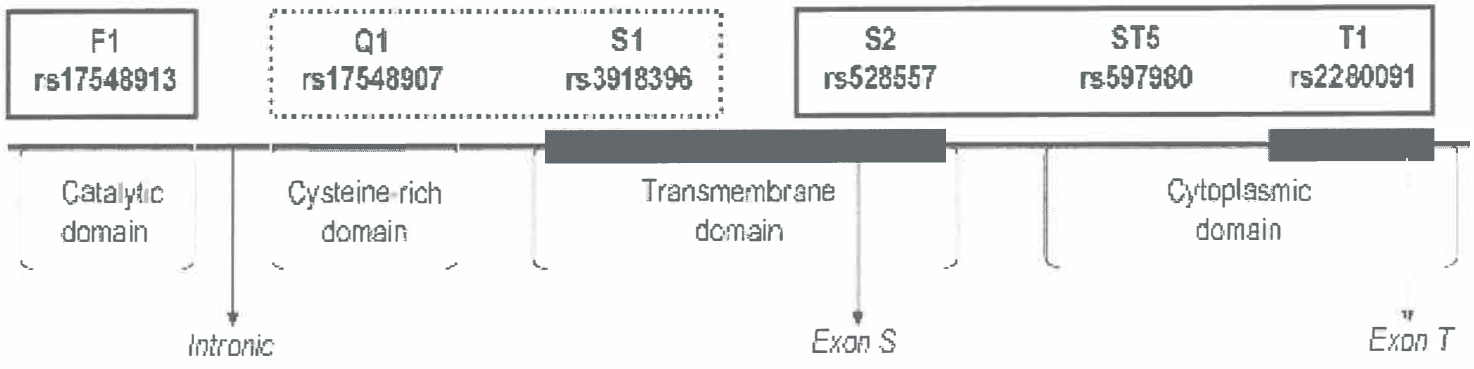
Following the idea that the genes are networking together as a unit, the third group is also of interest since this is represented by the *GSTT1*, *GSTM1* and *GSTP1* genes known to play important roles in the detoxification of many substances and organic pollutants. Moreover, combined presence of polymorphisms in these genes may have favourable effects on redox balance in COPD [20].
Of interest, *GSTs* and *GCLs*, that are both involved in the oxidative stress processes do not belong to the same group in DAVID but rather separate in group 3 and group 4 respectively (Table 3), suggesting that they are not totality sharing the same biological terms, yet glutathione metabolism is found in both groups. This was an unexpected finding, since we previously reported a significant interaction of the *GCLC* genotype with packyears smoked in individuals with the lowest vitamin C

intake in the general population [4] and an *in vivo* study indicated an interaction between vitamin C and glutathione [21], both being involved in tissue antioxidant defense [4]. These data suggest that there may exist a connection between GSTs and GCLs. Apparently SNPs in *GSTs* and *GCLs* may function together in COPD as observed in our factor analysis (Table 4), but at the same time they may function alone as signified by the DAVID gene functional classification (Table 3), highlighting that identifying the underlying mechanisms by which genes are clustering together is of importance in COPD.

Although not highly significant, the 5th group is of interest since *CHRNA3* and *CHRNA5* have been associated cross-sectionally with the level of lung function, COPD and nicotine dependence [22, 23]. Interestingly, in the gene functional classification *CHRNA3* and *CHRNA5* genes were linked to terms related to nicotine addiction and behavioural response to nicotine (Table 3), but not to lung function and COPD (Table 2). This is in line with a previous study in which we showed that SNPs in the *nAChRs* cluster might have a role in the development of COPD via smoking habits, but not via direct effects on lung function level and decline [8]. Since these variants are related to the onset of COPD via their association with smoking habits exclusively, we did not include them in the factor analysis.

To our knowledge this is the first study attempting to categorize SNPs in candidate genes previously associated with lung function decline and COPD [1-8] by factor analysis. This resulted in 10 groups of SNPs in which the *ADAM33* SNPs explained the largest part of variance in our data, indicating that SNPs in *ADAM33* are correlated with each other based on their possible functionality. Figure 2 highlights the SNPs in *ADAM33* corresponding to the first 2 factors. In the first factor, the SNPs rs17548907 and rs3918396 in *ADAM33* correspond to the Cysteine-rich domain and the Transmembrane domain respectively [24].

Figure 2: Location of the *ADAM33* SNPs with their corresponding gene domains and factors in the factor analysis



The square with the dashed line is represented by the SNPs on Factor 1 and the squares with the continuous line are represented by the SNPs on Factor 2 from the factor analysis.

These domains regulate ADAM function and play a role in regulating protease function in response to intracellular signalling events [25]. Interestingly the SNPs rs17548907 and rs3918396 are moderately correlated with each other (r^2=0.62) and since the Cysteine-rich domain is linked to cell adhesion this might indicate that these SNPS exert their effect in processes that determine the fate of primary ADAM33 transcripts [26, 27].

The second factor was represented by *ADAM33* SNPs that are located in different domains: rs17548913 in the catalytic domain, rs528557 in the transmembrane domain and rs2280091 and rs597980 in the cytoplasmic domain. Our data suggest that these variants can have a functional relevance together as a unit or, alternatively, can be influenced by each other. The SNP rs17548913 is thought to influence proteolytic activity [27] and non-coding intron SNPs like rs597980 can exert

their effects by influencing alternative splicing, splicing efficiency and messenger RNA turnover [24]. A study on the alternative splicing of *ADAM33* transcripts indicated that the 3'domains (transmembrane and cytoplasmatic) are present in all transcripts, and that variability in exon usage occurs in the 5'end of the gene, i.e. in the metalloprotease and prodomains including the catalytic domain corresponding to rs17548913 [26, 28]. Thus it is likely that the SNPs clustering together on factor 2 exhibit their effect by different complementary mechanisms.

Other clustering of SNPs on one factor occurred within the *MRP1* gene, i.e. rs4781699 and rs504348 as well as rs4148382, rs212093 and rs35621. Although it is not known if rs4781699 exhibits a functional role in *MRP1*, this SNP clustered together with a moderate correlation (r^2=0.44) with rs504348 known to result in a significant increase in *MRP1* promoter activity *in vitro* [29]. The other 3 SNPs (rs4148382, rs212093 and rs35621) in *MRP1* are located in the middle and end of the gene, and this needs clearly further research to elucidate whether they complement each other in their effect or have additive affects.

In conclusion, our current study was designed to identify how previously reported genes that are linked to COPD belong to the same pathway and whether SNPs in these genes cluster together. The pathway analysis allows genes to be assigned to one or more groups which align with their biological nature. Thus, we found that the most important cluster of genes functioning together was represented by *MMP1*, *MMP2*, *MMP9* and *MMP12* and *ADAM33*. Moreover *GSTs* and *GCLs* were clustering as oxidative stress genes and *SFTPs* as surfactant genes. Factor analysis showed that SNPs in the *ADAM33* cluster together on two factors based on their possible functional role. These factors explained most variance in our data set with COPD subjects from the general population. Making use of the bioinformatics tools as well as integrated databases will further lead to a better understanding how the genes, the SNPs and the pathways identified contribute to the development of COPD and other respiratory diseases based on their functionality and/or their shared biology.

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CHAPTER 8

Summary, conclusions and future perspectives

Summary of the findings

Chronic Obstructive Pulmonary Disease (COPD) is clinically characterized by irreversible airway obstruction and an accelerated decline in lung function generally with or without respiratory symptoms like cough, phlegm and dyspnea [1]. Cigarette smoking is the most important risk factor for the development of COPD and the low percentage of variance in pulmonary function explained by smoking suggests that there could be genetic differences in susceptibility to the effects of cigarette smoking [2]. The mechanisms contributing to structural and functional changes in the lungs of COPD patients are protease-antiprotease and oxidant-antioxidant imbalance, and increased inflammation. This thesis describes several genetic variants in genes involved in the above mentioned mechanisms in relation to the level and decline of lung function in the general population and to inflammatory markers in bronchial biopsies and induced sputum in patients with established COPD.

Chapter 2 describes a study investigating five single nucleotide polymorphisms (SNPs) in *multidrug resistance-associated protein-1, MRP1, (official name ABCC1)*. MRP1 is a member of the human ATP-binding cassette superfamily of transporters which regulates the traffic of molecules across cell membranes. A total of 51 SNPs with a minor allele frequency (MAF) > 5% are required to tag the entire *MRP1* gene in Caucasians [3]. Siedlinski et al. demonstrated that two SNPs in *MRP1* were significantly associated with a lower or higher level of FEV_1 in two independent population-based cohorts (Doetinchem and Vlagtwedde-Vlaardingen). Two additional SNPs had a significant effect of the same, negative magnitude on the level or decline of FEV_1. One SNP was a significant predictor of COPD in the general population [4]. We selected these five SNPs for genotyping in patients with COPD (GLUCOLD study). We found all 5 SNPs to be involved in the severity of COPD. One of these SNPs, rs212093 was associated with a higher level of FEV_1 and less inflammatory cells in bronchial biopsies. Additionally, the SNPs rs504348 and rs4781699 were associated with less airway wall inflammation and rs4148382 with a lower FEV_1 level and increased sputum cell numbers. On top of these findings, we have estimated the haplotypes of *MRP1* and assessed the effects of these haplotypes on FEV_1 level and inflammatory cells in bronchial biopsies and induced sputum.

We observed that the effects of *MRP1* haplotypes are due to a specific SNP e.g. rs4148382 constituting these haplotypes, and therefore *MRP1* haplotypes did not add new information.

Chapter 2 describes the associations of the before mentioned SNPs in *MRP1* with MRP1 protein in bronchial biopsies of COPD patients. We found that rs4148382 and rs4781699 were significantly associated with higher respectively lower levels of MRP1 protein in bronchial biopsies. The apparent differences with one of our previous study [5] might be due to underlying differences of *MRP1* genotypes distribution in the two populations, since we were able to observe that the previous low intensity of MRP1 staining was driven by wild type individuals of rs4148382 [5], suggesting the importance of the genotypes distribution in relation to MRP1 expression. We conclude that the findings of this study are an important step forward linking *MRP1* polymorphisms with the pathophysiology of COPD.

Since MRP1 is responsible for trans-membrane transport of organic anions, including clinical drugs and as *MRP1* polymorphisms as well as MRP1 protein expression in bronchial biopsies are of importance in COPD severity, we moved forward to the second study involving MRP1 in COPD patients, which is described in **chapter 3.** This study focused on the associations of *MRP1* SNPs and MRP1 protein expression in airway wall biopsies with the decline of lung function in the same population of COPD patients we previously studied with respect to anti-inflammatory and clinical effects of inhaled corticosteroids (ICS) with or without long-acting β-agonists (LABAs) [6]. We found significant associations of a higher MRP1 protein expression with smaller FEV_1 decline in COPD patients using long-term therapy with inhaled corticosteroids (ICS) and a significant association of higher MRP1 protein expression with a faster FEV_1 decline after withdrawal of ICS. These associations were not present in patients using placebo or the combination of long-acting β-agonists (LABAs) and ICS. We did not observe any significant associations of MRP1 protein expression at baseline, 6 or 30 months with the level of lung function at these time points within the specific treatment groups. This suggests that MRP1 protein expression may reflect one of the COPD phenotypes that is sensitive to inhaled corticosteroid therapy. Moreover, we concluded that these associations were not likely due to the genetic background in *MRP1*, since the

SNPs in *MRP1* that were previously associated with the level of FEV_1 in chapter 2, in the current study are not significantly associated with FEV_1 decline in the same group of COPD patients.

Chapter 4 describes a study concerning *TLR2* and *TLR4* genes and their role in patients with COPD (GLUCOLD study). Toll-like receptors (TLRs) participate in the defence against viral and bacterial infections and since infections in the airways amplify disease progression in the lungs of COPD patients, it might be that TLRs are of importance in COPD. We showed for the first time that 26 tagging SNPs in *TLR2* and *TLR4* are associated with both the level and decline of FEV_1, and with inflammatory cells in induced sputum at baseline and their changes from baseline over time. From 9 SNPs investigated in *TLR2*, 4 SNPs were associated with a higher or lower level of FEV_1, while others were associated with less respectively more rapid FEV_1 decline. One of the SNP in *TLR2* rs11938228 was consistently associated with a higher number of inflammatory cells in induced sputum (neutrophils, macrophages and eosinophils). Another SNP in *TLR2* (rs3804099) was significantly associated at baseline with the number of eosinophils and epithelial cells and with changes from baseline in the number of neutrophils and macrophages. Interestingly, none of the SNPs in *TLR4* was associated with the level of FEV_1, but 10 out of 17 SNPs genotyped in *TLR4* were associated with the decline of FEV_1. Two SNPs in TLR4 (rs12377632 and rs10759931) were consistently associated with inflammatory cell counts in induced sputum, while the other SNPs were associated either with the baseline level or with the changes from baseline of the number of inflammatory cells in induced sputum. More studies are needed to confirm these findings and to understand the mechanism by which the *TLR2* and *TLR4* polymorphisms affect the pathological role of TLRs in the signaling pathways involved in COPD.

Chapter 5 describes a study investigating one of the genes involved in lung developmental processes, namely *Hedgehog-interacting protein (HHIP)*. We found that the intergenic SNP rs13147758 near the *HHIP* gene is not significantly associated with FEV_1 level or decline in the total general population, but in smokers from the general population rs13147758 was significantly associated with a higher level of FEV_1. This might suggest that this is the ultimate effect of lung development

and smoking in relation to the *HHIP* gene variations. In the GLUCOLD study, we observed that rs13147758 was significantly associated with less accelerated decline in lung function (FEV_1 and FEV_1/IVC), an effect that was enhanced in smokers, suggesting that this gene might be involved in a balance between repair and cigarette induced lung injury. Besides the fact that this study corroborates the findings of previous studies, we additionally provided data that strengthen the idea that this developmental gene may play an important role in the level of FEV_1 and that *HHIP* may be involved in lung tissue repair processes in COPD.

In **chapter 6** we have described a controversial gene with respect to its association with COPD and smoking addiction, the *nicotinic acetylcholine receptor (nAChR)* gene. Since smoking is a risk factor for COPD itself, the previous cross-sectional studies [7-9] did not provide information whether the SNPs in the *nAChR* cluster are directly and independently a risk for COPD development or whether they are associated with COPD through their association with nicotine dependency and smoking habits. We investigated 3 SNPs in the *nAChR* cluster (rs569207, rs1051730 and rs8034191) in the population-based Vlagtwedde-Vlaardingen cohort. We observed that rs1051730 and rs569207 in the *nAChR* cluster are associated with an increased respectively decreased ability to quit smoking, but have no significant effect on the annual FEV_1 decline in smokers and ex-smokers separately, with or without correction for quitting respectively restarting smoking. Additionally, smokers who quit smoking had 20.3 ml less annual FEV_1 decline than subjects who continued smoking. Interestingly, current and ex-smokers that were homozygote for rs569207 had a lower number of packyears at the last survey as compared with wild type individuals. None of the SNPs was associated with number of cigarettes smoked per day at the last survey.
This is the first longitudinal study suggesting that SNPs in the *nAChR* cluster potentially have a causal role in COPD via smoking habits among smokers, independent of effects on lung function. Thus these variants are related to the onset of COPD via their association with smoking habits, and they are not independently associated with COPD.

In the final study of this thesis, described in **chapter 7**, we combined two different

methods that might provide a better understanding of how genes and their SNPs are functioning together. Firstly we explored via DAVID (the Database for Annotation, Visualization and Integration Discovery) a set of 26 genes previously shown to be significantly associated with lung function level and decline and with COPD in the general population-based cohort Vlagtwedde-Vlaardingen [10-15]. Using DAVID we adopted a strategy to systematically identify a number of genes that are associated with biological terms (e.g. lung function) and statistically highlight the most overrepresented or enriched biological terms [16]. We identified 5 functional groups having similar annotation profiles, being functionally related, i.e.1) *metalloproteinases*, including *ADAM33*, 2) *surfactants*, 3) *glutathione transferases*, 4) *glutamate-cysteine ligases* and 5) *cholinergic receptor, nicotinic, alpha* genes. The most relevant biological term was "lung function" that was associated with 10 genes: *ADAM33, GSTM1, GSTP1, GSTT1, HMOX1, MMP1, MMP12, MMP9, SOD2* and *SOD3*.

Secondly we investigated how the SNPs in these candidate genes were categorized together by factor analysis in these subjects with COPD. We selected 26 SNPs from 78 SNPs previously genotyped in these 26 genes. These 26 SNPs were previously found to be significantly associated with the level and/or decline of lung function in the general population. Factor analysis showed 10 factors to explain 62.6% of the total variance in our data set: 1) *ADAM33* (5 SNPs), 2) *ADAM33* (4 SNPs), 3) *TGF-β1* (2 SNPs), 4) *MRP1* (2 SNPs), 5) *SOD2* (2 SNPs), 6) *SFTPD* and *SFTPA2* (2 SNPs), 7) *MRP1* (3 SNPs), 8) *KEAP, TGF-β1* and *GCLC* (3 SNPs), 9) *GCLM, GCLC* and *GSTT1* (2 SNPs and a null allele) and 10) *NFE2L, GSTM1* and *SOD3* (2 SNPs and a null allele).

The results of the factor analysis are of importance since these are in line with our analysis using DAVID tools.

In conclusion, our study indicates that the combination of these two methods should lead to a better understanding how the genes, the SNPs and the pathways identified contribute to the development of respiratory diseases based on their functionality or their shared biology.

Conclusions

Advances in knowledge about the biology and genetics of COPD have been accompanied by improved understanding of the factors leading to this disease. The main goal of performing genetic research in complex diseases like COPD is to investigate and question why some people develop the disease while others do not. Another goal is to identify the best strategy to prevent the progression of the disease and minimize the detrimental effects of the genetic factors or take advantage of the beneficial effects some genes are displaying and incorporate this knowledge into the existing COPD treatment.

COPD is a heterogeneous disease. Some patients have large and/or small airway obstruction without emphysema, whereas others have small airways disease, emphysema or a combination. It has been demonstrated that a.o. the inter-individual differences in phenotypes may have a genetic background [17]. Therefore it is of importance to study different COPD phenotypes with a clear definition and validation that might be influenced by these genetic factors. We have studied genetic determinants of lung function level and lung function decline in COPD patients and in healthy individuals from the general population. Moreover, we used airway inflammation as an intermediate phenotype. For this purpose we studied different cohorts such as GLUCOLD, including COPD patients, and Doetinchem and Vlagtwedde-Vlaardingen, two independent general population-based Dutch cohorts. In this thesis we show that some genes are involved in the severity of COPD based on the significant associations of these genes with level of lung function (FEV_1 and FEV_1/FVC) and with airway inflammation in bronchial biopsies and induced sputum in COPD patients. Moreover, these genes are also involved in the progression of the disease since they are significantly associated with the decline in lung function and changes from baseline of inflammatory cells in bronchial biopsies and induced sputum in COPD patients. We also show that multiple genes are associated with lung function level and decline in the general population. Therefore, as studies were performed in COPD patients and the general population, the outcomes of these studies complement each other and provide a better understanding of how genes may affect different aspects of COPD development, severity and progression. It is

essential to identify and separate which genes are involved in COPD susceptibility and which in COPD severity since these two aspects of COPD may be different. Besides the identification of different genes involved in COPD, it is important to have a clear overview on how these genes are studied, in which populations and sample size, which COPD biomarkers are used, which variations in the genes are found, how the genes are selected and what they have in common. By carefully interpreting the results of the studies described in this thesis we provide new insights for future studies investigating the biologically plausible genetic candidates for COPD. We will point out in the following paragraphs the different interpretations of the results of this thesis. In addition some of the limitations of the described studies are discussed.

The importance of longitudinal data

In four of the studies (chapters 3, 4, 5 and 6) described in this thesis we have used longitudinal data. Longitudinal data are crucial if we want to study onset and progression of disease. As shown for example in our studies on (chapters 2 and 3) *MRP1,* this gene appears to play a role in COPD severity (e.g. lower baseline level of FEV_1 and more airway inflammation) without any further effects on lung function or inflammation over time. There is also a need for longitudinal data if we want to determine disease progression that is responsive to treatments of COPD. Since 2 longitudinal studies demonstrated that ICS might influence the natural course of the disease [6, 18], our findings from the study described in chapter 3 might contribute to potential future applications of pharmacogenetics in COPD management of mild to moderately severe COPD patients.

Our studies in the longitudinal population-based cohorts Doetinchem and Vlagtwedde-Vlaardingen are particularly useful in that they allow determining which genetic risk factors are important in an early stage of lung function loss before clinical symptoms appear. Moreover, they allow us to investigate the role of the genetic factors in the inter-individual variation to start and quit smoking and their effects on lung function. Using longitudinal data, we could clearly show that variants in the nicotinic acetylcholine receptor are related to the onset of COPD via their association with smoking habits, and are not independently associated with

COPD development, as was suggested by cross-sectional studies.

Different results in different populations

MRP1 may play a role in smoking related development of COPD and even severity and progression of the disease. However, investigating such a gene in different cohorts might lead to different results which require different interpretations. For example, different cohorts often have different histories of exposure to cigarette smoking or exposure to cigarettes in utero, youth or adulthood, which may change the effect of some variations in genes involved in smoking-related pathways to change. There is evidence that studies on the role of *MRP1* might render different results depending on how this gene is studied [4, 5, 19-21]. The discrepancy of the results may be explained by the different countries from which the cohorts have been obtained (British and Dutch) [4]. Thus, country-specific environmental and behavioural factors could influence the effect of the SNP differentially in two areas of residence. Not only the area of residence and possible cultural differences might influence the associations of the SNPs in *MRP1*, but also the selection of subjects with COPD appears to be of importance as we have shown in our study (chapter 2). We observed that our results are opposite to previous findings as described by Siedlinski et al in the general population of Doetinchem that originates from The Netherlands, just as the GLUCOLD participants [4]. The opposite effects are likely due to the fact that although the selected COPD subset of the Doetinchem general population and COPD patients from the GLUCOLD study had almost the same number of packyears, the matched COPD subset in the Doetinchem cohort had a higher lung function, suggesting that this COPD subset from the Doetinchem study, might be less susceptible to cigarette smoke than the patients with established COPD.

Candidate gene studies versus GWAs

The advantage of candidate gene studies is that they do not require large families with both affected and unaffected members but can be performed with unrelated cases and control subjects or with small families (e.g. a proband and parents). However, case-control studies may result in spurious associations if the controls are not appropriately matched to the cases with respect to ethnicity or factors that

influence disease occurrence. Contrary to candidate gene approaches, GWA studies are hypothesis free, because the markers that are set throughout the genome are merely used to identify loci associated with disease and are not selected based on their assumed biological function. Although GWAs provide strong evidence regarding the importance of different loci in COPD susceptibility [22-26], these studies are usually still underpowered to identify genetic determinants of small effects. Establishing a consortium of groups studying genetics in cigarette smokers and non-smokers may facilitate pooling large samples to identify genetic variants associated with COPD susceptibility [27]. Although larger sample sizes of cases and controls will definitely increase the power to detect COPD susceptibility loci, linking these genes to markers of inflammation in sputum and bronchial biopsies merely remains a power issue with respect to bronchial biopsy data. Apart from the ethical aspects, it is not feasible to perform bronchoscopy and sputum induction on a large scale. In the GLUCOLD population we had the possibility to directly link genes to inflammation in induced sputum and bronchial biopsies. Even though from a genetic point of view a sample size of 114 patients with COPD is relatively small, the GLUCOLD study is still one of the largest biopsy studies world-wide, giving us the unique opportunity to investigate in depth the associations of candidate genes for COPD with markers of inflammation that are sensitive measures of assessing COPD severity and disease progression.

Bronchial biopsies from COPD patients

COPD severity is graded by FEV_1 % predicted [28], but this grading does not take into account the range of pathophysiological abnormalities that may be present in this heterogeneous disease. The assessment of COPD progression typically requires studies using decline in FEV_1 that can be used for prognostic purposes as well. The main contribution of bronchial biopsies when compared to lung function grading is that they directly sample airways tissue, maintaining the structural components that may be important to functional changes [30, 31], and may provide information on cellular patterns and expression of inflammatory proteins in patients with COPD [30].

Obviously, more sensitive measures of assessing COPD severity and disease progression are needed. Such measures should ideally reflect several components

such as inflammation, structural changes and disease activity. There is much interest in improving the phenotypic description of COPD by the use of biomarkers that allow identification of distinct subgroups of patients with different prognosis or response to therapy [28, 29]. Studying e.g. induced sputum shows the potential to sub-categorize groups of COPD patients [28, 29]. In future, gene expression profiles, protein or peptides derived from bronchial biopsies, bronchoalveolar lavage, epithelial lining fluid and exhaled breath, are promising tools for identification of COPD sub-phenotypes.

Biomarkers are promising tools for future studies since they may be affected by genetic polymorphisms and this may contribute to new pathogenic insights into COPD or parameters that may be used for the assessment of disease severity or effects of therapy. For example, the simultaneous genome-wide assay of gene expression in bronchial biopsies from COPD patients can be coupled with the interrogation of the same patients' genetic variation. This allows the discovery of gene variants that contribute to inter-individual differences in gene expression (tissue-specific expression of quantitative trait loci (eQTLs)). Therefore, eQTL mapping is of interest since it can elucidate which transcripts or groups of transcript are associated with which (sets of) biomarkers.

Selection of the genes and their corresponding SNPs

Candidate genes are usually chosen based on molecular pathways or cellular mechanisms believed to be involved in the disease biology. Candidate gene association studies have been performed to elucidate the genetic determinants of COPD and related phenotypes. Bearing in mind current concepts of COPD pathogenesis, geneticists have studied genes involved in inflammation, immune response, antioxidant, xenobiotic metabolism and protease-antiprotease balance. Therefore, we have chosen the genes described in this thesis based on their involvement in well known mechanisms of COPD i.e. protease-antiprotease and oxidant-antioxidant imbalance or based on previous findings from the general population.

By investigating all tagging SNPs in a candidate gene we make sure that these SNPs cover all the genetic variation.

In chapter 4 we investigated all tagging SNPs in the *TLR2* and *TLR4* genes, thus

providing a comprehensive overview of the genetic contribution of *TLRs* to COPD severity and progression with respect to lung function and inflammation. We chose these genes based on their plausible biological role in COPD since the innate immune response in the airways involves the detection of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) by recognition receptors such as TLRs on cell surfaces [32]. TLRs participate in the defence against viral and bacterial infections and since infections in the airways worsen the disease and promote progression of COPD, TLRs are thus biological plausible genes to study in relation to COPD development and/or progression.

Such biologically plausible genetic candidates have been found for both the level and/or decline of lung function and COPD development in the general population [4, 10-15, 24]. However, since genes do not function on their own, gene pathway identification is useful for understanding the mechanisms of developing a complex disease like COPD. In chapter 7 we systematically mapped a number of genes that we previously showed to be associated with reduced lung function and COPD. We combined two methods to identify how genes and their corresponding variants are clustering together and thus are possibly functioning together. This study provided us the opportunity to identify how the genes, the SNPs and the pathways identified contribute to the development of COPD and other respiratory diseases based on their functionality and/or their shared biology.

Beside the candidate gene selection involved in the disease biology, in recent years the collection of large cohorts has allowed unbiased GWAs in individuals with COPD. Top hits from one of these GWAs were SNPs in the *nAChR* cluster and the *HHIP* region [24]. Because the GWAs did not provide information on the tagging SNPs, but on SNPs in genes' region, we chose to investigate these top hits from these GWAs in our two general populations-based cohorts and in COPD patients. This approach might lead to replication of previously reported findings, but future candidate gene studies should additionally focus on the tagging SNPs in the *nAChR* cluster and the *HHIP* region.

Future perspectives

Although genetic studies on COPD have some limitations and should be carefully interpreted, we believe that these studies have greatly contributed to the progress in elucidating the pathogenesis of COPD. Future studies will bring us insight into mechanisms underlying various phenotypes of COPD including emphysema, pulmonary hypertension and mucus hypersecretion, leading to the development of a specific treatment for each part of the disease process.

The candidate gene approach is useful for determining the association of a genetic variant with a disorder and for identifying genes of modest effect. Candidate gene association studies have been performed extensively, but the results have been largely inconsistent and often lack replication. A major problem in replications attempts is that they may result in spurious associations if the controls are not appropriately matched to the cases with respect to ethnicity or other factors that influence an individual's genetic make-up. In the current thesis the associations found are mostly novel, and therefore the challenge is to replicate these findings in further studies and move forward for a better understanding of COPD pathophysiology. To avoid false positive associations that may have been obtained by chance, future studies should take into consideration some methodological issues that might facilitate the replication of the results: phenotype definition, sample size, population stratification, publication bias, incorrect interpretation of data, multiple testing, genotyping errors, incomplete genetic coverage and genetic and environmental population heterogeneity.

The GLUCOLD study has unique data with airway wall biopsies taken at baseline, 6 months and 30 months following treatment with fluticasone (either 6 months followed by 24 months of placebo, or 30-month treatment), fluticasone/salmeterol combination for 30 months, or placebo for 30 months. However, it will be challenging to replicate our findings in future studies, since the GLUCOLD study remains one of the largest biopsy studies world-wide. Therefore, it will be of great interest to compare the outcome of our studies with a large group of lung tissues in individuals treated or not with the same drug regimens and followed for the same or a longer period of time.

Another replication issue is represented by the phenotyping difference that concerns the definition of COPD. COPD definition might be based on lung function measurements using different spirometry protocols or spirometers or different COPD sub-phenotypes such as emphysema scores or COPD symptoms. It is likely that the effect of functional variation in genes involved in destruction of lung parenchyma tissue will become more evident in studies involving COPD patients with severe emphysema than in studies investigating COPD patients with no emphysema, but with respiratory features such as mucus hypersecretion and airway obstruction. Some COPD patients may have signs of airway obstruction, yet they do not have emphysema as assessed by computer tomography (CT) scan. In a recent study it has been shown that extensive baseline low-attenuation areas at CT scanning are associated with a greater reduction in lung function in both non-obstructed and obstructed male heavy smokers [33]. Moreover, individuals with low-attenuation areas values at baseline showed a larger decline in lung function level over time [33]. These results indicate that CT-quantified emphysema may represent a form of subclinical COPD which may help to identify non-obstructive male smokers with a high risk of developing airway obstruction [33]. Therefore it would be of interest to apply genome wide association studies and identify genetic variants that contribute to these sub-phenotypes, i.e. chronic bronchitis, bronchiolitis and emphysema. This may help identifying a shared (or unshared) etiology of these distinct sub-phenotypes. This may lead to a better understanding of the underlying pathogenetic mechanism in COPD resulting in an effective and tailored treatment for COPD.

The genes described in this thesis showed significant associations with disease development, severity and/or disease progression implying a role in COPD pathogenesis, but whether this would be amenable to pharmacological intervention or represents a developmental abnormality less amenable to change is not yet known. Specific pharmacogenetic studies of COPD are currently missing from the medical literature but those for asthma are growing in number [34]. This suggests that similar work may follow for COPD, given that elements of treatment strategy, including bronchodilation, are the same for the two conditions. We can thus only speculate on the directions that pharmacogenetics of COPD may take, on the basis of current knowledge of the variation in treatment response to the classes of drug now used

for COPD. One recent GWA study of more than 530,000 SNP markers revealed a novel functional SNP in *GLCCI1*, rs37973 that causes a detrimental response to inhaled glucocorticoids in patients with asthma through changes in GLCCI1 expression [35]. Consequently, van den Berge et al. showed that the same variant in *GLCCI1* was not only associated with glucocorticoid response in asthma, but also in COPD [36]. The authors showed in the GLUCOLD study that the improvement in FEV_1 was greater in wild-type individuals for rs37973 than in the homozygotes individuals for the same SNP after 3 and 30 months ICS therapy [36]. The effect of treatment with ICS on the annual rate of decline in FEV_1 was larger in TORCH [18] and the GLUCOLD study [6] than in earlier studies [37, 38]. From these studies (TORCH and GLUCOLD) there is an indication that COPD is a treatable disease in a subset of patients. Therefore, it is of interest to identify in which subset of COPD patients the disease progression can be modified by treatment.

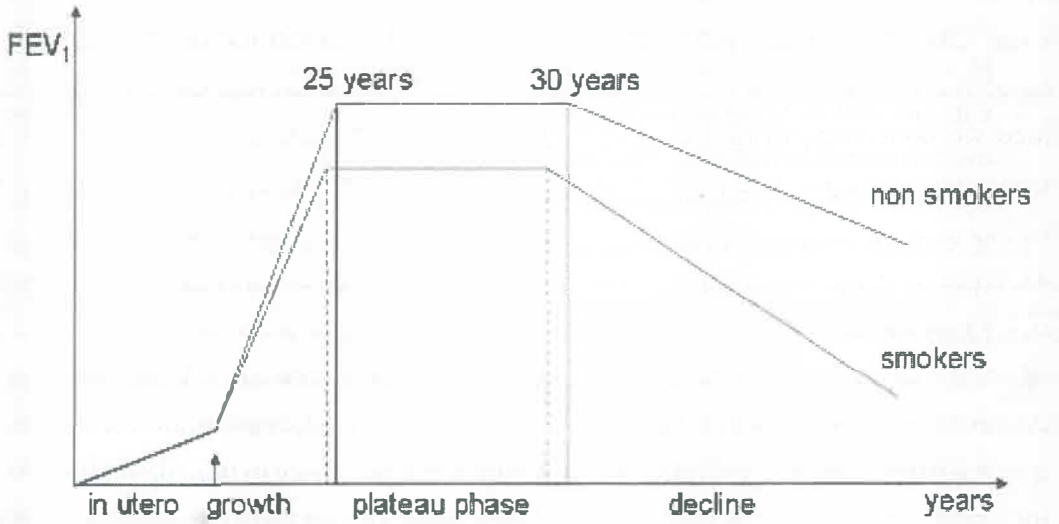
A previous study using gene expression microarrays identified a number of genes that were upregulated in patients with asthma, but not in healthy controls [39]. Furthermore, the baseline expression of these genes correlated directly with fluticasone-induced improvement of FEV_1 [39], indicating that this technique may reveal which asthma patients are likely to benefit most from ICS treatment. It is tempting to speculate that gene expression microarrays may also be useful in studies involving COPD patients, since it might identify those COPD patients that are responding or not to ICS treatment or identify those COPD patients that are more prone to a rapid progression of their disease.

Furthermore it might provide a better insight into the biological mechanism that underlies the responsiveness to treatment and progression of the disease. This could be investigated in the GLUCOLD study since that has comprehensive clinical, physiological and histological data and on top of this bronchial biopsies from which RNA can be extracted for studies with mRNA and microRNA arrays that have also been shown to play an important role in regulating gene expression responses [40]. It would be interesting to investigate if the specific genes that are differentially expressed in the bronchial biopsies of COPD patients are also associated with SNPs in these respective genes, as derived from candidate gene studies or GWAS findings. In this context, it would be interesting to assess the gene expression in pathways that are important because of their association with COPD such as the multidrug

resistance (MDR) pathway, in particular MRP1 (ABCC1) in combination with COPD severity [19], smoking [20, 41] and treatment [42]. Lung injury and repair pathways are also of interest since an imbalance of expression of repair genes in the small airway and lung parenchyma can lead to small airway remodelling respectively emphysema [43]. Therefore, the *HHIP* gene expression is of interest since we concluded in this thesis that this gene might be involved in a balance between repair and cigarette induced lung injury given its protective role in smokers. Besides the vivid interest in assessment of gene expression in bronchial biopsies of COPD patients, it would be interesting to further study this developmental gene (*HHIP*) in the context of lung growth and its interaction with environmental as well as personal smoke exposure, since we observed positive associations with the level and decline of FEV_1 especially in smokers. This specific association should be assessed in cohorts followed from birth up to the plateau phase in lung development, since cigarette smoking can operate in all life cycles: before birth (lower initial lung function), during growth phase (lower maximal attained lung function), plateau phase (earlier start of decline) and decline (an accelerated decline). (Figure 1)

Continuing elucidation of the mechanisms of lung development may identify novel therapeutic targets in the quest to prevent and treat COPD.
In summary, understanding the genetics and the mechanisms underlying COPD and the sub-phenotypes of COPD will help to establish whether exist identifiable groups of COPD patients who will respond differently to treatments. This is because their underlying genotype has the potential to determine not only the specific pathological processes underlying a clinical phenotype of disease, which may dictate treatment response, but also influences drug metabolism and thus efficacy. This makes the study of pharmacogenetics an exciting prospect for COPD in the years to come.

Figure 1: The effect of smoking on FEV_1



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Nederlandse samenvatting (summary in Dutch)

Chronic Obstructive Pulmonary Disease (COPD), oftewel "chronische obstructieve longziekte", wordt klinisch gekenmerkt door luchtwegverandering (verlaagde longfunctie) en een versnelde afname van de longfunctie, met of zonder luchtwegklachten als hoesten, slijm en kortademigheid. Roken is de belangrijkste oorzaak van het ontstaan van COPD, maar er wordt slechts een laag percentage van de variatie in longfunctie verklaard door roken. Dit suggereert dat er sprake zou kunnen zijn van genetische verschillen in gevoeligheid voor de effecten van het roken van sigaretten. Verschillende mechanismen dragen bij aan structurele en functionele veranderingen in de longen van COPD patiënten. Voorbeelden hiervan zijn een verhoogde staat van ontsteking en een verstoorde balans in protease-antiprotease en oxidant-antioxidant systemen. Dit proefschrift beschrijft in hoeverre en op welke wijze een aantal genetische varianten (zogenaamde single nucleotide polymorfismen, SNPs) bijdragen aan de ontwikkeling van COPD. Deze SNPs die aangeduid woorden met "rs" nummers kunnen betrokken zijn bij de hierboven genoemde mechanismen die samenhangen met het niveau en daling van de longfunctie in de algemene bevolking en bij patiënten met COPD en met inflammatoire markers in luchtwegbiopten en geïnduceerd sputum bij patiënten met COPD.

De genetische informatie van ieder organisme ligt opgeslagen in de chromosomen. Er zijn 23 paren van chromosomen die uit DNA zijn opgebouwd, waarvan 1 paar de geslachtchromosomen zijn (X en Y). Het DNA molecuul bestaat uit twee koordachtige strengen, lijkend op ladders, die om elkaar heen zijn gedraaid. DNA is opgebouwd uit 4 verschillende nucleotiden: Adenine (A), Cytosine (C), Thymine (T) en Guanine (G) en deze komen altijd in paren voor op tegenovergestelde strengen DNA zoals A+T en C+G. Een enkele streng van DNA bestaat dus uit deze 4 letters, b.v. ATGCTCGAA, die woorden maken van drie nucleotiden (b.v. ATG CTC GAA) en uiteindelijk vormen combinaties van deze woorden zinnen, de genen. Deze genen bevatten informatie over hoe eiwitten geproduceerd moeten worden. Eiwitten zijn nodig voor de structuur, functie en regulering van alle cellen, weefsels en organen van het lichaam.

De SNPs zijn veranderingen in het DNA in de chromosomen en komen voor bij tenminste 1% van de algemene bevolking. Dergelijke variaties kunnen bijdragen aan de ontwikkeling van ziekte, de ernst van ziekte, of hoe het lichaam reageert op medicatie. Van elk chromosoom hebben we er twee: 1 overgeërfd van de vader en 1 van de moeder (twee allelen). Het is dus mogelijk dat we van een SNP de normale variant hebben (zogenaamde "wild-type" allel) en op de andere allel de mutatie. Als een persoon twee verschillende allelen heeft, noemen we dat heterozygoot. Als we op beide allelen de mutante vorm dragen, noemen we dit een homozygote variant. Vaak komen SNPs in vaste combinaties over. Dan hoeft er maar één SNP van deze combinatie te worden gegenotypeerd. Dit noemen we een "tag" SNP.
Genen in dit proefschrift zijn geselecteerd op basis van hun biologische functie waardoor een rol zouden kunnen spelen in het ontstaan van COPD of de ernst van COPD ("kandidaat-genen"). In longitudinale studies van de algemene bevolking is het mogelijk om genetische effecten op te sporen die betrokken zijn bij een verlaagde longfunctie voordat klinisch manifeste ziekte is ontstaan. In longitudinale studies van COPD patiënten is het mogelijk de genen die een rol spelen bij de progressie van de ziekte te onderzoeken.

Eén van deze kandidaatgenen is beschreven **hoofdstuk 2** waarin vijf SNPs in *multidrug resistance-associated protein-1 (MRP1)* zijn onderzocht. MRP1 is verantwoordelijk is voor het transport van moleculen door de celmembranen. Onze onderzoek groep toonde eerder aan dat twee SNPs in *MRP1* significant geassocieerd zijn met een lager respectivelijk hoger niveau van longfunctie zoals bepaald door meting van de 1-seconde waarde (FEV_1), in twee onafhankelijk cohorten uit de algemene bevolking (Doetinchem en Vlagtwedde-Vlaardingen). Twee andere SNPs hadden een significant negatief effect van gelijke grootte op het niveau en afname van FEV_1. Eén SNP was een significante voorspeller van het risico op het krijgen van COPD in de algemene bevolking. We hebben deze vijf SNPs geselecteerd voor genotypering in patiënten met COPD. We vonden dat alle vijf SNPs betrokken zijn bij de ernst van COPD. Eén van deze SNPs, rs212093, was geassocieerd met een hoger niveau van FEV_1 en minder inflammatoire cellen in luchtwegbiopten. Bovendien waren rs504348 en rs4781699 geassocieerd met minder luchtwegwandontsteking en rs4148382 was geassocieerd met een lager niveau van FEV_1 en met toegenomen

sputum celaantallen. Daarnaast hebben wij een schatting gemaakt van *MRP1* haplotypes (een haplotype is de unieke combinatie van allelen zoals die voorkomen op een chromosoom) en de effecten van deze haplotypes op het niveau van FEV_1 en inflammatoire cellen in bronchiale biopten en geïnduceerd sputum onderzocht. Wij vonden dat de effecten van *MRP1* haplotypes zijn toe te wijzen aan slechts één SNP (e.g. rs4148382) die deel uitmaakt van deze haplotypes, en dus dat analyse van *MRP1* haplotypes geen nieuwe informatie toevoegde.

Hoofdstuk 2 beschrijft ook de associaties van de eerder genoemde SNPs in *MRP1* met MRP1 expressie in luchtwegbiopten van COPD patiënten. We vonden dat rs4148382 in het *MRP1* gen met een hoger MRP1 expressie geassocieerd was. Eerder vonden we dat MRP1 eiwit lager is bij COPD patiënten. De schijnbaar verschillende resultaten t.o.v. deze vorige studie zouden kunnen komen door onderliggende verschillen van *MRP1* SNPs in de twee populaties, want we zagen dat de vorige lage intensiteit van MRP1 kleuring werd bepaald door "wild type" individuen bij rs4148382, terwijl in onze huidige studie MRP1 kleuring werd bepaald door "heterozygote" individuen voor rs4148382. Samenvattend concludeerden we dat *MRP1* SNPs van belang zijn bij de ernst van COPD.

Omdat MRP1 verantwoordelijk is voor het trans-membraan transport van organische anionen met inbegrip van klinische geneesmiddelen is het interessant om de associaties van *MRP1* SNPs en MRP1 eiwit in de luchtwegbiopten met de afname van longfunctie in de aanwezigheid van medicatie in COPD patiënten te onderzoeken. Wij onderzochten dit nader in een studie die wordt beschreven in **hoofdstuk 3**.

We onderzochten de associaties van *MRP1* SNPs en MRP1 expressie in de luchtwegbiopten met de afname van longfunctie in de COPD patiënten in relatie tot de inflammatoire cellen en klinische effecten van inhalatiecorticosteroïden (ICS) met of zonder langwerkende β-agonisten (LABA). Inhalatiesteroïden worden veelvuldig voorgeschreven aan patiënten met COPD, o.a. omdat het bijdraagt aan een afname van het aantal exacerbaties. LABAs bewerkstelligen ook ontspanning van de kleine spiertjes rondom de luchtwegen en daardoor verbetert de longfunctie. We vonden significante associaties van een hogere MRP1 expressie in longweefsel

met een langzamere FEV_1 afname bij COPD patiënten die langdurig behandeld zijn met ICS en met een versnelde FEV_1 afname na het staken van ontstekingsremmende medicijnen. Deze associaties waren niet aanwezig bij de patiënten die met placebo of een combinatie van LABA en ICS waren behandeld. We vonden geen significante associaties tussen MRP1 expressie bij aanvang, 6- en 30-maanden na behandeling en het niveau van longfunctie binnen de behandelingsgroepen met placebo, ICS of ICS met LABA. Dit suggereert dat MRP1 eiwit expressie één van de COPD fenotypen die gevoelig is voor inhalatiecorticosteroïden kan weerspiegelen.

Hoofdstuk 4 beschrijft een onderzoek naar *TLR2* and *TLR4* genen en hun rol bij patiënten met COPD. Toll-like receptoren, zoals TLR2 en TLR4 zijn betrokken bij de verdediging tegen virale en bacteriële infecties. Omdat infecties van de luchtwegen de ziekte verergeren, kan het zijn dat *TLRs* van belang zijn bij COPD. Van 9 SNPs die werden onderzocht in *TLR2*, waren 2 SNPs geassocieerd met een hoger niveau van FEV_1 en 2 SNPs geassocieerd met een lager niveau van FEV_1, terwijl anderen geassocieerd waren met minder of meer afname van FEV_1. Eén van de SNPs in *TLR2* rs11938228 was significant geassocieerd met verschillende inflammatoire cellen in geïnduceerd sputum. Een andere SNP in *TLR2*, rs3804099, was significant geassocieerd met minder neutrofielen en macrofagen gedurende follow-up. Interessant is dat geen van de onderzochte genetische varianten (SNPs) in *TLR2* was geassocieerd met het niveau van FEV_1, maar 10 van 17 de gegenotypeerde SNPs in *TLR4* vertoonden een relatie met de afname van FEV_1. Twee SNPs in *TLR4* rs12377632 en rs10759931 waren significant geassocieerd met meer neutrofielen, macrofagen en eosinofielen in geïnduceerd sputum. Meer studies zijn nodig om deze bevindingen te bevestigen en om het mechanisme te ontrafelen dat de rol van de SNPs in *TLR2* en *TLR4* op de ernst en progressie van COPD verklaard.

Hoofdstuk 5 beschrijft een onderzoek waarbij één van de genen die betrokken is bij de longontwikkelingsprocessen, namelijk *Hedgehog-interagerende proteïne* (*HHIP*), onderzocht is. We vonden dat de intergenic SNP rs13147758 in de buurt van het *HHIP* gen niet geassocieerd was met het niveau of de afname van FEV_1 in de algemene bevolking. Echter, in rokers uit de algemene bevolking was rs13147758 significant geassocieerd met een hoger niveau van FEV_1. Dit zou kunnen betekenen

dat dit het uiteindelijke resultaat is van de longontwikkeling in de vroege jeugd. In de GLUCOLD studie zagen wij dat rs13147758 significant geassocieerd was met minder versnelde afname van longfunctie (FEV_1 en FEV_1/IVC), een effect dat was versterkt bij rokers. Dit suggereert dat dit gen betrok kan zijn bij de balans tussen schade en reparatie van longweefsel.

In **hoofdstuk 6** beschreven we een controversieel gen met betrekking tot COPD en rookverslaving, het *nicotinic acetylcholine receptor (nAChR)* gen. Roken versnelt de achteruitgang van de longfunctie en is daarmee een risicofactor voor COPD. We vonden in onze studie dat rokers die stoppen met roken jaarlijks 20,3 ml minder afname van FEV_1 hadden dan rokers die bleven roken. Eerdere studies suggereerden dat SNPs in het *nAChR* cluster met het ontwikkelen van COPD samenhangt. Bovendien werd aangetoond dat deze SNPs geassocieerd zijn met nicotineafhankelijkheid en rookgewoonten. Echter, deze studies gaven geen inzicht of de SNPs in het *nAChR* cluster zelfstandig met COPD geassocieerd zijn of door middel van hun associatie met nicotineafhankelijkheid en rookgewoonten. We onderzochten 3 SNPs in het *nAChR* cluster (rs569207, rs1051730 en rs8034191) in het Vlagtwedde-Vlaardingen cohort. Het bleek dat 2 SNPs in het *nAChR* cluster geassocieerd zijn met rookgewoonten. Rs569207 is geassocieerd met een hogere kans om te stoppen met roken en rs1051730 is geassocieerd met een lagere kans om te stoppen met roken. Geen van de onderzochten SNPs heeft echter een significant effect op de jaarlijkse afname van FEV_1 bij rokers of ex-rokers. Interessant is dat rokers en ex-rokers met de homozygote variant voor rs569207 een lager aantal pakjaren op het laatste onderzoek hadden in vergelijking met individuen die deze variant niet hadden (een pakjaar = een jaar lang 20 sigaretten per dag roken). Geen van de onderzochte genetische varianten was geassocieerd met het gemiddeld aantal sigaretten per dag die iemand rookte tijdens het laatste onderzoek.
Dit is de eerste longitudinale studie die liet zien dat SNPs in de *nAChR* cluster waarschijnlijk een causale rol spelen bij COPD via het rookgedrag en niet direct via de ontwikkeling van COPD.

In de laatste studie beschreven in dit proefschrift (**hoofdstuk 7**), combineerden we twee verschillende methoden die een beter inzicht zouden kunnen geven over hoe

genen en hun SNPs naast elkaar functioneren. Ten eerste hebben we via DAVID (Database for Annotation, Visualization and Integration Discovery) een set van 26 genen verkend waarvan eerder aangetoond was dat ze significant geassocieerd waren met het niveau en de afname van longfunctie en de aanwezigheid van COPD in de algemene bevolking (Vlagtwedde-Vlaardingen). Met behulp van DAVID hebben we met een verkennende strategie een aantal genen geïdentificeerd met behulp van de bijbehorende biologische termen en vervolgens de meest oververtegenwoordigde of verrijkte biologische termen statistisch gemarkeerd. We identificeerden 5 functionele genengroepen: 1) *metalloproteinases*, inclusief *ADAM33*, 2) *surfactants*, 3) *glutathione transferases*, 4) *glutamate-cysteine ligases* en 5) *cholinergic receptor, nicotinic, alpha* genen. De meest relevante biologische term was "longfunctie" die met 10 genen geassocieerd was (*ADAM33, GSTM1, GSTP1, GSTT1, HMOX1, MMP1, MMP12, MMP9, SOD2 en SOD3*).
Ten tweede hebben we onderzocht hoe de SNPs in deze kandidaat-genen bij elkaar werden ingedeeld door middel van factor analyse. We hebben 26 van 78 SNPs geselecteerd die eerder in deze 26 genen gegenotypeerd waren. Deze 26 SNPs waren significant geassocieerd met het niveau en afname van longfunctie in de algemene populatie in de eerdere studies. Factor analyse toonde 10 factoren aan die ongeveer 62,6% van de totale variatie verklaarden in onze dataset: 1) *ADAM33* (5 SNPs), 2) *ADAM33* (4 SNPs), 3) *TGF-β1* (2 SNPs), 4) *MRP1* (2 SNPs), 5) *SOD2* (2 SNPs), 6) *SFTPD* en *SFTPA2* (2 SNPs), 7) *MRP1* (3 SNPs), 8) *KEAP*, *TGF-β1* en *GCLC* (3 SNPs), 9) *GCLM*, *GCLC* en *GSTT1* (2 SNPs en één nul allel) en 10) *NFE2L*, *GSTM1* en *SOD3* (2 SNPs en één nul allel). De resultaten van deze factor analyse waren in overeenstemming met onze verkennende analyse die gebruik maakte van DAVID tools.
Tot slot wees onze studie uit dat de combinatie van deze twee methoden kan leiden tot een beter begrip over hoe de genen en de SNPs een bijdrage leveren aan de ontwikkeling van COPD op basis van hun werking en hun gedeelde biologie.

We bestudeerden genetische determinanten van de ernst en progressie van de longfunctie bij COPD patiënten, maar ook genetische determinanten in de algemene bevolking. Het is essentieel om vast te stellen welke genen betrokken zijn bij ontstaan van COPD en welke bij de ernst van COPD, omdat de genetische

achtergrond van deze twee aspecten van COPD van elkaar kunnen verschillen, zoals wij in dit proefschrift laten zien.

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