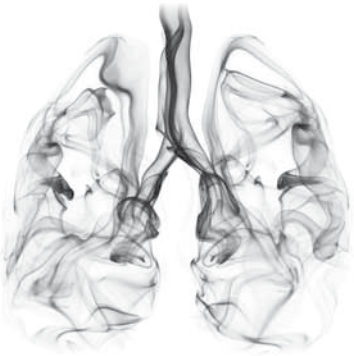
Epidemiology of Lung Function and Chronic Obstructive Pulmonary Disease



Daan Willem Loth

Epidemiology of Lung Function and Chronic Obstructive Pulmonary Disease

Epidemiologie van longfunctie en chronisch obstructieve longziekte

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

prof. dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 30 oktober 2013 om 15.30 uur

Daan Willem Loth
geboren te Breda

Promotoren:	Prof.dr. B.H.Ch. Stricker
	Prof.dr. G.G.O. Brusselle
	Prof.dr. H.G.M. Leufkens
Overige leden:	Prof.dr. C.M. van Duijn
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Financial support by the Netherlands Health Care Inspectorate
for the publication of this thesis is gratefully acknowledged

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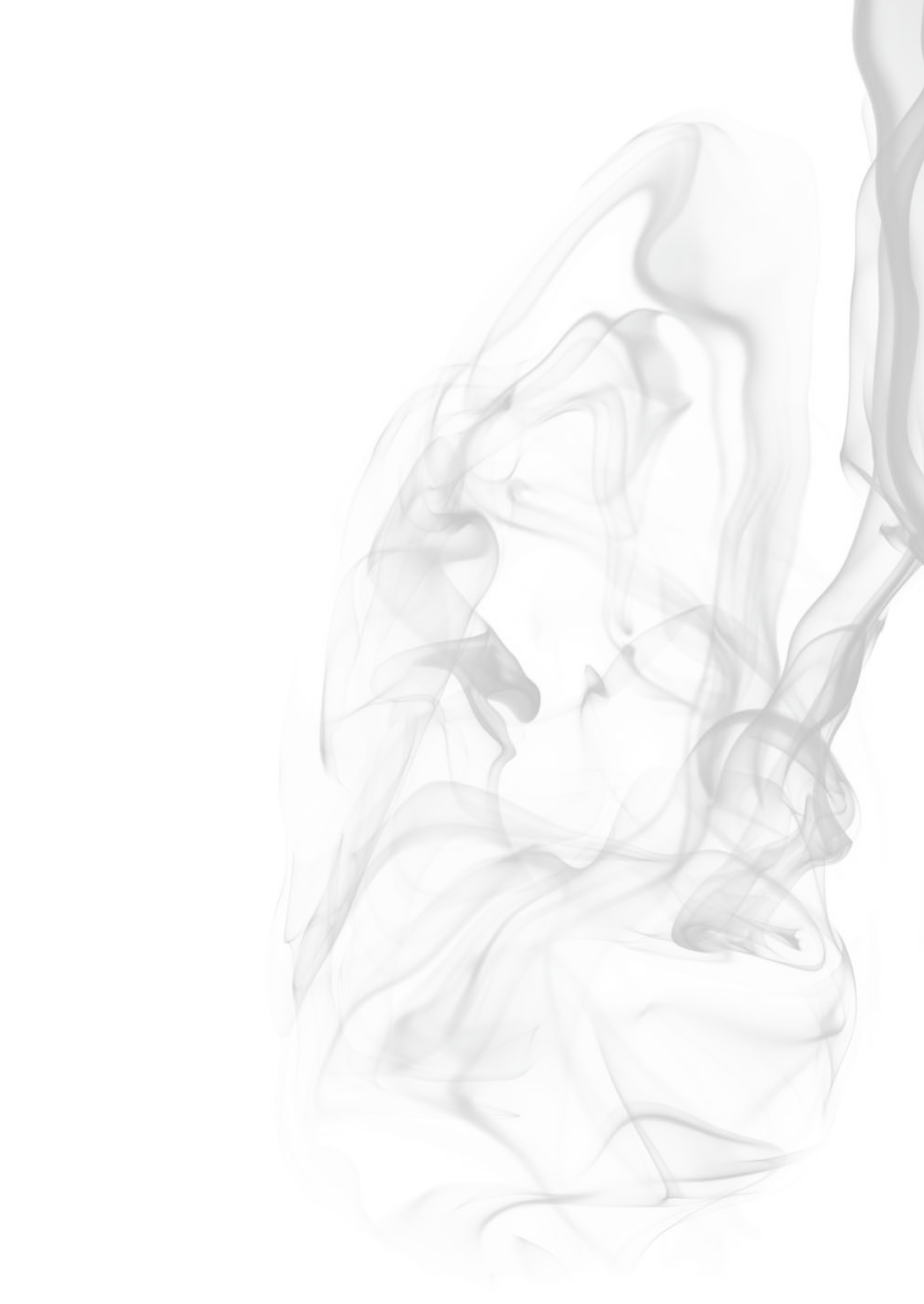
PART I

General Introduction



CHAPTER 1 Introduction

SPIROMETRY

Spirometry is a technique to evaluate the pulmonary ventilatory function and is a reflection of several forces implied in lung volumes. The lung volume is dependent on the elastic recoil of the lungs and chest wall and the muscular efforts of the chest wall, diaphragm, and abdomen. Contraction of the inspiratory muscles, such as the diaphragm, expands the thorax, creating an increased negative pressure. This pressure is lower than the atmospheric pressure, drawing air into the lungs. Expiration is mainly a passive process. When the muscles relax, due to elastic recoil of the lungs, air will flow out. This process can be assisted by contraction of intercostal and abdominal wall muscles, resulting in a positive pleural pressure [1]. Using the spirogram, several different types of lung volume can be evaluated.

Apart from the tidal volume, a person is capable of recruiting the expiratory residual volume and inspiratory residual volume, e.g. during exercise but also when the participant performs a specific diagnostic maneuver (figure 1). The figure below (figure 2) shows three flow-volume curves. To assess airway obstruction, the patient needs to take a deep breath, then exhales as much air as possible as quickly as possible for at least six seconds; lastly, he inhales deeply again when the end of the expiratory capacity is reached.

Two of the most important measures are the Forced Expiratory Volume in the first second, (FEV_1) and the Forced Vital Capacity (FVC). The FEV_1 provides information about the

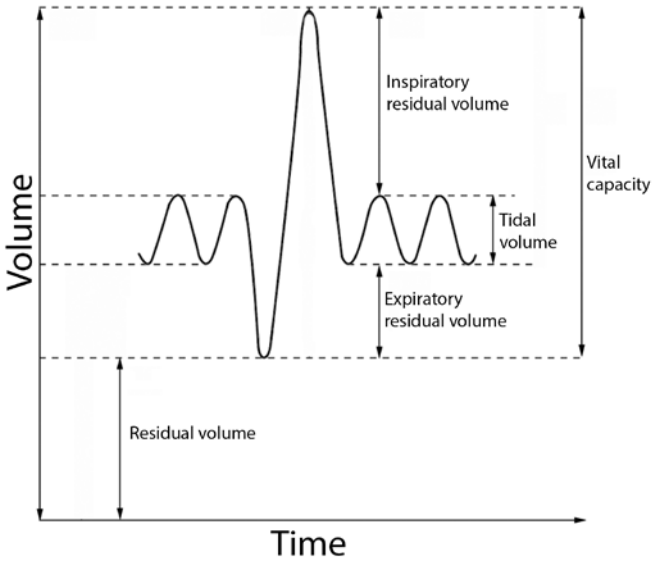


Figure 1. Normal spirogram with normal breaths, 1 maximal expiration and inspiration.

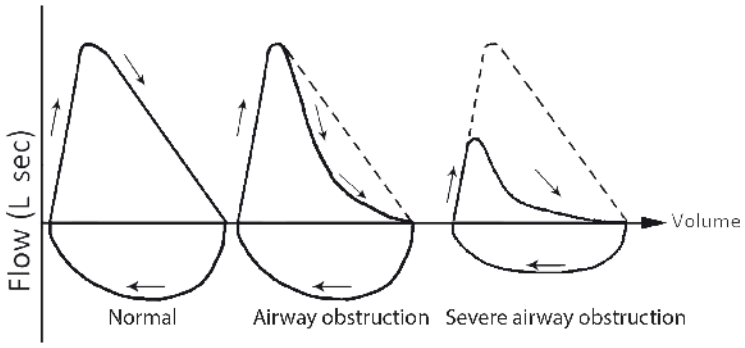


Figure 2. Three flow-volume curves; one normal curve, one showing airflow obstruction and one curve showing severe airflow obstruction

airflow, while the FVC provides an estimate of the vital capacity. In obstructive disease, the FEV_1 is disproportionally decreased in comparison to FVC, resulting in a decreased ratio. In restrictive disease the FEV_1 and FVC are often equally decreased, resulting in a normal ratio. In early adulthood, the peak levels of lung function are attained, after which they gradually decline with age [2,3]. The individual maximal lung capacity is dependent on genetic factors and environmental exposures, e.g. parental smoking during childhood[4]. Similarly, the rate of lung function decline is dependent on a plethora of factors, of which smoking is one of the strongest risk factors for an accelerated decline [2].

Both FEV_1 and FVC are predictors of mortality, even in subjects without pulmonary disease [5-12].

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic Obstructive Pulmonary Disease (COPD) is the most frequently occurring chronic disease of the lungs and is estimated to become the third cause of death worldwide in 2020 [13]. According to the definition by the Global Initiative For Chronic Obstructive Lung Disease (GOLD), COPD is "a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients." [14]. Although the definition of disease is based on airflow obstruction, exacerbations and co-morbidities contribute to the overall severity, prognosis and, eventually, mortality [14,15]. Spirometry, or pulmonary function testing, is an essential tool in diagnosing COPD and evaluating the effect of treatment of COPD [16].

PATHOPHYSIOLOGY AND PATHOGENESIS OF COPD

COPD is a complex disease on a clinical, cellular and molecular level [17]. The most well-studied cause of COPD is tobacco smoking [18]. The inhaled cigarette smoke causes damage to the airways and lung parenchyma via several pathways. The damage can occur directly through epithelial cell destruction, exaggerated inflammatory responses and oxidative stress [19,20]. However, only approximately 20 percent of the smokers actually develop COPD [21]. Conversely, not all COPD patients have smoked [22], leading to the hypothesis that COPD is a consequence of synergy between several direct or indirect risk factors. Other risk factors which are thought to play a role in the development of COPD are airway hyper-responsiveness, environmental and occupational exposure and genetic determinants [23-27].

GENETICS

Until recently, the genetic variation causing alpha-1-antitrypsin deficiency was the most well-known genetic mutation causing COPD. The prevalence of this disease is approximately 1 in 2,000 to 1 in 5,000 and the effects are enhanced by cigarette smoking, leading to emphysema at a young age [28]. Since the prevalence of COPD is much higher than that and because of the above-mentioned discrepancy between smokers and the development of COPD, we hypothesized that other genetic variations exert their effects on the development of COPD or the decline of lung function. With the introduction of Genome-Wide Association studies, various efforts have been made to identify these variants [25,26,29-32]. While these projects have proven to be successful in identifying common variants underlying COPD and spirometry measurements (FEV_1 and FEV_1/FVC) in general, the proportion of variance explained by these common variant alleles was low, implicating that a large part of the heritability is still unexplained.

DIAGNOSIS

Although COPD is mainly characterized by not fully reversible airflow limitation, patients can present themselves with a wide array of symptoms, including coughing, dyspnea, respiratory mucus production and recurrent lower respiratory tract infections. To define airflow limitation in a patient with clinical symptoms, the most widely used diagnostic lung function test for COPD is spirometry. Spirometry is the most reproducible and objective measurement of airflow limitation currently available [14,16]. Airflow limitation is currently defined by the Global initiative of Obstructive Lung disease (GOLD) as an

FEV_1/FVC below 70% (measured post-bronchodilator). For staging of COPD, FEV_1 percent predicted is used. Together with frequency of exacerbations and amount of symptoms, spirometry is used in the recently updated GOLD staging [33].

THERAPY

Essential in treatment of COPD is smoking cessation. It is important to emphasize that it is never too late to stop smoking.

Besides smoking cessation, there are several pharmacological and non-pharmacological treatment options to reduce symptoms, but also to prevent exacerbations. Bronchodilating agents, such as β_2-agonists and anticholinergics, play a central role in the treatment of COPD symptoms, but there is also evidence that these products may improve survival.

Recent evidence shows that in COPD patients with frequent exacerbations, chronic treatment with low dose macrolide antibiotics may reduce exacerbations [34]. However, the adverse events caused by these antibiotics and the microbial resistance will need to be weighed against the benefits.

PHARMACOGENETICS

Genetic association testing not only provides insight into biological processes influencing the lungs and their function, it may point to possible targets for new therapies. Furthermore, identifying genetic modifiers for drug response and the occurrence of adverse events can help in the goal to tailor specific treatments to individual patients (i.e. personalized medicine). This might pertain to the preferable drug choice, as well as to the recommended starting dose. The most well-known gene influencing one of the therapeutic strategies in pulmonary function is the *ADRB2*-gene, coding for the β_2-adrenergic receptor. This receptor is the target of the inhaled β_2-agonists, which can be distinguished into two groups according to their duration of action: Long- and Short-Acting β_2-agonists (LABA & SABA). These products cause airway relaxation through decrease of airway smooth muscle tone activity. The differences in therapeutic responses in patients have been associated with polymorphisms in *ADRB2*, but the results have been inconclusive so far [35]. Another treatment strategy of asthma and COPD is focused on the reduction of inflammation (e.g. inhaled corticosteroids). An interesting target would be the gene encoding for the protein histone deacetylase 2 (*HDAC2*), possibly influencing glucocorticoid resistance [36]. More efforts are needed to further elucidate the highly variable responses to treatment.

OBJECTIVES

The main objectives of this PhD thesis are 1) to explore the epidemiology of spirometric measurements, 2) to identify genetic determinants for lung function, smoking susceptibility, lung function decline and COPD, 3) to assess the effects of drugs on lung function or disease progression and to elucidate the genetic and environmental modifiers for drug response. In part I, we provide a general introduction and some background to spirometry. Part II focuses on the epidemiologic exploration of pulmonary function. Chapter 2.1 describes our efforts to redefine the reference values for spirometry in the general (elderly) population and chapter 2.2 describes the association between spirometric measures and pulmonary artery systolic pressure, investigating the lung-heart interaction. Part III consists of several studies aimed to identify genetic determinants for lung function, specifically FEV_1 and FEV_1/FVC (chapter 3.1) and FVC (chapter 3.2) using genome-wide association studies. Furthermore, we investigated common variants and their association with FEV_1 decline over time (chapter 3.3) using GWAs. To better understand the interaction between smoking and lung function, we investigated the interaction between genetic variants and cigarette smoke exposure (chapter 3.4) by a joint-meta-analysis of genome-wide main and interaction effects. Lastly, our objective was to identify genetic determinants for COPD (chapter 3.5). Part IV of this thesis focuses on pharmaco-epidemiology, encompassing two studies in which we investigated the effects of β-blocker exposure on pulmonary function (chapter 4.1) in the general population and the effect of statin use on survival in COPD patients (Chapter 4.2). The final part (Part V) will provide a general discussion.

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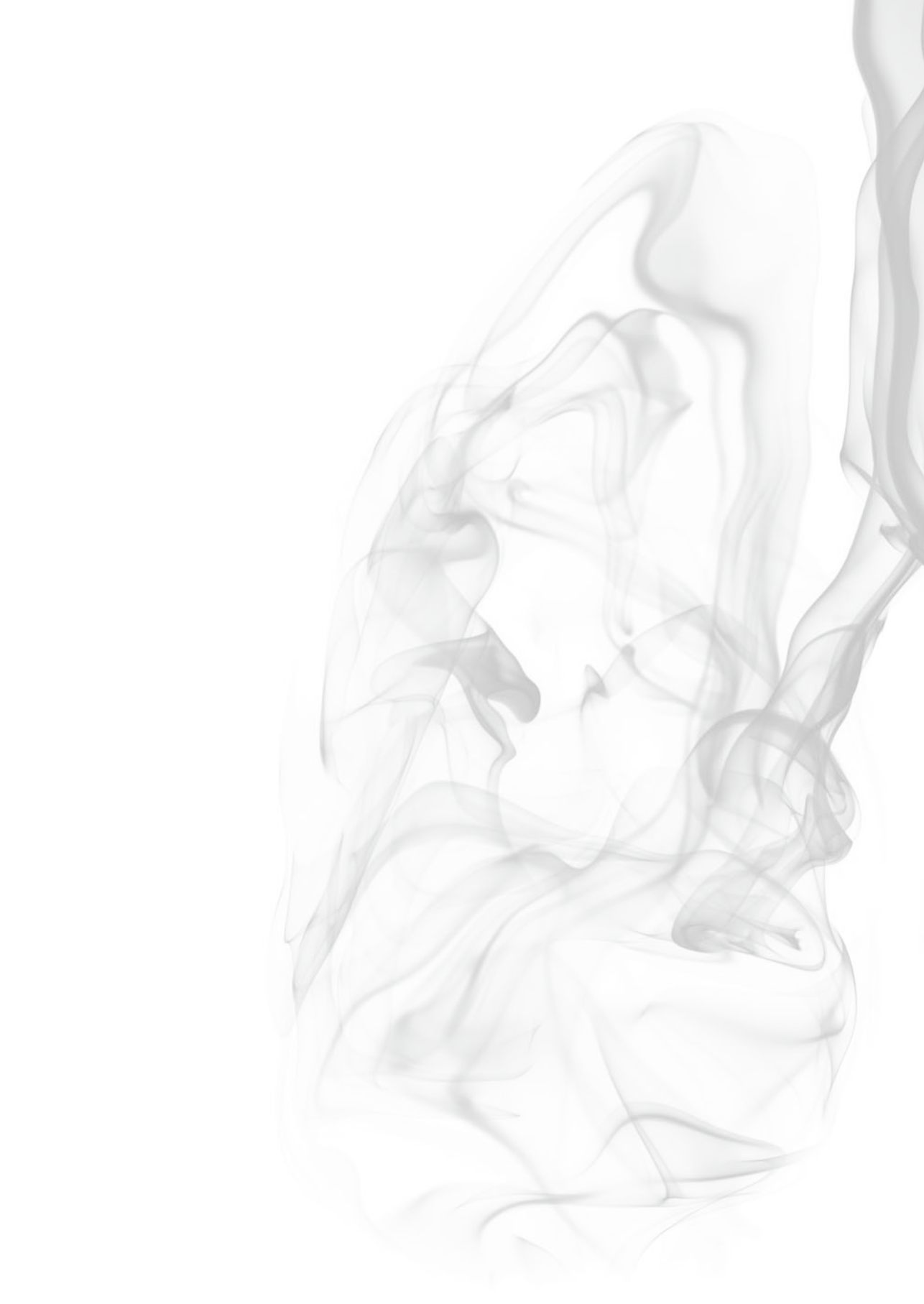
PART II

Epidemiology of Lung Function



CHAPTER 2.1

Normal spirometry values in healthy elderly: The Rotterdam Study

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Published in Eur J Epidemiol.
2013 Apr; 28(4):329-34.
doi: 10.1007/s10654-013-9800-4.
Epub 2013 Mar 29. PubMed PMID: 23539359.

ABSTRACT

Introduction Although many different reference values for spirometry are available from various studies, the elderly are usually underrepresented. Therefore, our objective was to assess reference values in a sample of healthy participants from a prospective population-based cohort study, including a large proportion of elderly.

Methods We included spirometry measurements of healthy, never smokers, from the Rotterdam Study and excluded participants with respiratory symptoms or prescriptions for respiratory medication. Age- and height-specific curves for the 5th (lower limit of normal) and the 50th (median) percentile of Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC), and the ratio (FEV_1/FVC) were calculated by quantile regression models.

Results The group of healthy elderly study subjects consisted of 1,125 individuals, with a mean age of 68 years, ranging from 47 to 96 years of age. Sex stratified equations for the median and the lower limit of normal were calculated adjusted for age and height. Conclusions In this study, we report age- and height-dependent reference limits for FEV_1, FVC, and FEV_1/FVC in a large population, and prediction equations for the lower limit of normal and median values for a sample containing a large proportion of healthy elderly.

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INTRODUCTION

Pulmonary function testing plays a vital role in diagnosing obstructive airway diseases such as asthma or chronic obstructive pulmonary disease (COPD), assessing disease severity, and monitoring treatment responses [1-3]. Pulmonary function also predicts mortality in the general population, even among people who have never smoked, who have only modestly reduced pulmonary function, and who have no respiratory symptoms [4]. Studies consistently find that reduced pulmonary function (usually Forced Expiratory Volume in 1 second or FEV_1) is associated with an increased risk of mortality from respiratory and non-respiratory diseases [5,6]. The peak level of pulmonary function is attained in early adulthood and declines subsequently over time [7]. When measured at some point in a person's lifetime and reduced levels of FEV_1, Forced Vital Capacity (FVC) and FEV_1/FVC are found, these can be due to impaired lung growth during fetal life, childhood or adolescence and/or due to an accelerated decline of lung function in adulthood. Both of these processes can be influenced by genetics [8-11], or can be due to exposure to noxious substances, e.g. cigarette smoke or fine particulate matter from combustion of fossil fuels [12].

During the last decade, many worldwide efforts have been made to calculate reference values for pulmonary function tests, resulting in several equations [13-20]. Many of the currently used reference values were calculated several decades ago, but also more recent studies demonstrate the clinical interest in this topic [14,15,17,19,21]. However, studies performed in the elderly are scarce and usually comprise only a few dozen people aged above 70 years [16,18,19]. Extrapolation of reference equations calculated in relatively young participants to an elderly population may lead to misclassification of disease. Therefore, international guidelines discourage extrapolation of values to elderly when calculating reference values.

Recently the authors of the Global Lung Initiative, European Respiratory Society Task Force concluded, in their large effort to establish global reference values, that data on participants with ages of 75 years and older is scarce. Consequently, it is necessary to collect more data from healthy elderly to obtain more precise reference values in this group [16-18,21,22]. Therefore, the objective of our study was to evaluate normal values of spirometry measurements in the elderly. Hereto, we used spirometry measurements from a prospective population-based cohort study, to calculate reference equations for spirometric measures in healthy elderly.

METHODS

Setting

The Rotterdam Study is a prospective population-based cohort study, which was initiated in 1990 in Ommoord, a suburb of Rotterdam, the Netherlands. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, approved the study. At baseline, all participants were visited for a standardized questionnaire, and were subsequently examined at the Rotterdam Study's research centre, located in Ommoord, Rotterdam. The initial cohort (RS I) consists of 7,983 participants, aged 55 years and over. Since the start of the first cohort, two more cohorts have been defined in the same suburb. The second cohort (RS II) started in 2000 and included 3,011 participants aged 55 years and over. The third cohort (RS III) was enrolled in 2006 and included 3,932 participants aged 45 years and over. As of 2009, the total of the combined cohorts encompassed 14,926 subjects aged 45 years or over. The main focus of the Rotterdam Study is on studying common chronic diseases in the elderly. During a follow-up of up to 22 years, extensive data on morbidity, mortality, and medical interventions have been gathered. Main objectives and methods of the Rotterdam Study have been described earlier [23,24].

Study population

The source population consisted of all participants of the Rotterdam Study with a valid pulmonary function test. We used the most recent pulmonary function test, performed at any study round. The tests were analysed by two research physicians, and validated by a specialist in pulmonary medicine (GB). During the validation process the quality control was performed by two researchers. Reproducible and interpretable spirometry recordings were classified and the values for the separate pulmonary function measures were stored digitally in a database. For the selection of the study population of healthy elderly, we excluded current and ex-smokers and participants with symptoms of respiratory diseases, participants taking inhaled short-acting β-adrenoreceptor-agonists (SABAs), long-acting β-adrenoreceptor-agonists (LABAs), anticholinergic agents, inhaled glucocorticosteroids, or combinations of the above mentioned substances, xanthine derivatives, leukotriene-receptor antagonists and β-blockers.

Spirometry

All measurements were performed pre- bronchodilator. Spirometry was performed by trained paramedical personnel using a SpiroPro® portable spirometer (Erich Jaeger, Hoechberg, Germany) from 2002 to 2009, and with the Jaeger Masterscreen PFT (Care Fusion, the Netherlands) since 2009, according to American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines [2]. FEV_1, FVC and FEV_1/FVC-ratio were measured. Quality control of pulmonary function tests was done by a researcher (DL or LL) and a

respiratory specialist (GB). Quality assessment includes assessment of the flow-volume loop including reproducibility and volume-time loop or maximal expiration. Spirometry results that did not meet ATS/ERS criteria for acceptability and reproducibility were classified as not interpretable [25,26].

Covariables

Information on potential confounders or effect modifiers such as age, sex, height, weight and body mass index (BMI) was gathered at the centre visit during which the pulmonary function test was conducted.

Statistical analyses

Statistical analyses were performed using STATA/SE 12 for Windows (StataCorp, College Station, Texas, USA). For the evaluation of reference values we used quantile regression analysis, a statistical method for estimating models for the conditional quantile functions instead of the conditional mean of the response variable. This method is more robust against outliers in the outcome measurements[27]. The prediction equations were calculated using the median, whereas the 5th percentile was chosen as cut off to define the lower limit of normal (LLN), as previously proposed [28]. For the prediction equations the median (50th percentile) was chosen.

Fractional Polynomials (FP) were applied to explore and graph nonlinear associations [29] between continuous exposure variables (age, height, weight) and the respective outcome. The dose-response relation was found using FP up to degree 2 with all possible combinations of powers selected from the set (-2, -1, -0.5, 0, 0.5, 1, 2, 3). The best fitting two-factorial FP was compared with the best fitting one-factorial FP by a deviance test. If the goodness of fit was not significantly better in the model with the two-factorial FP ($p>0.1$), we tested by a deviance test whether the model with the best fitting one-factorial FP was significantly better than the model with the untransformed exposure variable. If none of the FP models with the transformed exposure variable fitted the data significantly better than the model with the untransformed exposure variable, the exposure variable remained untransformed. Otherwise the exposure variable was transformed.

RESULTS

Of the total of 14,926 subjects from the Rotterdam Study and since the introduction of spirometry in the cohort study in 2002, in 6,959 individuals one or more pulmonary function tests were performed. Of these, a total of 4,324 spirometry tests were of sufficient quality. A total of 1,125 participants were never smokers, had no prescription for

pulmonary medication or beta-blockers, and did not have symptoms indicating respiratory diseases. The spirometry results of these participants were used for our analyses.

Subject characteristics

The population consisted of 334 men (29.7%) and 791 (70.3%) women with a mean age of 68.2 years, ranging from 47.1 years to 94.6 years old. As expected, the inter-quartile ranges of age for males and females differed and are shown separately (Table 1).

Table 1. Baseline characteristics

Characteristics	Male	Female
N (%)	334 (29.7%)	791 (70.3%)
Mean age Years (SD)	64.7 (10.0)	69.7 (10.0)
Age in years		
1st quartile	47.1 - 56.5	47.1 – 61.7
2nd quartile	56.5 – 64.6	61.7 – 71.2
3rd quartile	64.9 – 72.0	71.2 – 77.0
4th quartile	72.0 – 88.4	77.0 – 94.6
Height cm (SD)	176.4 (7.1)	162.8 (6.7)
Weight kg (SD)	84.2 (11.9)	71.4 (12.3)
BMI kg/m^2 (SD)	27.0 (3.3)	26.9 (4.3)
FEV_1 L (SD)	3.51 (0.72)	2.32 (0.57)
FVC L (SD)	4.46 (0.93)	3.00 (0.70)
FEV_1/FVC (SD)	79.2 (6.7)	78.7 (6.8)
FEV_1 % predicted* (SD)	109.1 (15.4)	109.9 (21.0)

Continuous variables are listed as mean (SD). FEV_1: Forced Expiratory Volume in 1 second (FEV_1), FVC: Forced Vital Capacity. *According to the European Community for Coal and Steel/ European Respiratory Society (ECCS/ERS)

Reference values

Prediction equations are shown in table 2. We calculated sex specific prediction equations for FEV_1, FVC and FEV_1/FVC by age adjusted for height. Age was linearly associated with FEV_1 and FVC, for both the lower limit of normal (5th percentile) and the median (50th percentile). Reference curves for age are shown in figures 1 and 2.

For the LLN of FEV_1/FVC in males, the prediction equation followed a more complex correlation with transformations for height (figure 3). In females, age was linearly associated with LLN and the median of FVC and FEV_1. Height was non-linearly associated with these two outcomes (Table 2, Figures 1 and 2) and FEV_1/FVC, for both the lower limit of normal and the predicted values. Figures 1, 2 and 3 show the individual measurements and the regression curves for the lower limit of normal and the median values in males and females.

Table 2. Equations derived from individual measurements.

	Lower Limit of Normal	Median
Males		
FEV_1	-0.4982893 - 0.0307421 × A + 0.0298244 × H_{cm}	0.5688802 - 0.0428957 × A + 0.0325003 × H_{cm}
FVC	0.4088557 - 0.0331373 × A + 0.0294108 × H_{cm}	-2.888613 - 0.0418384 × A + 0.0568359 × H_{cm}
FEV_1/FVC	$-6574.755 - 0.1581655 \times A - 12824.17 \times \frac{1}{\sqrt{H_{trans}}} + 14207.67 \times \frac{1}{\sqrt{H_{trans}}} \times \ln(H_{trans})$	101.4621 - 0.1405519 × A - 0.0724344 × H_{cm}
Females		
FEV_1	3.576269 - 0.0247535 × A - 6.258029 × $(1/H_m^2)$ + 3.528179 × $(1/H_m)$	8.055922 - 0.0309816 × A - 7.279633 × $(1/H_m^2)$ - 1.655085 × $\ln(H_m)$
FVC	-0.6304702 - 0.0306052 × A + 3.520033 × H_m - 0.3267878 x H_m^2	-3.745469 - 0.0332075 × A - 32.68719 × $(1/H_m)$ + 37.12663 × $(1/\sqrt{H_m})$
FEV_1/FVC	43.11051 + 394.8654 × $(1/A_{trans}^2)$ + 42.18434 × $(1/H_m^2)$	79.76162 - 0.1353647 × A + 22.90965 × $(1/H_m^2)$

$A = Age_{years}$
$A_{trans} = Age_{years} / 10$
H_{cm} = *Height in centimetres*
$H_{trans} = H_{cm} / 10$
H_m = *Height in meters*

In this table, we give prediction equations for the 50th percentile (Predicted Values, or Median) and the 5th percentile (Lower Limit of Normal). These equations are based on measurements in healthy elderly individuals.

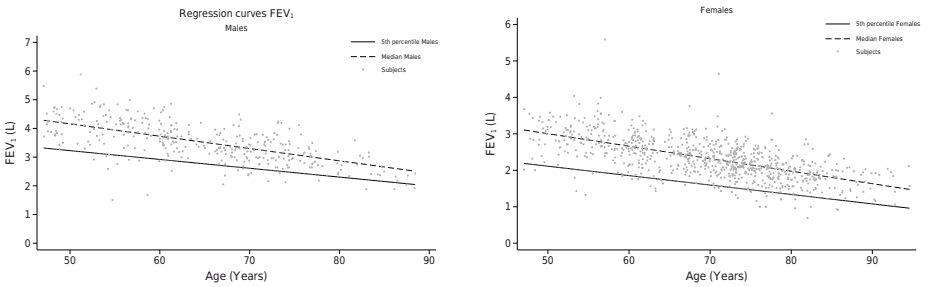

Figure 1. This figure shows individual measurements for FEV_1 for males (a) and females (b)separately. The solid line shows the equation for the lower limit of normal or 5th percentile. The dashed line shows the prediction equation for the median or 50th percentile. Both these regression curves are corrected for age and height and their respective transformations.

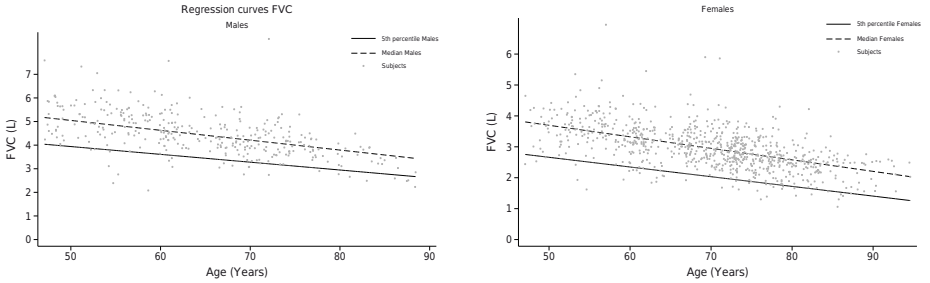

Figure 2. This figure shows individual measurements for FEV_1 for males (a) and females (b)separately. The solid line shows the equation for the lower limit of normal or 5th percentile. The dashed line shows the prediction equation for the median or 50th percentile. Both these regression curves are corrected for age and height and their respective transformations.

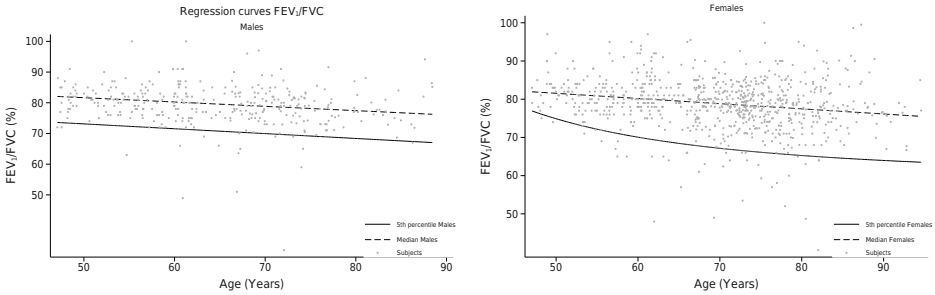

Figure 3. This figure shows individual measurements for FEV_1 for males (a) and females (b)separately. These values are not a derivative from the predicted values for FEV_1 and FVC but predicted directly from the ratio of FEV_1 to FVC. The solid line shows the equation for the lower limit of normal or 5th percentile. The dashed line shows the prediction equation for the median or 50th percentile. Both these regression curves are corrected for age and height and their respective transformations.

Comparison to other reference values

In the supplementary figures we show comparisons with existing reference equations. As can be seen in the graphs, our prediction equations allow for lower values in the elderly than both the Hankinson and standard output equations and have a more narrow range of values for different heights.

These supplementary figures can be found via
http://link.springer.com/article/10.1007%2Fs10654-013-9800-4

DISCUSSION

In this study, we calculated spirometry reference values for elderly without respiratory disease. These data are relevant because there are hardly precise reference values for spirometry in elderly. These analyses were done in a large prospective population-based cohort study in which assessment of pulmonary function is routinely performed since 2002. To assess spirometry measures in a sample of healthy elderly, we excluded participants with a history of smoking, pulmonary medication or respiratory symptoms. Although height decreases in elderly during ageing, this change over time is slow compared to growth spurts during adolescence. Therefore, we think that quantile regression analysis is a valid approach. Furthermore, we show a comparison between our equations, the set of prediction equations by Hankinson e.a. [15] and the "standard" or pre-installed formula's output from our spirometry-device. Our prediction equations allow for lower values in the elderly than both the Hankinson and standard output equations and have a more narrow range of values (e.g. FEV_1) for different heights. Especially a physiologically lower FEV_1, which is in fact normal for a person of a certain age and height, would be interpreted differently. Using reference values which are unsuitable for the higher age categories, could lead to misclassification of disease.

The importance of reference values for spirometry is underlined by the large combined effort which has recently been made by the Global Lung Initiative (GLI) European Respiratory Society Task Force to calculate multi-ethnic equations across an age-range from 3 to 95 years old [21]. This extensive overview provides reference values for subjects of four different ethnicities and across a broad age spectrum. However, the authors conclude in their paper that predicted values for participants over the age of approximately 75 years old should be used and interpreted with caution, because of the relatively low number of participants in that particular age-group the authors conclude that additional data about this age-group is required.

The strengths of our study include the high quality prospectively gathered data in a large sample of the aging population with standardized assessments of height, weight, and spirometry. Due to collection of several covariables and the continuous collection of medication, we were able to include a healthy population for our analyses, of which 25% of males was 72 years of age or older, and 25% of females 77 years of age or older. Taking into account one of the conclusions of the GLI European Respiratory Society Task Force [21], this underlines the importance of our findings. A limitation of calculating reference values in a population such as the Rotterdam Study may be that the calculated reference equation is population specific. These could arise due to different population characteristics. Therefore, there is a need for validation of our equations to evaluate

generalizability across populations and especially across the higher age categories. We were not able to include this in our manuscript because, to our knowledge, there are no studies with a similar design and such a large proportion of healthy elderly in the higher age categories. Perhaps in the future, an approach where these formulas could be compared in a different (e.g. clinical) setting would be very interesting.

In conclusion we show normal values for FEV_1, FVC and FEV_1/FVC in a large population with a large proportion of elderly subjects. Furthermore we show equations for the lower limit of normal and prediction values.

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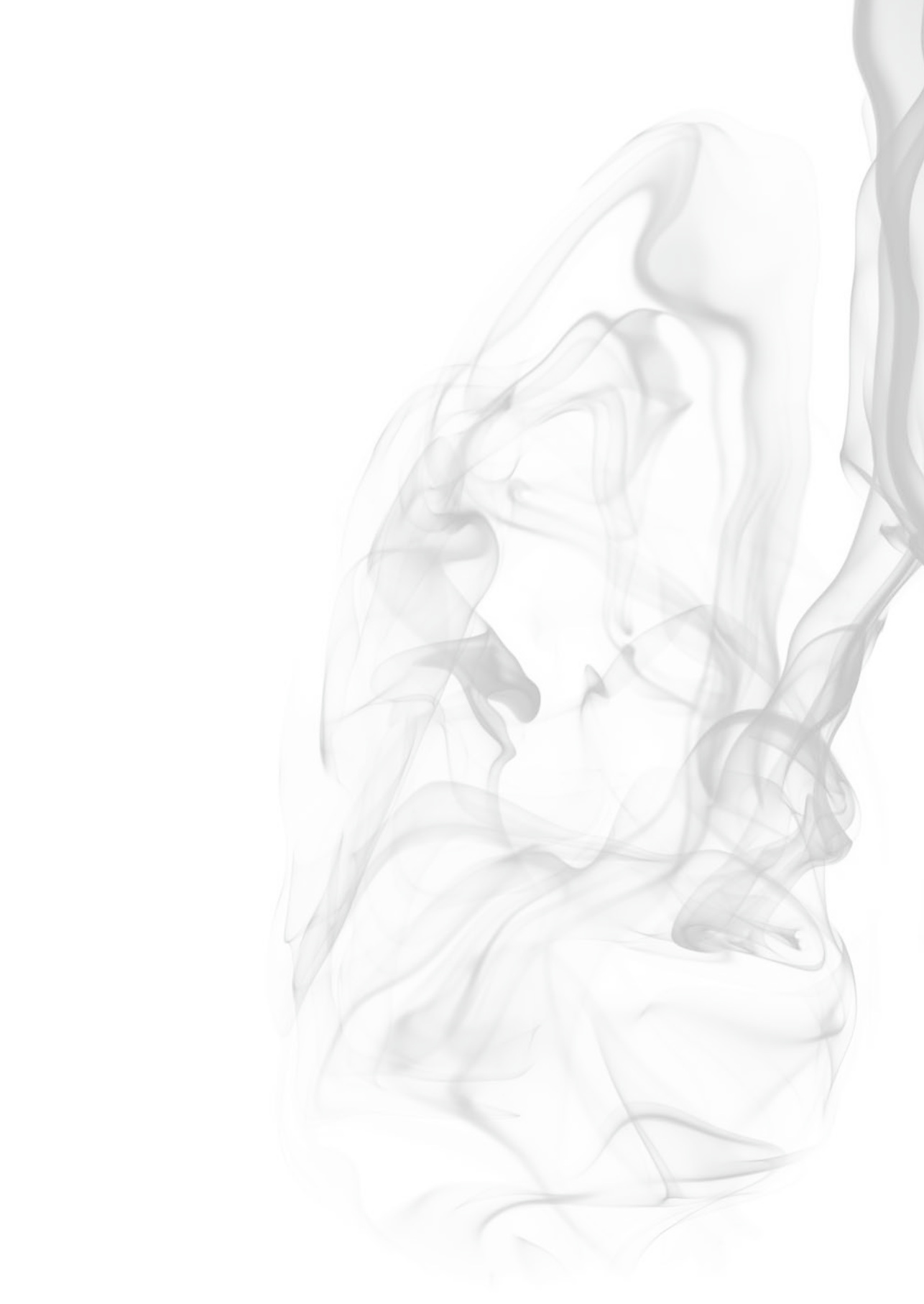

CHAPTER 2.2

Pulmonary Function is Associated with Pulmonary Artery Systolic Pressure in the General Population: The Rotterdam Study

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Manuscript in preparation

ABSTRACT

Background Pulmonary hypertension is a progressive and fatal heterogeneous syndrome, characterized by elevated pulmonary arterial (systolic) pressure and can lead to right ventricular cardiac failure. Although the presence of elevated pulmonary arterial systolic pressure (PASP) in patients with a lung disease is a well-known occurrence, little is known about the association between spirometry and PASP. We hypothesized that spirometry and PASP are associated, irrespective of pulmonary disease.

Methods This study was performed within the Rotterdam study, a prospective population-based cohort study. The study population included all participants with a spirometry, performed and interpreted according to ATS/ERS guidelines and echocardiography according to the ASE/EAE/CSE guidelines. We analyzed the association between Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) and FEV_1/FVC. Furthermore we investigated the association between spirometry measures and pulmonary hypertension defined by echocardiography.

Results We report significant associations for spirometry measures and PASP. Per 10% decrease FEV_1 % predicted was significantly associated with a PASP increase of 0.46 mmHg (95%CI: 0.32; 0.62). Similarly, per 10% decrease, FVC % predicted was significantly associated with an increased ePASP (+ 0.42 mmHg, 95%CI: 0.25; 0.60). Lastly, FEV_1/FVC showed an association of 1.01 mmHg (95%CI: 0.58; 1.45) increase in ePASP per 10% decrease in FEV_1/FVC. Lastly, we found a significant association for FEV_1 and FVC with pulmonary hypertension.

Conclusion We found a clear association between spirometry and PASP. Furthermore, we show a significant association for FEV_1 and pulmonary hypertension and FVC and pulmonary hypertension. Although physiologically interesting, the effect estimates are small and the clinical relevance of these findings remains unknown for now.

INTRODUCTION

Pulmonary hypertension is a progressive and fatal heterogeneous syndrome, characterized by elevated pulmonary arterial (systolic) pressure and can lead to right ventricular cardiac failure. Pulmonary hypertension can be classified into five groups, based on the etiology [1,2]. The main categories are: 1) Pulmonary Arterial Hypertension (PAH), e.g. idiopathic PH, heritable PH, PH induced by drugs and toxins, or PH associated with diseases influencing small pulmonary arterioles such as connective tissue diseases or Human Immunodeficiency Virus (HIV) infection; 2) Pulmonary hypertension owing to left heart disease; 3) Pulmonary hypertension due to lung diseases or chronic hypoxemia; 4) Chronic thromboembolic pulmonary hypertension; and 5) Pulmonary hypertension with a multifactorial origin. The prevalence of pulmonary hypertension differs per etiologic category and epidemiologic data are lacking [2].

Several causes of pulmonary hypertension are lung diseases and/or hypoxemia, which are classified into etiologic group 3. Pulmonary hypertension is a common co-morbidity, with a prevalence of up to 60% in patients with Chronic Obstructive Pulmonary Disease (COPD)[3]. Pulmonary hypertension in COPD patients seems be influenced by two mechanisms, obliteration of the vascular bed and hypoxia-induced vasoconstriction [4]. Its presence is associated with an impaired course of disease and decreased survival in comparison to COPD patients without pulmonary hypertension [5].

Although the presence of elevated pulmonary arterial systolic pressure (PASP) in patients with a lung disease is a well-known occurrence, the relation between the lung's ventilatory function and PASP is less well investigated. Furthermore, data on both pulmonary arterial pressure and spirometry measurements, from the general population are scarce.

Spirometry is a technique to evaluate the pulmonary ventilatory function and is a reflection of several forces implied in lung volumes. Two of the most important measures are the Forced Expiratory Volume in the first second, (FEV_1) and the Forced Vital Capacity (FVC). The FEV_1 provides information about the airflow, while the FVC provides an estimate of the vital capacity. In obstructive disease, the FEV1 is disproportionally decreased in comparison to FVC, resulting in a decreased ratio. In restrictive disease the FEV1 and FVC are often equally decreased, resulting in a normal ratio.

We hypothesized that spirometry measures and PASP) are associated. The objective of our study was to investigate the association of PASP) and FEV_1, FVC and FEV_1/FVC in a general population of older subjects.

METHODS

This study was performed in the Rotterdam Study I, a prospective population-based cohort study which started in 1990 in Ommoord, a suburb of Rotterdam. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, the Netherlands, approved the study. The cohort consists of 7,983 participants, aged 55 years and over. At baseline, all participants were visited at home for a standardized interview, and underwent a wide array of examinations at the research center. These study rounds are repeated every 3-5 years. In order to monitor the cohort for major diseases and mortality, the database is linked to the electronic records of the general practitioner, who acts as a gatekeeper to specialized medicine, as well as to the national death registry. All prescriptions dispensed to the participants are collected by linkage to the seven pharmacies covering the region. Main objectives and methods of the Rotterdam Study have been described elsewhere [6].

Study population

The study population consisted of all participants of the Rotterdam Study with both spirometry and echocardiography. These were gathered during the most recent (fifth) study round.

Echocardiography

Echocardiographies were made using a commercially available ultrasonography system (Vivid I, GE Healthcare), with a 2.5 MHz transducer according to a standardized protocol. Extensive left- and right-sided measurements and measures of both systolic and diastolic function were obtained. All images obtained at echocardiography were digitally stored and assessed offline by the specialized researchers. Pulmonary arterial systolic pressure (PASP, in mmHg) was calculated using the recommendations by the ASE/EAE/CSE as the sum of the estimated right atrial pressure (based on the diameter of the inferior vena cava and forced respiratory collapse) and the pressure gradient over the tricuspid valve. The pressure gradient was computed from the highest Doppler tricuspid regurgitation velocity gathered from several windows using the simplified Bernoulli equation ($4v^2$, where v is tricuspid regurgitation peak velocity in m/sec) [7]. In those with sufficient tricuspid regurgitation to estimate PASP, a 40 mmHg cut-off was set to define echocardiographic pulmonary hypertension. The definition of echocardiography-based pulmonary hypertension (ePH) is based on the measurements of TRV (TRV; elevated TRV is defined as TRV > 3, corresponding to a PASP of > 40 mmHg) and right ventricular end-diastolic dimension (RVEDD; elevated RVEDD is defined as RVEDD > 42 mm) [2,7,8]. If the TRV was too small to measure, but not missing, or normal, but a participant had an elevated RVEDD, this was considered to be indicative of PH (Supplementary Table 1).

Spirometry

Spirometry was performed by trained paramedical personnel using a Jaeger Masterscreen PFT (Care Fusion, the Netherlands), according to American Thoracic Society (ATS)/ European Respiratory Society (ERS) guidelines [9]. FEV_1, FVC and the ratio of FEV_1 over FVC (FEV_1/FVC) were measured. For practical reasons, reversibility testing could not be carried out. Participants were asked to refrain from using any prescribed pulmonary medication one day before the center visit. The spirometry tests were analyzed by two researchers (DL or LL), and verified by a specialist in pulmonary medicine (GGB). Spirometry procedures that yielded results that did not meet ATS/ERS criteria for acceptability and reproducibility were classified as not interpretable [10,11].

Covariables

Information on several potential confounders or effect modifiers such as age, sex, height, weight and body mass index (BMI) was gathered at the study in the same round as the spirometry and echocardiography. Furthermore, we collected indicators or left ventricular diastolic dysfunction in order to adjust for possible left heart failure leading to an increased PASP.

RESULTS

From the most recent cross-sectional examination of the Rotterdam Study (the 5th round), a total of 2,903 individuals had underwent assessment of both spirometry and echocardiography (44% males, mean age 75 years). In 1,829 participants, tricuspid regurgitation was present and the PASP could be estimated in 1,660 individuals. The baseline characteristics of these participants can be found in table 1. In 2,380 individuals the definition of pulmonary hypertension could be made according to the algorithm in Supplementary Table 1.

In this dataset, a total of 284 participants had COPD according to the spirometry-based GOLD definition, of whom none had GOLD Stage IV. When we evaluated these participants according to the, in 2011 updated, multidimensional COPD staging system [12], we were able to classify 195 participants according to the four categories (GOLD 2011: COPD A, B, C and D).

Using the algorithm described above (Supplementary table 1), 99 (4.2%) participants and 25 (5.9%) COPD subjects had echocardiographic signs of pulmonary hypertension.

Table 1. Baseline characteristics

Characteristics		Total
Sex N (%)	Male (%)	605 (36.4 %)
	Female (%)	1055 (63.6 %)
Age Years (SD)		75.7 (6.3)
Height cm (SD)		165.9 (9.0)
Weight kg (SD)		77.0 (13.6)
BMI kg/m^2 (SD)		26.9 (4.0)
Smoking Status† N (%)	Never	612 (36.9 %)
	Past	877 (52.8 %)
	Current	142 (8.6 %)
Pack Years mean (SD)		21 (21)
Blood Pressure mmHg (SD)	Systolic	151 (22)
	Diastolic	85 (11)
Echocardiography	ePASP mmHg (SD)	26.1 (6.9)
	LV mass grams (SD)	129.6 (38.1)
	LVED mm (SD)	51.1 (5.1)
	Fractional Shortening % (SD)	41.5 (5.7)
	E/A-Ratio	0.90 (0.35)
Echographic Pulmonary Hypertension* N (%)		99 (4.2 %)
Pulmonary Function	FEV_1 L (SD)	2.29 (0.66)
	FEV_1 percent predicted % (SD)	104 (22)
	FVC L (SD)	3.02 (0.83)
	FVC percent predicted % (SD)	109 (20)
	FEV_1/FVC % (SD)	76 (8)
Obstructive Pulmonary Disease		
GOLD Classification¶, N (%)	No obstruction‡	1305 (78.6)
	I	139 (8.4 %)
	II	125 (7.5%)
	III	20 (1.2%)
GOLD ABCD classification§, N (%)	No obstruction‡	1305 (78.6)
	A	99 (6.0 %)
	B	70 (4.2 %)
	C	9 (0.5 %)
	D	17 (17 %)

BMI: Body Mass Index, ePASP = estimated Pulmonary Artery Systolic Pressure, LV = left ventricular, LVED = Left ventricular end diastolic dimension, E/A-ratio: ratio of early and late ventricular filling velocity, SD: Standard Deviation.† Smoking status missing: n=29.* Based on Tricuspid valve Regurgitation Velocity and ePASP and/or secondary signs of PH such as Right Ventricle End Diastolic Dimension, total sample = 2380. ¶ GOLD classification based on spirometry only. § Classification based on Spirometry, Symptoms of Dyspnoea and Frequency of COPD Exacerbations. Available in n = 195. ‡ Spirometry suggestive for a restrictive syndrome: n=71.

Association of spirometric measures (FEV_1, FVC and FEV_1/FVC) with ePASP

Using linear regression per 10% decrease, FEV_1 % predicted was significantly associated with an ePASP increase of 0.46 mmHg (95%CI: 0.32; 0.62). Similarly, per 10% decrease, FVC % predicted was significantly associated with an increased ePASP (+ 0.42 mmHg, 95%CI: 0.25; 0.60). Lastly, FEV_1/FVC showed an association of 1.01 mmHg (95%CI: 0.58; 1.45) increase in ePASP per 10% decrease in FEV_1/FVC. (Table 2)

Table 2. Effect estimates on ePASP in mmHg

Pulmonary function test	Effect, crude	95%CI	Effect, adjusted‡	95%CI	P-value, adjusted
FEV_1 % predicted*	0.60	0.45; 0.75	0.46	0.32; 0.62	<0.0001
FVC % predicted *	0.62	0.46; 0.78	0.42	0.25; 0.60	<0.0001
FEV_1/FVC*	1.04	0.62; 1.47	1.01	0.58; 1.45	<0.0001
Excluding COPD					
FEV_1 % predicted*	0.49	0.29; 0.70	0.39	0.18; 0.59	<0.0001
FVC % predicted *	0.51	0.31; 0.71	0.33	0.12; 0.54	0.002
FEV_1/FVC*	0.73	-0.03; 1.48	0.72	-0.01; 1.47	0.054

* Per 10% decrease

‡ Adjusted for: sex, age, smoking status, pack-years, left ventricular fractional shortening, left ventricular mass, E/A-ratio, BMI, mean heart rate, use of beta-blocking agents, use of AT2-agonists, use of high ceiling diuretics, use of digoxin.

As a sensitivity analysis, we excluded participants with COPD defined on the basis of spirometry (FEV_1/FVC < 0.70). After this exclusion the associations persisted with similar effect estimates. For FEV_1 % predicted the ePASP was associated with an increase of 0.39 (95%CI: 0.18; 0.59); for FVC this increase in ePASP was 0.33 (95%CI: 0.12; 0.54). For FEV_1/FVC a numerical increase in ePASP was seen; however, this was not significant with an effect estimate of 0.72 (95%CI -0.01; 1.47).

Spirometry and pulmonary hypertension

When we analyzed pulmonary hypertension dichotomously, FEV_1 % predicted, FVC % predicted and FEV_1/FVC were significantly associated with an increased risk of pulmonary hypertension, defined as an ePASP over 40 mmHg and or an increased RVEDD (supplementary table 1). Per 10% percent decrease, the FEV_1 % predicted was associated with an adjusted odds ratio of pulmonary hypertension of 1.18 (95%CI 1.04; 1.34). FVC was associated with a similar adjusted odds ratio of 1.31 (95%CI 1.02; 1.36). For FEV_1/FVC the adjusted OR was 1.30 (95%CI 0.94; 1.79). (Table 3)

Table 3. Effect estimates of Pulmonary Function on risk of pulmonary hypertension

Pulmonary function test	OR, Crude	95%CI	OR, adjusted‡	95%CI	P-value, adjusted
$FEV_{1\ \%\ predicted}$*	1.26	1.15; 1.38	1.18	1.04; 1.34	0.008
$FVC_{\ \%\ predicted}$*	1.33	1.20; 1.48	1.18	1.02; 1.36	0.029
FEV_1/FVC*	1.27	1.01; 1.59	1.30	0.94; 1.79	0.110

* Per 10% decrease

‡ Adjusted for: sex, age, smoking status, pack-years, left ventricular fractional shortening, left ventricular mass, E/A-ratio, BMI, mean heart rate, use of beta-blocking agents, use of AT2-agonists, use of high ceiling diuretics, use of digoxin.

DISCUSSION

In this cross-sectional analysis population-based prospective cohort, we observed that pulmonary function was associated with PASP. There were significant associations for FEV_1 % predicted, FVC % predicted and FEV_1/FVC with ePASP as continuous outcome and pulmonary hypertension as dichotomous outcome. Per 10 % decrease, FEV_1 % predicted is associated with an increase in ePASP of 0.46 mmHg and an OR of 1.18 for pulmonary hypertension. Per 10% decrease, FVC % predicted is associated with an increase in ePASP of 0.42 mmHg and an OR of 1.18 for pulmonary hypertension. Lastly, per 10% decrease, FEV_1/FVC was associated with an increase in ePASP of 1.01 mmHg and an OR of 1.30. Interestingly, neither COPD according to the previous GOLD guidelines (severity defined by spirometry) showed a significant association, nor did COPD according to the 2011 GOLD classification. Across GOLD stages there seems to be an inconsistency in the direction of the ORs. This is most likely due to insufficient power.

The associations of PASP with FEV_1, FVC and FEV_1/FVC seem to be independent of the presence of COPD. Although the risk estimates of these associations are modest, they seem to be robust. To our knowledge this is the first time these associations are shown in the general population, rather than in specifically designed studies on pulmonary hypertension and/or COPD. This is in line with results from Lam e.a. who has previously shown that PASP increases in the aging population independent of cardiopulmonary disease [13]. Furthermore, they showed an increased risk for all-cause mortality by increased PASP.

Although interesting from the point of view of physiology, considering the small effect estimates one might question whether this relationship between spirometry and PASP is clinically relevant. Therefore, it is important to emphasize that although in general COPD patients will have a relatively elevated PASP compared to non-COPD patients, a small proportion of COPD patients may present itself with a disproportionally high PASP [14] with respect to their obstructive disease. This group might benefit from treatment for

pulmonary hypertension in addition to COPD treatment [14-16], whereas the only available treatment now is oxygen therapy.

Like every study, our study has its limitations. The major limitation is of course the fact that we are not able to use the gold standard for the definition of pulmonary hypertension, which is right-sided heart catheterization. We are however quite conservative with respect to the definition of pulmonary hypertension based on echocardiography, which would more likely lead to an underestimation of the prevalence of pulmonary hypertension. Another limitation, due to the cross-sectional design of the study, is that it is difficult to disentangle cause and consequence. By adjusting by indicators of left ventricular failure, we aimed to eliminate left-sided heart failure as a possible cause for pulmonary hypertension.

The strengths of our study are the prospectively gathered population-based data, which reduces the risk of selection bias. Furthermore we have gathered a wide range of detailed information in this large group of older subjects and, especially with respect to pulmonary hypertension and spirometry, population-based data are scarce.

In conclusion we show an association of FEV_1 % predicted, FVC % predicted and FEV_1/FVC with pulmonary arterial systolic pressure. Furthermore, FEV_1 and FVC are associated with an increased risk of pulmonary hypertension. The implications of these findings remain unclear for now, but may be important in the development of new diagnostic algorithms and treatment regimes in the future.

Supplementary table 1. Definition of pulmonary hypertension based on echocardiography (ePH)

	TRV missing	**TRV too small to measure, but non-missing**	**TRV normal, corresponding to PASP =< 40 mmHg**	**TRV elevated, corresponding to PASP > 40 mmHg**
RVEDD missing	Missing	No ePH	No ePH	ePH
RVEDD normal	Missing	No ePH	No ePH	ePH
RVEDD elevated	ePH	ePH	ePH	ePH

RVEDD: Right Ventricle End Diastolic Diameter, TRV: Tricuspid valve regurgitation velocity, PASP: Pulmonary Artery Systolic Pressure.

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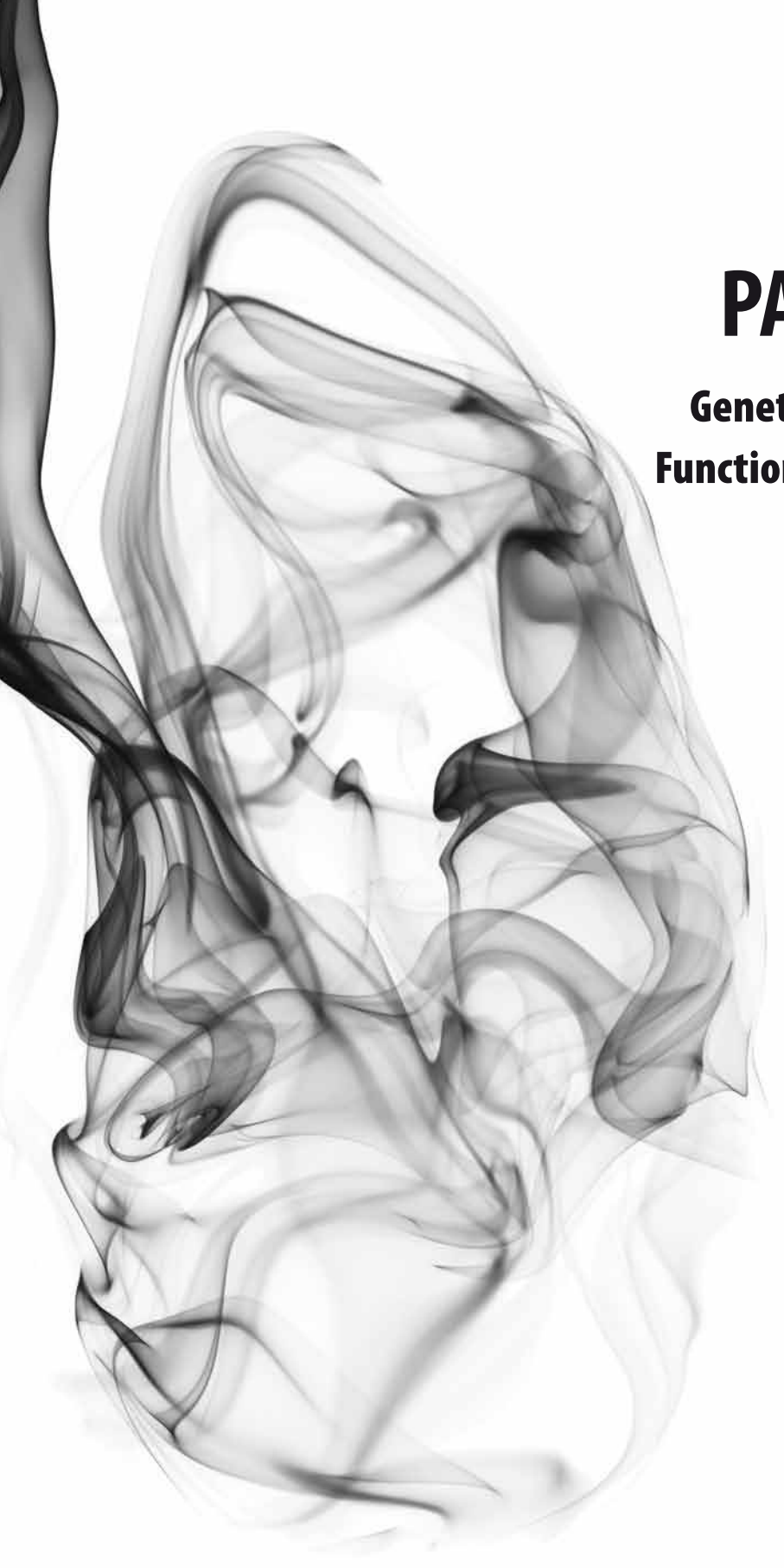
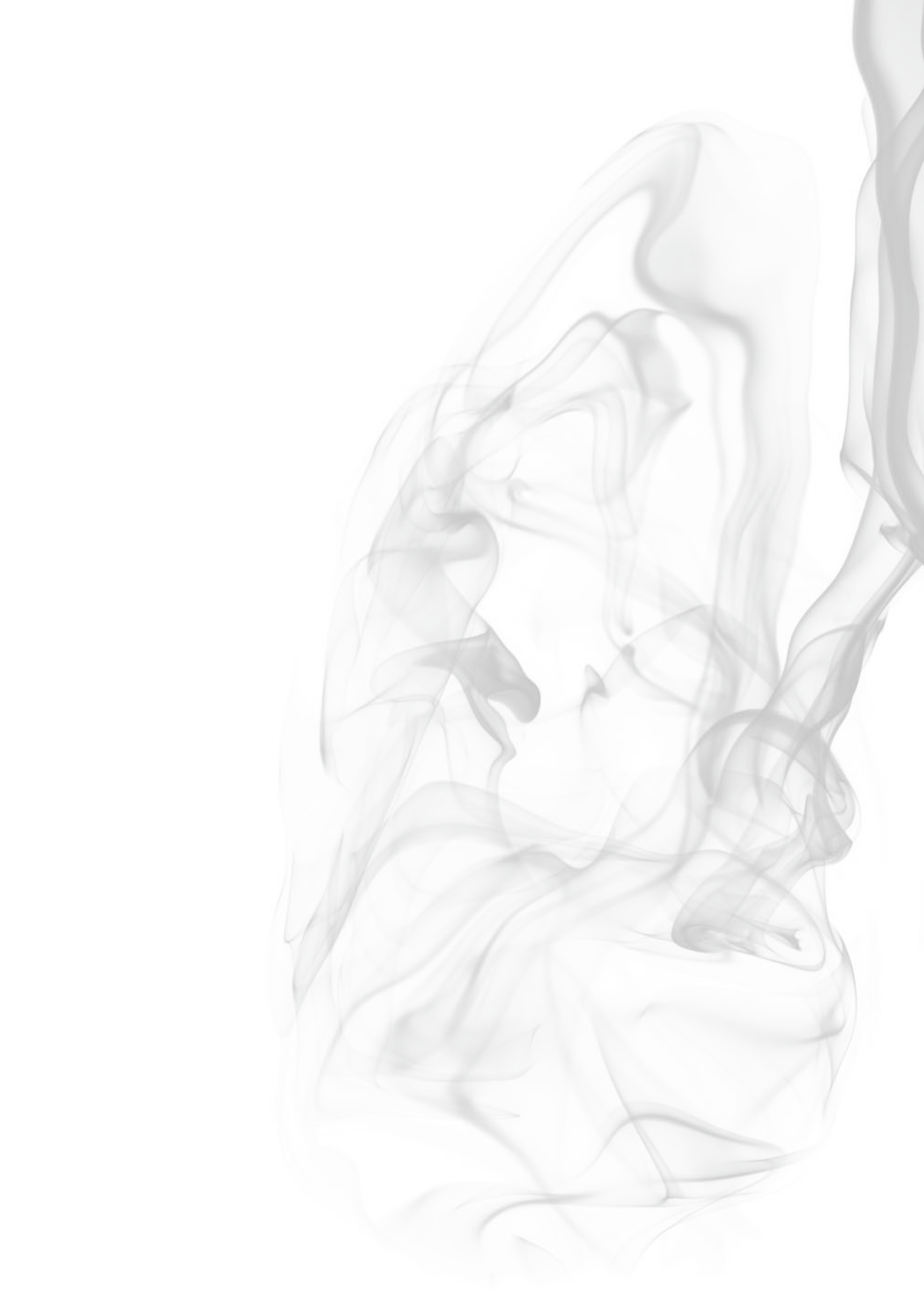


PART III

Genetics of Lung Function and COPD



CHAPTER 3.1

Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function

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Published in Nature Genetics, 2011 Sep 25;43(11):1082-90. doi: 10.1038/ng.941. PubMed PMID: 21946350

ABSTRACT

Pulmonary function measures reflect respiratory health and are used in the diagnosis of chronic obstructive pulmonary disease. We tested genome-wide association with forced expiratory volume in 1 second and the ratio of forced expiratory volume in 1 second to forced vital capacity in 48,201 individuals of European ancestry with follow up of the top associations in up to an additional 46,411 individuals. We identified new regions showing association (combined $P < 5 \times 10^{-8}$) with pulmonary function in or near *MFAP2*, *TGFB2*, *HDAC4*, *RARB*, *MECOM* (also known as *EVI1*), *SPATA9*, *ARMC2*, *NCR3*, *ZKSCAN3*, *CDC123*, *C10orf11*, *LRP1*, *CCDC38*, *MMP15*, *CFDP1* and *KCNE2*. Identification of these 16 new loci may provide insight into the molecular mechanisms regulating pulmonary function and into molecular targets for future therapy to alleviate reduced lung function.

INTRODUCTION

Pulmonary function, reliably measurable by spirometry, is a heritable trait reflecting the physiological state of the airways and lungs[1]. Pulmonary function measures are important predictors of population morbidity and mortality[2–4] and are used in the diagnosis of chronic obstructive pulmonary disease (COPD), which ranks among the leading causes of death in developed and developing countries[5,6]. A reduced ratio of forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) is used to define airway obstruction, and a reduced FEV_1 is used to grade the severity of airway obstruction[7].

Recently, two large genome-wide association studies (GWAS), each comprising discovery sets of more than 20,000 individuals of European ancestry, identified new loci for lung function[8,9]. Recognizing the need for larger data sets to increase the power to detect loci of individually modest effect size, we conducted a meta-analysis of 23 lung function GWAS comprising a total of 48,201 individuals of European ancestry (stage 1) and followed up potentially new loci in 17 further studies comprising up to 46,411 individuals (stage 2). We identified 16 additional new loci for lung function and provided evidence corroborating the association of loci previously associated with lung function[8–11]. Our findings implicate a number of different mechanisms underlying regulation of lung function and highlight loci shared with complex traits and diseases, including height, lung cancer and myocardial infarction.

RESULTS

Genome-wide analysis (stage 1)

We undertook meta-analyses for cross-sectional lung function measures for approximately 2.5 million genotyped or imputed SNPs across 23 studies with a combined sample size of 48,201 adult individuals of European ancestry. Characteristics of the cohort participants and the genotyping are shown in Supplementary Table 1a and b. We adjusted FEV_1 and FEV_1/FVC measures for ancestry principal components, age, age^2, sex and height as covariates. Our association testing of the inverse-normal–transformed residuals for FEV_1 and FEV_1/FVC assumed an additive genetic model and was stratified by ever-smoking versus never-smoking status. We performed the meta-analyses of the smoking strata within each study and of the study-specific results using inverse-variance weighting (and used the inverse of the standard error squared as the weight). We applied genomic control twice at the study level (to each smoking stratum separately and to the study-level pooled estimates) and also at the meta-analysis level to avoid inflation of the test statistics caused by cryptic population structure or relatedness (see Supplementary Table 1a for study-level estimates). Our application of genomic control at the three stages is likely to be overly conservative because it has recently been shown that in large meta-analyses, test statistics are expected to be elevated under polygenic inheritance even when there is no population structure[12]. The test statistic inflation (λ_{GC}) before applying genomic control at the meta-analysis level was 1.12 for FEV_1 and 1.09 for FEV_1/FVC. Genomic inflation estimates increase with sample size, as has been shown for other traits[13–15]; the standardized estimates to a sample of 1,000 individuals ($\lambda_{GC\_1,000}$) were 1.002 for FEV_1 and 1.002 for FEV_1/FVC. Plots of the meta-analysis P values for FEV_1 and FEV_1/FVC against a uniform distribution of P values expected under the null hypothesis showed deviations which were attenuated, but which persisted, after removal of SNPs in loci reported previously, consistent with additional loci being associated with lung function (Supplementary Fig. 1a).

Follow-up analysis (stage 2)

Twenty-nine new loci showing evidence of association with lung function ($P < 3 \times 10^{-6}$) in stage 1 were followed up in stage 2 by using in silico data from seven studies and by undertaking additional genotyping in ten studies for the ten highest ranked SNPs (Fig. 1). Full details of the SNP selection are given in the Online Methods. We performed an inverse-variance–weighting meta-analysis across stages 1 and 2 and obtained two-sided P values for the pooled estimates. Sixteen new loci reached genome-wide significance ($P < 5 \times 10^{-8}$) and showed consistent direction of effects in both stages, comprising 12 new loci for FEV_1/FVC, 3 new loci for FEV_1 and 1 new locus reaching genome-wide significance for both traits (Fig. 2 and Table 1). To assess the heterogeneity across the

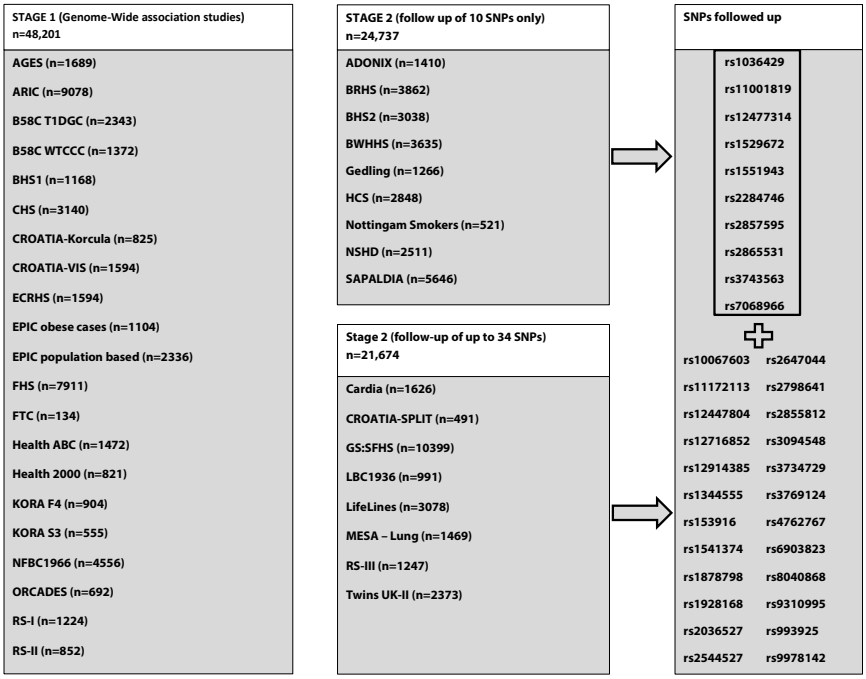

Figure 1. Study design.
We followed up in stage 2 a total of 34 SNPs showing new evidence of association ($P < 3 \times 10^{-6}$) with FEV_1 and/or FEV_1/FVC in a meta-analysis of the stage 1 studies. Studies with a combined total of 24,737 individuals undertook genotyping and association testing of the top ten SNPs. Seven studies (marked with an asterisk) with a combined total of 11,275 individuals had genome-wide association data and provided results for up to 34 SNPs. Researchers from GS: SFHS (marked with #) undertook genotyping on a 32-SNP multiplex genotyping platform and so included the 32 top ranking SNPs (including proxies and both SNPs from regions that showed association with both FEV_1 and FEV_1/FVC). This assay failed for one SNP (rs3769124), which was subsequently replaced with the thirty-third SNP (rs4762767). We excluded rs2284746 because of poor clustering. Although rs3743563 was chosen as proxy for rs12447804, which had an effective N < 80% in the stage 1 meta-analysis, researchers from BHS2 were unable to genotype rs3743563 and so undertook genotyping for rs12447804 instead. See Supplementary Table 1 for definitions of all study abbreviations.

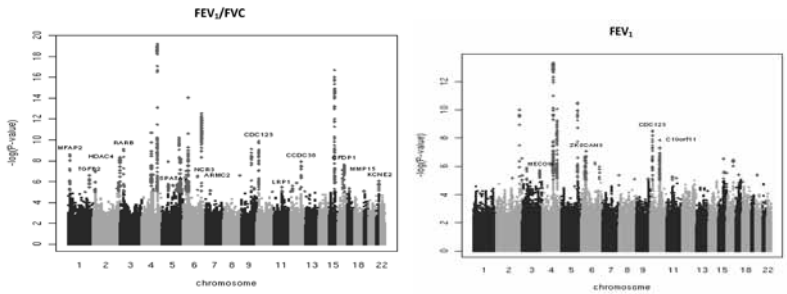

Figure 2. Manhattan plots of association results for FEV_1/FVC and FEV_1 (analysis stage 1).
The Manhattan plots for FEV_1/FVC (a) and FEV_1 (b) are ordered by chromosome position. SNPs for which $-\log_{10} P > 5$ are indicated in red. Newly associated regions that reached genome-wide significance after meta-analysis of stages 1 and 2 are labeled.

Table 1. Loci associated with lung function.

				Stage 1				Stage 2				Joint meta-analysis of all stages	
Chr	**SNP (position), function**	**Coded Allele**	**Measure**	**Beta (s.e.m.)**	***P***	**Allele freq.**	***N***	**Beta (s.e.m.)**	***P***	**Allele freq.**	***N***	**Beta (s.e.m.)**	***P***
1	rs2284746(17179262), *MFAP2*(intron)	G	FEV_1/FVC	-0.042 (0.007)	2.47E-09	0.516	45944	-0.038 (0.007)	**2.64E-07**	0.522	35371	-0.04 (0.005)	**7.50E-16**
			FEV_1	0.008 (0.007)	2.78E-01			0.006 (0.007)	3.70E-01			0.007 (0.005)	1.48E-01
1	rs993925(216926691), *TGFB2*(downstream)	T	FEV_1/FVC	0.04 (0.007)	2.54E-07	0.308	42402	0.023 (0.01)	1.76E-02	0.348	21414	0.034 (0.006)	**1.16E-08**
			FEV_1	0.025 (0.007)	1.51E-03			0.003 (0.007)	7.29E-01			0.014 (0.005)	8.71E-03
2	rs12477314(239542085), *HDAC4*(downstream)	T	FEV_1/FVC	0.052 (0.008)	4.48E-09	0.202	45585	0.031 (0.008)	**8.41E-05**	0.206	45821	0.041 (0.006)	**1.68E-12**
			FEV_1	0.032 (0.008)	2.77E-04			0.025 (0.007)	**1.82E-04**			0.028 (0.005)	1.02E-07
3	rs1529672(25495586), *RARB*(intron)	C	FEV_1/FVC	-0.06 (0.009)	7.75E-10	0.829	40624	-0.038 (0.009)	**1.16E-05**	0.831	45466	-0.048 (0.006)	**3.97E-14**
			FEV_1	-0.037 (0.009)	1.78E-04			-0.011 (0.007)	9.33E-02			-0.02 (0.006)	2.16E-04
3	rs1344555(170782913), *MECOM*(intron)	T	FEV_1/FVC	-0.019 (0.008)	2.61E-02	0.205	46067	-0.017 (0.012)	1.55E-01	0.209	21313	-0.018 (0.007)	6.65E-03
			FEV_1	-0.042 (0.008)	1.91E-06			-0.025 (0.009)	6.44E-03			-0.034 (0.006)	**2.65E-08**
5	rs153916(95062456), *SPATA9*(upstream)	T	FEV_1/FVC	-0.033 (0.007)	2.06E-06	0.552	47530	-0.025 (0.009)	6.67E-03	0.535	21647	-0.031 (0.005)	**2.12E-08**
			FEV_1	-0.001 (0.007)	8.91E-01			0.004 (0.007)	6.22E-01			0.001 (0.005)	8.20E-01

6	rs6903823(28430275), *ZKSCAN3*(intron)/*ZNF323*(intron)	G	FEV_1/FVC	-0.027 (0.008)	2.28E-03	0.209	47057	-0.013 (0.011)	2.34E-01	0.246	21489	-0.021 (0.007)	1.19E-03
			FEV_1	-0.046 (0.008)	2.00E-07			-0.029 (0.008)	**4.75E-04**			-0.037 (0.006)	**2.18E-10**
6	rs2857595(31676448), *NCR3*(upstream)	G	FEV_1/FVC	0.049 (0.009)	7.86E-08	0.809	45540	0.028 (0.008)	**5.36E-04**	0.796	46107	0.037 (0.006)	**2.28E-10**
			FEV_1	0.04 (0.009)	1.46E-05			0.017 (0.007)	9.41E-03			0.025 (0.005)	1.30E-06
6	rs2798641(109374743), *ARMC2*(intron)	T	FEV_1/FVC	-0.047 (0.009)	2.81E-07	0.183	46369	-0.03 (0.012)	1.57E-02	0.179	21173	-0.041 (0.007)	**8.35E-09**
			FEV_1	-0.046 (0.009)	5.39E-07			-0.009 (0.01)	3.35E-01			-0.03 (0.006)	4.69E-06
10	rs7068966(12317998), *CDC123*(intron)	T	FEV_1/FVC	0.045 (0.007)	1.28E-10	0.519	47085	0.023 (0.006)	**3.86E-04**	0.518	46067	0.033 (0.005)	**6.13E-13**
			FEV_1	0.04 (0.007)	1.19E-08			0.022 (0.005)	**3.56E-05**			0.029 (0.004)	**2.82E-12**
10	rs11001819(77985230), *C10orf11*(intron)	G	FEV_1/FVC	-0.019 (0.007)	6.50E-03	0.522	45546	-0.006 (0.006)	3.17E-01	0.506	45932	-0.012 (0.005)	7.58E-03
			FEV_1	-0.041 (0.007)	1.42E-08			-0.022 (0.005)	**3.10E-05**			-0.029 (0.004)	**2.98E-12**
12	rs11172113(55813550), *LRP1*(intron)	T	FEV_1/FVC	-0.035 (0.007)	1.36E-06	0.607	45387	-0.026 (0.01)	5.83E-03	0.59	20509	-0.032 (0.006)	**1.24E-08**
			FEV_1	-0.021 (0.007)	3.55E-03			-0.003 (0.007)	6.94E-01			-0.013 (0.005)	1.19E-02
12	rs1036429(94795559), *CCDC38*(intron)	T	FEV_1/FVC	0.049 (0.008)	1.24E-08	0.2	47814	0.028 (0.008)	**3.35E-04**	0.214	46311	0.038 (0.006)	**2.30E-11**
			FEV_1	0.01 (0.008)	2.67E-01			0.004 (0.006)	5.38E-01			0.006 (0.005)	2.26E-01

Table 1. (Continued)

				Stage 1				Stage 2				Joint meta-analysis of all stages	
Chr	**SNP (position), function**	**Coded Allele**	**Mea-sure**	**Beta (s.e.m.)**	***P***	**Allele freq.**	***N***	**Beta (s.e.m.)**	***P***	**Allele freq.**	***N***	**Beta (s.e.m.)**	***P***
16	rs12447804(56632783), *MMP15*(intron)	T	FEV_1/FVC	-0.053 (0.009)	7.12E-08	0.208	35123	-0.021 (0.01)	4.20E-02	0.222	24398	-0.038 (0.007)	**3.59E-08**
			FEV_1	-0.017 (0.009)	8.02E-02			0.004 (0.007)	5.71E-01			-0.004 (0.006)	4.73E-01
16	rs2865531(73947817), *CFDP1*(intron)	T	FEV_1/FVC	0.039 (0.007)	2.30E-08	0.418	47594	0.024 (0.006)	**1.94E-04**	0.409	46304	0.031 (0.005)	**1.77E-11**
			FEV_1	0.024 (0.007)	6.30E-04			0.011 (0.005)	3.89E-02			0.016 (0.004)	1.09E-04
21	rs9978142(34574109), *KCNE2*(upstream)	T	FEV_1/FVC	-0.048 (0.009)	8.23E-07	0.156	44577	-0.031 (0.013)	1.75E-02	0.149	20944	-0.043 (0.008)	**2.65E-08**
			FEV_1	-0.012 (0.009)	2.47E-01			-0.015 (0.01)	1.35E-01			-0.013 (0.007)	5.57E-02

Shown are FEV_1 and FEV_1/FVC results for the leading SNPs, ordered by chromosome and position for each independent locus associated ($P < 5 \times 10^{-8}$) with FEV_1 or FEV_1/FVC in a joint analysis of up to 94,612 individuals of European ancestry from the SpiroMeta-CHARGE GWAS (stage 1) and follow up (stage 2). Two-sided *P* values are given for stage 1, stage 2 and the joint meta-analysis of all stages. *P* values reaching genome-wide significance ($P < 5 \times 10^{-8}$) in the joint meta-analysis of all stages are indicated in bold. SNPs reaching independent replication in stage 2 ($P = 0.05/34 = 1.47 \times 10^{-3}$) are indicated with their stage 2 *P* value in bold. The sample sizes (*N*) shown are the effective sample sizes. The effective sample size within each study is the product of sample size and the imputation quality metric. The joint meta-analysis includes data from stage 1 and stage 2. β values reflect effect-size estimates on an inverse-normal transformed scale after adjustments for age, age2, sex, height and ancestry principal components. The estimated proportion of the variance explained by each SNP can be found in S**upplementary** T**able 6**. Chr., chromosome; freq., frequency.

studies included in stage 1 and 2, we performed χ^2 tests for all 16 SNPs, and none of these SNPs was statistically significant after applying a Bonferroni correction for 16 tests. The sentinel SNPs at these loci were in or near *MFAP2* (1p36.13), *TGFB2-LYPLAL1* (1q41), *HDAC4-FLJ43879* (2q37.3), *RARB* (3p24.2), *MECOM* (also known as *EVI1*) (3q26.2), *SPATA9-RHOBTB3* (5q15), *ARMC2* (6q21), *NCR3-AIF1* (6p21.33), *ZKSCAN3* (6p22.1), *CDC123* (10p13), *C10orf11* (10q22.3), *LRP1* (12q13.3), *CCDC38* (12q22), *MMP15* (16q13), *CFDP1* (16q23.1) and *KCNE2- LINC00310* (also known as *C21orf82*) (21q22.11) (Supplementary Fig. 1b,c). The strongest signals in *AGER* (rs2070600)8,9 and two of the new signals (rs6903823 in *ZKSCAN3* and rs2857595, upstream of *NCR3*) lie within a ~3.8-Mb interval at 6p21.32-22.1 that is characterized by long-range linkage disequilibrium (LD). Nevertheless, the leading SNPs in these regions, which are within the major histocompatibility complex (MHC), were statistically independent (Supplementary Note).

Gene expression

We investigated mRNA expression of the nearest gene for each of the 16 new loci in human lung tissue and a range of human primary cells including lung, brain, airway smooth muscle cells and bronchial epithelial cells. We detected transcripts for all the selected genes in lung tissue except *CCDC38*, and we also detected transcripts for most genes in airway smooth muscle cells and in bronchial epithelial cells (Table 2). As we were unable to detect expression of *CCDC38* in any tissue, we also examined expression of SNPRF, which is the gene adjacent to *CCDC38* (Table 2), and found its expression in all four cell types. *TGFB2*, *MFAP2*, *EVI1* and *MMP15* were expressed in one or more lung cell types but not in peripheral blood mononuclear cells, providing evidence that these genes may show tissue-specific expression. We assessed whether SNPs in these new regions or their proxies ($r^2 > 0.6$) were associated with gene expression using a database of expression-associated SNPs in lymphoblastoid cell lines16. Four loci showed regional (cis) effects on expression ($P < 1 \times 10^{-7}$; Supplementary Note). A proxy for our sentinel SNP in *CFDP1*, rs2865531, coincided with the peak of the expression signal for *CFDP1*, and the strongest proxy for rs6903823 in *ZKSCAN3* coincided with the peak of expression for *ZSCAN12*.

Plausible pathways for lung function involving new loci

The putative function of the genes within, or closest to, the association peaks identify a range of plausible mechanisms for affecting lung function. The most statistically significant new signal for FEV_1/FVC ($P = 7.5 \times 10^{-16}$) was in the gene encoding *MFAP2*, an antigen of elastin-associated microfibrils[17], although correlated SNPs in the region potentially implicate other genes that could plausibly influence lung function, such as *CROCC*, which encodes rootletin, a component of cilia[18]. Our second strongest new signal, also for FEV_1/FVC, was in *RARB*, the gene encoding the retinoic acid receptor β.

Table 2. Expression profiling of candidate genes in the lung and periphery

Sentinel SNP (relationship to gene)	Chr.	Gene	Putative Function of encoded protein	Tissue			
				Lung	HASM	HBEC	PBMC
rs993925 (intron)	1	***TGFB2***	Cytokine with roles in pro-fibrotic cytokine modulating epithelial repair mechanisms and extracellular matrix homeostasis including collagen deposition[40]	+	+	-	-
rs2284746 (intron)	1	***MFAP2***	Major antigen of elastin-associated microfibrils[17] and a candidate for involvement in the etiology of inherited connective tissue diseases.	+	+	+	-
rs12477314 (downstream)	2	***HDAC4***	Deacetylase of histone surrounding DNA thus influencing transcription factor access to the DNA possibly repressing gene transcription.	+	+	+	+
rs1344555 (intron)	3	***EVI1***	Zinc finger transcription factor, encoded as part of *MDS1/EVI1* complex locus (*MECOM*).	+	+	+	-
rs1529672 (intron)	3	***RARB***	Nuclear retinoic acid receptor responsive to retinoic acid, a vitamin A derivative and which also controls cell proliferation and differentiation.	+	+	+	+
rs153916 (intron)	5	***SPATA9***	Initially identified as a mediator of spermatogenesis, other family members may have a role in pancreatic development and β-cell proliferation[41]	+	+	+	+
rs2798641 (intron)	6	***ARMC2***	Function unknown although other family members have been identified as having roles in cell signaling, protein degradation and cytoskeleton functions[42]	+	+	+	+
rs2857595 (upstream)	6	***NCR3***	Required for efficient cytotoxicity responses by natural killer cells against normal cells and tumours[43]	+	-	-	+
rs6903823 (intron)	6	***ZKSCAN3***	Transcription factor involved in cell growth/cell cycle/signal transduction	+	+	+	+
rs7068966 (intron)	10	***CDC123***	Homologue in yeast shown to be a critical control protein modulating Eukaryotic initiation factor 2 in times of cell stress	+	+	+	+
rs11001819 (intron)	10	***C10orf11***	Function unknown	+	+	+	+
rs11172113 (intron)	12	***LRP1***	Potentially diverse roles including cell signaling and migration[44]	+	+	+	+

rs1036429 (intron)	12	***CCDC38***	Function unknown although other family members involved in a diverse array of functions skeletal and motor function[45]	-	-	-	-
rs1036429 (r^2=0.96 with rs4762633 in *SNRPF*)	12	***SNRPF***	Small nuclear ribonucleoprotein F	+	+	+	+
rs12447804 (intron)	16	***MMP15***	Member of a large protease family with diverse functional roles via protease activity and specificity including; tissue remodeling, wound healing, angiogenesis, and tumor invasion.	+	+	+	-
rs2865531 (intron)	16	***CFDP1***	Craniofacial Development Protein 1	+	+	+	+
rs9978142 (upstream)	21	***KCNE2***	KCNQ1-*KCNE2* K+ channels may modulate transepithelial anion secretion in Calu3 airway epithelial cells[46].	+	-	-	+
Reference gene	12	***GAPDH***		+	+	+	+

+ indicates the gene is expressed in the cell type used, and – indicates that we did not detect the gene expression at the mRNA level following 40 cycles of PCR. PCR profiling of gene transcripts in the human lung showed expression of all candidates except *CCDC38*, for which two sets of primers were designed and tested under different optimization conditions. None of these assays detected expression of *CCDC38* in the cell types analyzed. We instead assayed *SNRPF*, which neighbors *CCDC38* and harbors SNPs in strong LD with *CCDC38*'s sentinel SNP. All PCR products were sequence verified. We used *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) as a positive control for the complementary DNA, and this gene was expressed in all tissues. Chr., chromosome; HASM, human airway smooth muscle; HBEC, human bronchial epithelial cells; PBMC, peripheral blood mononuclear cells.

Rarb-null knockout mice have premature alveolar septation[19]. The third most statistically significant new signal for FEV_1/FVC, and the most statistically significant new signal for FEV_1, was in *CDC123*.

This was the only new region to show genome-wide association with both traits. *CDC123* encodes a homolog of a yeast cell-division–cycle protein that plays a critical role in modulating eukaryotic initiation factor 2 in times of cell stress[20]. The fourth signal for FEV_1/FVC is downstream of *HDAC4*, which encodes a histone deacetylase; reductions in the expression of other histone deacetylases (specifically *HDAC2*, *HDAC5* and *HDAC8*) have been noted in COPD[21]. The regions we observed in the MHC are much more difficult to localize, with multiple genes being tagged by the top SNP, including non-synonymous SNPs in *ZKSCAN3*, *PGBD1*, *ZSCAN12*, *ZNF323*, *TCF19*, *LTA*, *C6orf15* and *GPANK1* (also known as *BAT4*) (Supplementary Table 2). At 6p21.33, we observed the strongest association with lung function for rs2857595, which is in LD (r^2 = 0.47) with a non-synonymous SNP

in *LTA* (encoding lymphotoxin α) and with a SNP in the upstream promoter region of *TNFA* (encoding tumor necrosis factor α) ($r^2 = 0.86$), both of which are plausible candidates[22,23]. Our top SNP in *MMP15* is in strong LD ($r^2 = 1$) with a non-synonymous SNP (rs3743563, which has an association with FEV_1/FVC at $P = 1.8 \times 10^{-7}$) within the same gene. Among the plausible mechanisms implicated by the other new signals of association with lung function reported here is TGF-β signaling; *TGFB2* expression is up regulated in bronchial epithelial cells in asthma[24]. The putative function of key genes (as defined by LD with the leading SNP) in each of the 16 loci, and relevant findings from animal models, are summarized in Table 2 and are detailed in Supplementary Table 2.

Associations with lung function in children

Alleles representing 11 of the 16 new loci showed directionally consistent effects on lung function in 6,281 children (7–9 years of age) (Supplementary Table 3a), suggesting that genetic determination of lung function in adults may in part act through effects on lung development, or alternatively, that some genetic determinants of lung growth and lung function decline are shared.

Association of lung function loci with other traits

Although we stratified for ever smoking versus never smoking, we did not adjust for the amount smoked. In order to investigate the possibility that the associations at any of our 16 new regions were driven by an effect of the SNP on smoking behavior, we evaluated in silico data for associations with smoking amount from the Oxford-GlaxoSmithKline (Ox-GSK) consortium[25] for the leading SNPs in these 16 regions. None of these 16 SNPs showed statistically significant association with the number of cigarettes smoked per day (Supplementary Table 3b). In addition, in our stage 1 and 2 datasets combined, we assessed whether the estimated effect sizes of the variants on lung function phenotypes differed substantially between ever smokers and never smokers (Supplementary Table 4) across the 16 loci. For the most strongly associated trait at each locus, we tested the SNP interaction with ever smoking versus never smoking. None of the 16 new loci showed a significant interaction (Bonferroni-corrected threshold for 16 independent SNPs $P = 0.003125$). These analyses suggest that the genetic effects we have identified underlie lung function variability irrespective of smoking exposure.

We adjusted our lung function associations for height, but there are some overlaps between loci associated with height and those associated with lung function. Therefore, we evaluated in silico data for height associations of our new regions in the GIANT consortium[14] dataset. The G allele of rs2284746 (in an intron of *MFAP2*), which was associated with decreased FEV_1/FVC, was associated with increased height (Supplementary Table 3c).

Given reported associations between lung cancer and either COPD or lung function decline, we also assessed in silico data for sentinel or proxy SNPs in these 16 regions for associations with lung cancer in the International Lung Cancer Consortium (ILCCO) GWAS meta-analysis[26]. Alleles associated with reduced lung function were associated with risk of lung cancer at the strongest available proxy SNP for rs2857595 (upstream of *NCR3*) at 6p21.33 (rs3099844, $r^2 = 0.67$) and for the strongest proxy SNP for rs6903823 (a SNP in an intron of *ZKSCAN3* and *ZNF323*) at 6p22.1 (rs209181, $r^2 = 0.69$) (lung cancer associations at $P = 2.2 \times 10^{-7}$ and $P = 3.4 \times 10^{-5}$, respectively; Supplementary Table 3d). We saw no significant associations with lung cancer at the other new loci (proxy SNPs were available for 15 of the 16 loci, Bonferroni-corrected $P < 0.0033$).

In addition to the effects on height, smoking and lung cancer described above, we examined the literature for evidence of associations with other traits for each of the 16 new loci (detailed in Supplementary Table 2). Genome-wide significant associations ($P < 5 \times 10^{-8}$) have been reported in *KCNE2* with myocardial infarction[27] and at 6p21.33 near *NCR3-AIF1* with neonatal lupus[28] and systemic lupus erythematosus[29]. Other significant complex disease associations have also been noted in the regions of *CDC123* (type 2 diabetes[30]), *CFDP1* (type 1 diabetes[31]) and *MECOM* (blood pressure[32,33]), but with weaker LD ($r^2 < 0.3$) being seen between the reported SNP and the sentinel SNP for lung function in the region (Supplementary Table 2).

Proportion of variance explained by loci discovered to date

Associations in ten loci previously reported for lung function[8,9] reached genome-wide significance ($P < 5 \times 10^{-8}$) in our stage 1 data, namely loci in or near *TNS1*, *FAM13A*, *GSTCD-NPNT*, *HHIP*, *HTR4*, *ADAM19*, *AGER*, *GPR126*, *PTCH1* and *TSHD4* (Supplementary Table 5a). Thus, a total of 26 regions showed genome-wide significant association with lung function in our study. In aggregate, variants at these 26 regions explain approximately 3.2% of the additive polygenic variance for FEV_1/FVC and 1.5% of the variance for FEV_1 (Supplementary Note). Following the approach previously described[34], we estimated that there are a total of 102 (95% confidence interval 57–155) independent variants with similar effect sizes to the 26 variants we report here. In combination, these 102 variants, comprising 26 discovered variants and 76 putative undiscovered variants, collectively explain around 7.5% of the additive polygenic variance for FEV_1/FVC and 3.4% of the variance for FEV_1 (Supplementary Table 6 and Supplementary Note)

DISCUSSION

In meta-analysis of 23 studies comprising 48,201 individuals of European ancestry and follow up in 17 studies comprising up to 46,411 individuals, we report genome-wide

significant associations with an additional 12 regions for FEV_1/FVC, an additional 3 regions for FEV_1 and 1 additional region associated with both FEV_1 and FEV_1/FVC. We also confirmed genome-wide association with ten regions previously associated with lung function, bringing to 26 the total number of loci associated with lung function from analyses of these datasets. Most of the new loci are in regions not previously suspected to have been involved in lung development, the control of pulmonary function or the risk of developing COPD. Elucidating the mechanisms through which these regions influence lung function should lead to a more complete understanding of lung function regulation and the pathogenesis of COPD. Four of the new loci (*MFAP2*, *ZKSCAN3*, near *NCR3* and near *KCNE2*) that we showed to be associated with lung function are also associated with other complex traits and diseases (with $P < 5 \times 10^{-8}$ for the other trait at a SNP having $r^2 > 0.3$ with the top lung function SNP in the region). Understanding the intermediates underlying these pleiotropic effects could also lead to crucial insights into the pathophysiology of lung disease. One potential explanation is that these loci underlie control of the mechanisms regulating the development and resolution of inflammation and subsequent tissue remodeling in a range of tissues.

The effect sizes of the variants in the 26 loci associated with lung function collectively explain a modest proportion of the additive genetic variance in FEV_1/FVC and in FEV_1, even after accounting for putative undetected variants with a similar distribution of effect sizes[34]. Our findings are consistent with those from other common complex traits, where it is thought that many as yet unidentified common and rare sequence variants, and potentially structural variants, could explain the remaining heritability[35]. That our study more than doubled the number of loci known to be associated with lung function underlines the utility of large sample sizes to achieve the power to detect common variants associated with complex traits. Nevertheless, it is likely that additional variants with similar effect sizes remain undiscovered[14]. In addition, our study was not designed to detect rare variants or structural variants associated with lung function. Identification of rare variants associated with lung function could be helpful in narrowing the scope of ongoing functional work to those genes most likely to be causally related to the association signals we detected.

Our study focused on cross-sectional measures of lung function. Adult lung function at a particular time point is influenced by the peak lung function achieved by 25–35 years of age as well as the rate of decline of lung function after that peak[36]. The 26 loci now confirmed to be associated with lung function could affect either pre- or post-natal lung development and growth or decline in lung function during adulthood, or both. We showed consistent directions of estimated effects on lung function between adults and children 7–9 years of age for SNPs at 11 of the 16 new loci and 8 of the 10 previously re-

ported loci (Supplementary Table 3a). The results we show for lung function in children provide some indication that these loci affect lung function development, although studies in larger populations of children would provide greater clarity for SNPs in the new loci. Further investigations will be required in large populations with longitudinal data to delineate the influence of these variants on the rates of development of, and decline in, lung function and on the risk of developing COPD.

Of the sentinel SNPs at the 16 new loci associated with lung function, only rs2284746 (*MFAP2*) was associated with height in the GIANT consortium[14] dataset. The G allele of rs2284746 was associated with both increased height and reduced lung function. A similar relationship between lung function and height was previously reported for the G allele of rs3817928 in *GPR126* [refs. 8,14], which is associated with decreased height but with increased FEV_1/FVC. A further 3 of the 180 loci found to be associated with height[14] showed association (for the 180 loci, we used a Bonferroni-corrected threshold of $P = 2.8 \times 10^{-4}$) with either FEV_1 (*CLIC4* and *BMP6*) or FEV_1/FVC (*PIP4K2B*) (Supplementary Table 3e). In each case, the allele associated with an increase in height was associated with a decrease in lung function. This is not the case for the association of rs1032296 near *HHIP*, which has shown consistent directions of effects on lung function and height[11,14]. However, the strongest SNP associated with height in the *HHIP* region lies within an intron of *HHIP* but shows no association with FEV_1 or FEV_1/FVC. Furthermore, although height is an important predictor of FEV_1, this is not true for its ratio to FVC[37]. These observations argue against the associations with lung function at these loci being simply caused by incomplete adjustment for height.

We stratified by ever- and never-smoker status in our analyses, and in our investigation of amount smoked in the Ox-GSK consortium[25], none of the sentinel SNPs in the 16 new regions showed association with the number of cigarettes smoked per day. Additionally, none of these regions was associated with ever smoking in the Ox-GSK consortium data (Supplementary Table 3b). Thus, the SNP associations with lung function we observed are unlikely to have arisen simply as a consequence of inadequate adjustment for smoking.

We did not observe any interactions with ever smoking for any of the sentinel SNPs in the 16 new regions that exceeded a Bonferroni-corrected significance level (for 16 SNPs). Thus, the effects on lung function of the newly associated variants we identified are apparent in both ever smokers and in never smokers, and the effects of smoking and of these genetic variants may be independent and additive.

In other common complex diseases, follow-up studies that incorporate common genetic risk variants into models to predict disease have not been shown to add substantially

to existing risk models, particularly when such models already include family history[38,39]. The same may also prove to be true for the 26 genetic variants described in this paper, as the effect size of any individual variant is small, but further work is required in this area. The major utility of our findings will be in the knowledge they provide about previously unknown pathways underlying lung function. Elucidating the mechanisms that these genes are involved in will lead to improved understanding of the regulation of lung function and potentially to new therapeutic targets for COPD.

METHODS

Study design

The study consisted of two stages. Stage 1 was a meta-analysis conducted on directly genotyped and imputed SNPs from individuals of European ancestry in 23 studies with a total of 48,201 individuals. Supplementary Table 1a gives the details of these studies. Thirty-four SNPs selected according to the results in stage 1 were followed up in stage 2. The ten leading SNPs were followed up in up to 46,411 individuals of European ancestry, and the remaining 24 SNPs were followed up in a subset of up to 21,674 individuals (Fig. 1).

Stage 1 samples

A total of 23 studies, 17 from the SpiroMeta consortium and 6 from the CHARGE consortium, formed stage 1: AGES, ARIC, B58C T1DGC, B58C WTCCC, BHS1, CHS, ECRHS, EPIC (obese cases and population-based studies), the EUROSPAN studies (CROATIA-Korcula, ORCADES and CROATIA-Vis), FHS, FTC (incorporating the FinnTwin16 and Finnish Twin Study on Aging), Health 2000, Health ABC, KORA F4, KORA S3, NFBC1966, RS-I, RS-II, SHIP and TwinsUK-I (see Supplementary Table 1 for the definitions of all abbreviations). Measurements of spirometry for each study are described in the Supplementary Note. The genotyping platforms and quality-control criteria implemented by each study are described in Supplementary Table 1b.

Imputation

Imputation of non-genotyped SNPs was undertaken with MACH[47], IMPUTE[48] or BIMBAM[49] with pre-imputation filters and parameters as shown in Supplementary Table 1b. SNPs were excluded if the imputation information, assessed using r2.hat (MACH), .info (IMPUTE) or OEvar (BIMBAM), was <0.3. In total, 2,706,349 SNPs were analyzed.

Transformation of data and genotype-phenotype association analysis

Linear regression of age, age^2, sex, height and ancestry principal components was undertaken on FEV_1 (milliliters) and FEV_1/FVC (percent). The residuals were transformed to ranks and then transformed to normally distributed z-scores. These transformed residuals were then used as the phenotype for association testing under an additive genetic model, separately for ever smokers and never smokers. The software used is specified in Supplementary Table 1b. Appropriate tests for association in related individuals were applied where necessary, as described in the Supplementary Note.

Meta-analysis of stage 1 data

All stage 1 study effect estimates, both for ever smokers and never smokers, were corrected using genomic control[50] and were oriented to the forward strand of the NCBI build 36 reference sequence of the human genome, consistently using the alphabetically higher allele as the coded allele. Study-specific λ estimates are shown in Supplementary Table 1. For each study, effect estimates and standard errors for ever smokers and never smokers were meta-analyzed using inverse-variance weighting. Genomic control was applied again to the pooled effect-size estimates for each study. Finally, effect-size estimates and standard errors were combined across studies using an inverse-variance–weighting meta-analysis, and genomic control was applied to the pooled effect-size estimates. To describe the effect of imperfect imputation on power, for each SNP we report the effective sample size (N effective), which is the sum of the study-specific products of the sample size and the imputation quality metric. Meta-analysis statistics and figures were produced using R version 2.9.2 (see URLs).

Selection of SNPs for stage 2

All regions selected for follow up in stage 2 contained a lead SNP with new evidence of association (all with $P < 3 \times 10^{-6}$) with FEV_1 and/or FEV_1/FVC, an N effective ≥70% of the total stage 1 sample size and association signals from the surrounding SNPs that were consistent with their correlation (LD) with the leading SNP. Twenty-nine independent regions with a leading SNP meeting these criteria were assessed in stage 2. Regions were defined as independent if the leading SNP from one region was >500 kb from the leading SNP of any other region. Long-range LD was also investigated between leading SNPs of regions in or near the MHC on chromosome 6 (Supplementary Note). For two regions, the leading SNP had an N effective ≥70% but <80% of the stage 1 sample size and, therefore, a proxy SNP from each region ($r^2 = 1$ and $r^2 = 0.97$) was also taken forward. For three regions, there were different leading SNPs for FEV_1 and FEV_1/FVC, and so both leading SNPs were assessed. A total of 34 SNPs were analyzed in stage 2 and are listed in Supplementary Table 5b. Previously reported regions[8–11,51,52] were not followed up. We

present in Supplementary Table 5a association test statistics in stage 1 only for relevant SNPs from previously reported regions.

Stage 2 samples

The 34 SNPs were followed up in up to 11,275 individuals from seven studies with in silico data: CARDIA, CROATIA-Split, LifeLines, LBC1936, MESA-Lung, RS-III and TwinsUK-II (Supplementary Table 1). rs2647044 was not available from TwinsUK-II.

The 34 SNPs were ranked by P value (for association with either FEV_1 or FEV_1/FVC), and the top ten leading SNPs were selected for follow up by genotyping in up to 35,136 individuals from ADONIX, BHS2, BRHS, BWHHS, Gedling, GS: SFHS, HCS, Nottingham Smokers, NSHD and SAPALDIA (Supplementary Table 1). If a SNP in the top ten had an N effective <80%, only the proxy SNP was included in the top ten for follow up. For regions that showed association with both FEV_1 and FEV_1/FVC, only the leading SNP with the lowest P value for either trait was included if it was within the top ten SNPs. The study design is illustrated in Figure 1.

Meta-analysis of stage 2 data

All stage 2 studies provided effect estimates for ever smokers and never smokers, apart from Nottingham Smokers, as that study only included smokers. Studies with family data (BHS2 and GS: SFHS) analyzed ever smokers and never smokers together to account for the family correlation, adding the smoking status as a covariate in the model, and therefore provided smoking-adjusted effect estimates. All stage 2 study effect estimates were oriented to the forward strand of the NCBI build 36 reference sequence of the human genome, consistently using the alphabetically higher allele as the coded allele. For each study with separate results for ever smokers and never smokers, effect estimates and standard errors for ever smokers and never smokers were meta-analyzed using inverse-variance weighting. Genomic control was applied to the pooled effect sizes of those studies with in silico data that undertook the analysis genome wide. Effect estimates and standard errors were combined across the stage 2 studies using an inverse-variance–weighting meta-analysis.

Combined analysis of stage 1 and stage 2 samples

A meta-analysis of stage 1 and 2 results was undertaken using inverse-variance weighting. We described associations as genome-wide significant if they had $P < 5 \times 10^{-8}$.

PCR expression profiling

The mRNA expression profiles of *TGFB2*, *MFAP2*, *HDAC4*, *EVI1*, *RARB*, *SPATA9*, *ARMC2*, *NCR3*, *CDC123*, *LRP1*, *CCDC38*, SNRPF, *MMP15*, *CFDP1*, *ZKSCAN3*, *KCNE2* and *C10orf11*

were determined in human lung tissue and primary cell samples using RT-PCR, including RNA from lung (Ambion/ABI), brain, airway smooth muscle cells and human bronchial epithelial cells (Clonetics42). Primer sequences are listed in Supplementary Table 2. Full details are provided in the Supplementary Note.

Lung function associations in our data of SNPs previously associated with lung function

In order to permit comparison of findings with recent studies of relevance to the field, we present association test statistics (in stage 1 only) for relevant SNPs from previously reported regions (Supplementary Table 5a). We included the regions (i) reported as showing genome-wide significant association ($P < 5 \times 10^{-8}$) with lung function, (ii) reported as showing genome-wide significant association with COPD, providing that there was additional evidence of association with lung function and (iii) *DAAM2*, which reached borderline significance in the SpiroMeta consortium[9]. Within each of these regions, if multiple SNPs had been reported, we included all relevant SNPs and also the SNP that showed the strongest association in our data.

Association to other traits of lung-function–associated SNPs

Regions associated ($P < 5 \times 10^{-8}$) with lung function or COPD (and also associated with lung function) were looked up for other traits. Where multiple SNPs were reported for different traits or by different investigators, we aimed to include all relevant SNPs, except those having $r^2 > 0.9$ with another SNP in the region. We also included the SNPs that showed the strongest association in our data for each region. The following related traits were assessed: (i) lung function in children (Supplementary Table 3a); (ii) smoking amount and ever smoking versus never smoking in the Ox-GSK consortium25 dataset (Supplementary Table 3b); (iii) height in the GIANT consortium14 dataset (Supplementary Table 3c,e); and (iv) lung cancer in the International Lung Cancer Consortium (ILCCO) GWAS meta-analysis26 (Supplementary Table 3d).

Estimation of the number of undiscovered variants and calculation of the proportion of variance explained

We used the approach previously proposed[34] to estimate the number of independent variants associated with lung function measures that have similar effect sizes to the variants already reported and to calculate the proportion of the variance explained by them. We excluded discovery data when estimating effect sizes to avoid winner's curse bias and obtained the number of undiscovered variants using the discovery power to detect the unbiased effect sizes (Supplementary Table 6 and Supplementary Note).

Additional analyses

The top SNPs from our new loci and their proxies were searched for correlation with known common copy number variants and expression SNPs. Analyses to identify common pathways underlying the association signals for lung function were undertaken using MAGENTA v2 [53] and GRAIL[54].

Full methods and results are given in the Supplementary Note.

http://www.nature.com/ng/journal/v43/n11/extref/ng.941-S1.pdf

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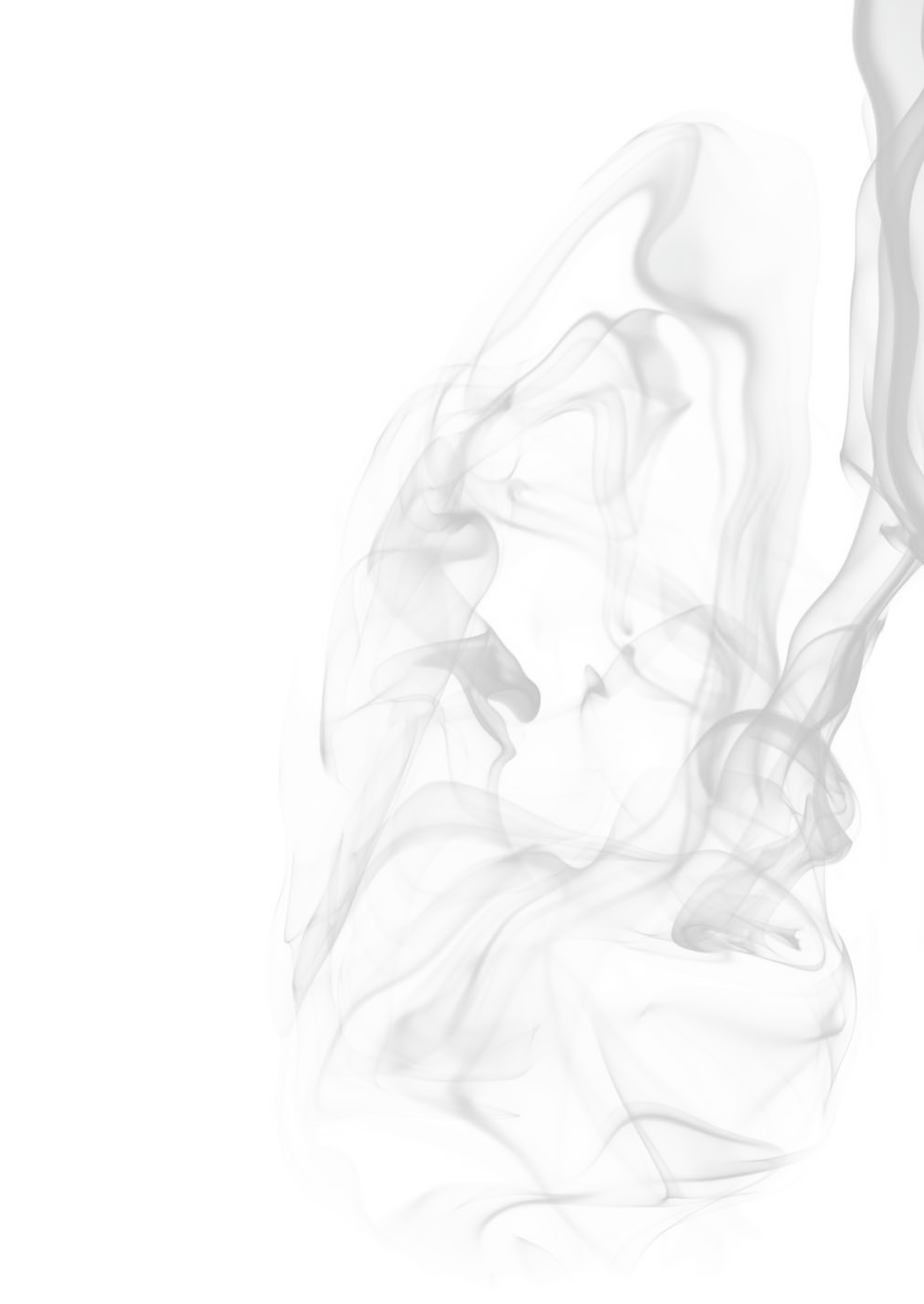

CHAPTER 3.2

Genetics of forced vital capacity: genome-wide association study meta-analysis and follow-up identifies six new loci

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ABSTRACT

Forced vital capacity (FVC), a spirometric measure of pulmonary function, reflects lung volume and is used in the diagnosis and monitoring of various lung diseases. We performed a genome-wide association study meta-analysis of FVC in 52,253 individuals from 26 studies and followed up the top associations in 24,840 additional individuals all of European ancestry. We found six novel regions associated at genome wide significance ($P < 5 \times 10^{-8}$) with FVC. Besides lung tissue gene expression and expression Quantitative Trait Locus (eQTL)-analysis in whole blood, we evaluated these loci in African Americans. The new loci may inform molecular mechanisms involved in lung development, lung growth or pathogenesis of restrictive lung disease.

INTRODUCTION

Pulmonary function is a heritable trait that can be reliably measured by spirometry and reflects the physiological state of the lungs and airways [1]. Forced vital capacity (FVC), one of the most widely used pulmonary function measures, approximates vital capacity. In conjunction with FEV_1, FVC is used to diagnose various respiratory diseases. A reduced ratio of FEV_1 to FVC (FEV_1/FVC) indicates airflow obstruction when FEV_1 is reduced disproportionately relative to FVC. In contrast, a decreased FVC in the face of a normal to elevated FEV_1/FVC suggests a restrictive ventilatory defect. In clinical practice, FVC is often used as a surrogate measure of disease progression in patients with established restrictive lung disorders, such as idiopathic pulmonary fibrosis [2,3]. Reduced FVC is a strong predictor of mortality in the general population, independently of the forced expiratory volume in 1 second (FEV_1) and standard risk factors such as age and cigarette smoking[4-8].

Pulmonary function measures show familial aggregation, with evidence for genetic effects in twin and family studies[9,10]. We previously reported associations between FEV_1 or FEV_1/FVC and at least 26 genetic loci using large-scale meta-analyses of genome-wide association studies (GWAS) [11-14]. To date, the genetic determinants of FVC have not been studied using GWAS methods. We conducted a comprehensive GWAS meta-analysis across two large consortia of participants of European ancestry—the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) and SpiroMeta—to identify common genetic variants associated with cross-sectional measures of FVC in 52,253 subjects of European descent. We confirmed expression levels of genes at six loci nearest the novel variants in lung tissue, performed expression Quantitative Trait locus (eQTL)-analyses in 762 whole blood samples and evaluated the novel loci in 5,497 African Americans in the National Heart, Lung and Blood Institute (NHLBI)-sponsored Candidate gene Association Resource (CARe) Project.

METHODS

Study design

The study consisted of two stages. Stage 1 was a meta-analysis of study-specific genome wide analyses of FVC conducted in 26 studies with a total of 52,253 individuals of European ancestry. The study characteristics are shown in table 1. In stage 2 we followed-up SNPs showing association with FVC ($P < 5 \times 10^{-7}$) and meta-analysed betas and standard errors across stages 1 and 2. Stage 2 encompassed 24,840 subjects of European ancestry from 7 independent cohorts.

Table 1. Baseline characteristics of the studies included in stage 1.

	N total	N male	N female	Age range (year) at FVC measurement	Mean age, y (s.d.)	Height, cm (s.d.)	Mean FEV_1, L (s.d.)	Mean FVC, L (s.d.)	Mean FEV_1/ FVC (s.d.)	N never-smokers	N ever-smokers	Genomic inflation factor (λ) FVC	
Stage 1: GWAS												**Ever-Smokers**	**Never-smokers**
AGES	1,620	618	1,002	66-95	76.2 (5.6)	166 (9)	2.10 (0.68)	2.83 (0.84)	0.74 (0.11)	737	883	1.01	1.02
ARIC	9,078	4,279	4,799	44-66	54.3 (5.7)	169 (9)	2.94 (0.78)	3.99 (0.98)	0.74 0.08)	3,620	5,458	1.03	1.01
B58C-T1DGC	2,343	1,131	1,212	44–45	44.5 (0)	169 (9)	3.31 (0.78)	4.19 (0.96)	0.79 (0.08)	692	1,651	1.00	1.01
B58C-WTCCC	1,372	691	681	44–45	44.5 (0)	169 (9)	2.93 (0.75)	4.18 (0.96)	0.79 (0.08)	394	978	1.01	1.00
CARDIA	1,626	768	858	17-32	25.6 (3.3)	171 (9)	3.68 (0.81)	4.70 (1.00)	0.82 (0.06)	932	694	0.97	0.99
CHS	3,140	1,226	1,914	65-95	72.3 (5.4)	160 (9)	2.12 (0.66)	3.00 (0.87)	0.71 (0.11)	1,543	1,597	1.03	1.02
CROATIA-Korcula	825	300	525	18–90	55.5 (13.5)	168 (9)	2.84 (0.81)	3.37 (0.93)	0.84 (0.09)	397	428	1.03	1.06
CROATIA-Vis	769	323	446	18–88	56.3 (15.3)	168 (10)	3.39 (1.22)	4.38 (1.43)	0.77 (0.09)	328	441	1.04	1.02
ECRHS	1,594	784	810	19-48	33.9 (7.2)	171 (9)	3.78 (0.82)	4.59 (1.03)	0.83 (0.07)	699	895	1.01	1.01
EPIC obese cases	1,104	476	628	39–76	59.1 (8.8)	166 (9)	2.35 (0.69)	2.84 (0.87)	0.82 (0.17)	489	615	1.00	1.02

EPIC population based	2,336	1,100	1,236	39–77	59.2 (9.0)	167 (9)	2.50 (0.72)	3.04 (0.90)	0.85 (0.16)	1,061	1,275	1.01	1.01
FHS	7,692	3,544	4,148	19-92	51.9 (14.6)	169 (10)	3.04 (0.94)	4.03 (1.14)	0.75 (0.08)	3,554	4,138	1.04	1.04
FTC	134	13	121	23–76	57.4 (19.3)	158 (6)	2.69 (0.94)	2.93 (0.61)	0.79 (0.09)	104	30	1.04	1.01
Health 2000	821	394	427	30-75	50.5 (10.9)	169 (9)	3.29 (0.90)	4.16 (1.07)	0.79 (0.07)	249	572	0.99	1.02
Health ABC	1,475	786	689	70-79	73.7 (2.8)	167 (9)	2.30 (0.70)	3.11 (0.81)	0.74 (0.08)	643	832	1.00	1.00
HCS	1,820	915	905	55-86	66.0 (7.4)	166 (9)	2.44 (0.69)	2,97 (0,83)	0.83 (0.08)	1,012	808	0.99	1.00
KORA S3	555	261	294	29–73	47.6 (9.0)	170 (9)	3.43 (0.78)	4.18 (0.99)	0.83 (0.07)	266	289	1.00	1.01
MESA	1,433	728	705	48-90	66.1 (9.7)	169 (10)	2.57 (0.76)	3.52 (0.99)	0.73 (0.09)	615	818	1.03	1.05
NFBC1966	4,556	2,182	2,374	31–31	31.0 (0)	171 (9)	3.96 (0.79)	4.73 (0.99)	0.84 (0.06)	1,648	2,908	1.02	1.01
NSPHS	549	255	294	18-91	50.0 (19.1)	164 (10)	3.02 (0.95)	3.68 (1.12)	0.82 (0.09)	464	85	1.01	1.00
ORCADES	692	322	370	19–93	54.9 (15.3)	167 (9)	2.88 (0.84)	3.58 (0.98)	0.80 (0.09)	404	288	1.02	1.07
RS-I	1,152	502	650	72-96	79.3 (4.6)	166 (9)	2.19 (0.65)	2.90 (0.83)	0.75 (0.08)	362	790	0.99	1.00
RS-II	862	386	476	58-88	67.2 (6.3)	168 (9)	2.71 (0.78)	3.61 (1.08)	0.76 (0.09)	290	572	1.01	0.99
RS-III	1,043	464	579	46-88	56.5 (5.4)	171 (9)	3.19 (0.81)	4.06 (1.04)	0.79 (0.07)	354	689	1.00	1.00

Table 1. (Continued)

	N total	N male	N female	Age range (year) at FVC measurement	Mean age, y (s.d.)	Height, cm (s.d.)	Mean FEV_1, L (s.d.)	Mean FVC, L (s.d.)	Mean FEV_1/ FVC (s.d.)	N never-smokers	N ever-smokers	Genomic inflation factor (λ) FVC	
Stage 1: GWAS												**Ever-Smokers**	**Never-smokers**
SHIP	1,777	870	907	25–85	52.3 (13.7)	170 (9)	3.28 (0.89)	3.87 (1.03)	0.87 (0.06)	773	1,004	1.00	1.00
Twins UK-I	1,885	0	1,885	18–79	48.4 (12.2)	162 (6)	2.73 (0.56)	3.40 (0.61)	0.80 (0.08)	943	942	1.00	1.00

Sample population characteristics for each study. Characteristics are shown for studies analyzed in Stage 1 (GWAS meta-analysis) Stage 1 studies: AGES, Age, Gene/Environment Susceptibility; ARIC, Atherosclerosis Risk in Communities; B58C-T1DGC, British 1958 Birth Cohort–Type 1 Diabetes Genetics Consortium; B58C-WTCCC, British 1958 Birth Cohort–Wellcome Trust Case Control Consortium; CARDIA: Coronary Artery Risk Development in Young Adults; CHS, Cardiovascular Health Study; the CROATIA- Korcula study; the CROATIA-Vis study; ECRHS, the European Community Respiratory Health Survey; EPIC obese cases, European Prospective Investigation into Cancer and Nutrition, Obese Cases; EPIC population based, European Prospective Investigation into Cancer and Nutrition Cohort; FHS, Framingham Heart Study; FTC, Finnish Twin Cohort incorporating FinnTwin16 and FITSA; H2000, Finnish Health 2000 survey; Health ABC, Health, Aging, and Body Composition; HCS, Hunter Community Study; KORA S3, Cooperative Health Research in the Region of Augsburg; MESA, Multi-Ethnic Study of Atherosclerosis; NFBC1966, Northern Finland Birth Cohort of 1966; NSPHS: The Northern Swedish Population Health Study; ORCADES, Orkney Complex Disease Study; RS-I, RS-II, RS-III, Rotterdam Study; SHIP, Study of Health in Pomerania; the TwinsUK-I study. Definition of abbreviations: N, number; cm, centimeter; s.d.; standard deviation; L, liters; FEV_1, Forced Expiratory Volume in 1 second; FVC, Forced Vital Capacity.

Cohorts included in stage 1

Stage 1 included a total of 26 studies, 15 from the SpiroMeta consortium and 11 from the CHARGE consortium: AGES, ARIC, B58C T1DGC, B58C WTCCC, CARDIA, CHS, ECRHS, EPIC (obese cases and population-based studies), the EUROSPAN studies (CROATIA-Korcula, ORCADES and CROATIA-Vis), FHS, FTC (incorporating the FinnTwin16 and Finnish Twin Study on Aging), Health 2000, Health ABC, HCS, KORA S3, MESA, NFBC 1966, NSPHS, RS-I, RS-II, RS-III, SHIP and Twins UK-I.

Cohorts included in stage 2

A total of 8 studies were included in our stage 2 follow-up: BHS 1 & 2, CROATIA-Split, KORA F4, LBC1936, LifeLines, LLFS, Pivus, Twins UK-II & III. Study descriptions can be found in the Supplementary Table 1.

Imputation

Imputation to the HapMap CEU panel was conducted using either MACH [15], IMPUTE [16], Beagle [17,18] or BIMBAM [19] with filters and quality control parameters as shown in Supplementary Table 1. SNPs were excluded on a cohort basis if the imputation score, assessed using r2.hat (MACH), .info (IMPUTE) or OEvar (BIMBAM), was <0.3. In total, 2,706,349 SNPs were analyzed.

Statistical analysis

Individual studies performed a GWAS analysis using linear regression models with FVC (in millilitres) as the outcome, stratified by never/ever smoking (Supplementary Figure 1). Adjustment factors included age, age^2, sex, height, height ^{2} and weight. If applicable, cohorts adjusted for centre, cohort or principal components to adjust for population stratification. The follow-up studies used the same models. Effect estimates for each study were corrected using genomic control [20] separately within smoking strata. Study-specific lambda estimates are shown in Table 1.

Meta-analysis of stage 1 data

Variants with imputation quality below 0.3 or minor allele frequency below 0.03 were excluded from each dataset before the meta-analysis. For each study, effect estimates and standard errors for ever-smokers and never-smokers were meta-analysed with METAL using fixed effects inverse variance. Genomic control was applied again to the resulting combined (ever-smokers and never-smokers) effect estimates for each study. The effect estimates across smoking strata were then combined across studies, again using fixed-effect inverse variance weighting meta-analysis, and genomic control was applied to the combined (across studies) effect estimates. Manhattan plots, quantile-quantile-plots, forest-plots, gene annotation and additional statistics were produced using R version 2.9.2 [21]. Regions were defined as independent if the leading SNP from one region was > 500 kilobases (kb) from the leading SNP of any other region.

Combined analysis of stage 1 and stage 2

We performed an inverse-variance–weighting fixed effects meta-analysis across stages 1 and 2 and obtained two-sided P values for the resulting effect estimates (fig 1)

Lookup replication in the CARe consortium

To evaluate these loci across ethnicities, we performed a lookup replication of FVC in the National Heart, Lung, and Blood Institute (NHLBI)-sponsored Candidate gene Association Resource (CARe) Project [22,23], which genotyped African-Americans in ARIC, MESA, CHS, CARDIA, the Jackson Heart Study and the Cleveland Family Studies. We assessed the sentinel SNPs from the HapMap CEU reference panel that were available on the Affymetrix 6.0 platform used in CARe. For SNPs not genotyped we looked-up a proxy SNP based on $r^2 > 0.5$. We also evaluated associations in the regions of the identified loci based on location of the sentinel SNPs ± 250 kb and $r^2 \geq 0.30$. The regional analysis included 1,478 SNPs. The P value threshold for this regional analysis was determined by the number of independent SNPs based on the covariance matrix generated using "matSpDlite" [24-26]. We estimated the number of independent tests at 886 leading to a Bonferroni P value threshold of 5.79×10^{-5}.

Gene expression analysis

Following the meta-analysis, we investigated mRNA expression of the nearest gene for each of the new SNPs in: human lung tissue, human airway smooth muscle cells (HASM), human bronchial epithelial cells (HEBC) and peripheral mononuclear blood cells (PMBC). Lung resection specimens were obtained from patients diagnosed with solitary pulmonary tumors at Ghent University Hospital (Ghent, Belgium). Primary human bronchial epithelial cells (HBEC) and human airway smooth muscle cells (HASM) were prepared from lung resection specimens (n = 64) obtained from anonymous donors during

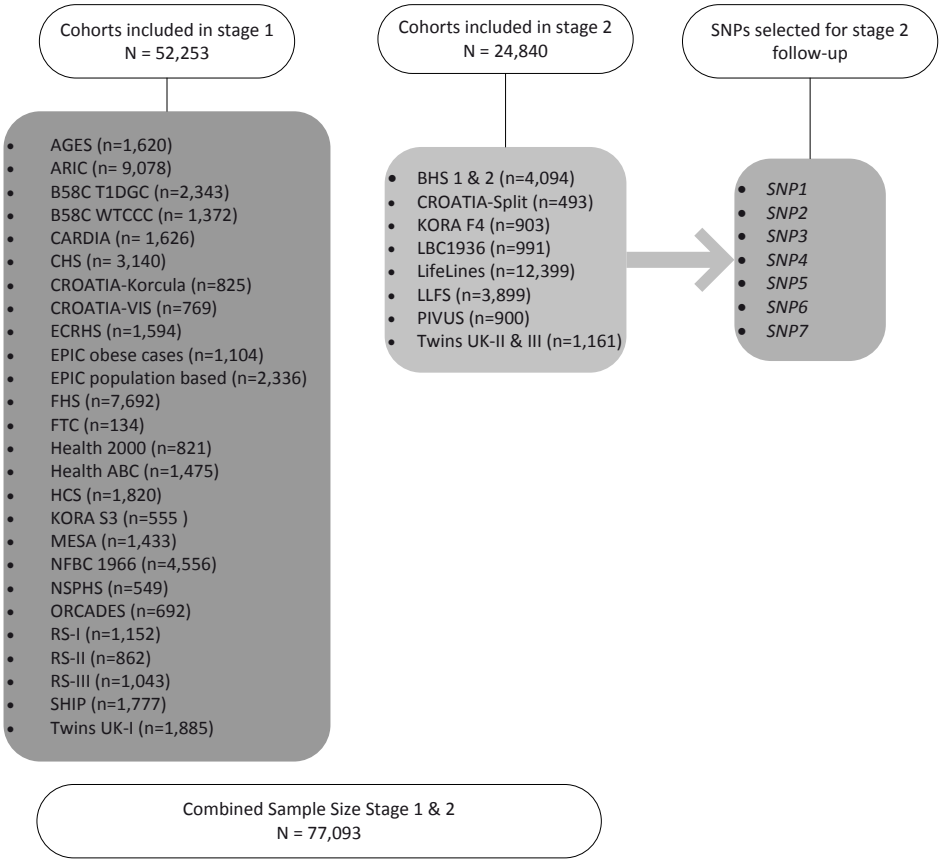


Figure 1. Study design
Overview of our staged analysis to identify new variants influencing FVC. After a large-scale metaanalyzed GWAS of stage 1 cohorts (n = 52,253), we followed a total of 7 SNPs showing evidence of association with FVC ($P < 5 \times 10^{-7}$) in Stage 2. The studies included in stage 2, encompassing a total sample size of n = 24,840, undertook in silico testing of the 7 loci, which were not previously associated to any pulmonary phenotype. See Table 1 for definitions of all study abbreviations.

surgery for lung cancer at the Leiden University Medical Center (LUMC, Leiden, The Netherlands). All assays were done at the Ghent University Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll gradients. Written informed consent was obtained from all subjects according to protocols approved by the local ethics committees. Total RNA was extracted from samples using the miRNeasy Mini kit (Qiagen) and cDNA was prepared from 1µg RNA template using the Transcriptor Universal cDNA Master kit (Roche,) following manufacturer's instructions. Expression of the candidate genes and the housekeeping gene *GADPH* was analyzed using TaqMan Gene Expression Assays (Applied Biosystems, Forster City, CA, USA).

We assessed whether top SNPs or their proxies, based on an $r^2 > 0.7$, in these new regions were associated with gene expression in whole blood cells in a sample of 762 individuals from the Rotterdam Study III (RS-III) [27]. Expression was assessed using Illumina Whole-Genome Expression Beadchips (HumanHT-12 v4). For eQTL-analysis, we used the eQTL mapping pipeline called MegaQTL [28]. eQTLs were deemed cis when the distance between the SNP chromosomal position and the probe midpoint was less than 250 kb. eQTLs were mapped using Spearman's rank correlation, using the imputation dosage values as genotypes. Resultant correlations were then converted to P values and their respective z-scores weighted with the square root of the sample size. The model was adjusted for the first 40 eigenvectors of the principal component analysis. We corrected for multiple testing by using Bonferroni correction: associations with $P < 0.0001$ were considered statistically significant.

Finally, we also queried publicly available eQTL databases derived from multiple cell and tissue types (lymphoblastioid cell lines, brain tissue, and human fibroblasts) [29-31].

RESULTS

There were 9 regions containing at least one SNP associated with FVC at $P < 5x10^{-7}$. Six loci reached genome-wide significance ($P < 5 \times 10^{-8}$) for FVC in the meta-analysis of stages 1 and 2 (table 2, fig. 2).

We evaluated the effect of the six novel loci was assessed separately in ever-smokers and in never-smokers and the effect sizes were consistent across smoking strata for all the variants.

Lookup replication in African Americans

The baseline characteristics of the African-Americans in CARe are shown in Supplementary Table 1. For SNPs which were not represented on the platform, we looked up the best proxy for that SNP.

Only one of the six loci associated with FVC in subjects of Caucasian descent was directly genotyped using the Affymetrix 6.0 array used in CARe (SNP3). The P value for association of this SNP with FVC was nominally significant at $P = 0.03838$. The effect estimate was similar to that in Caucasians. For three SNPs we analyzed a proxy (SNP1, SNP4, and SNP5). None of these showed a statistically significant association with FVC in African Americans.

Table 2. Main results from stage 1, stage 2 and the meta-analysis of stage 1 and 2 for loci associated with FVC.

	Stage 1				Stage 2				Meta-Analysis			
SNP	**Allele**	**β (mL)**	**S.E.$_{gc}$**	**P-value**	**Allele freq.**	**β (mL)**	**S.E.**	**P-value**	**Allele freq.**	**β (mL)**	**S.E.**	**P-value**
SNP1	T	-23.7498	4.022	3.52E-09	0.37	-18.6481	5.115	**0.000267**	0.361	-21.8007	3.162	**5.365e-12**
SNP2	G	-19.9428	3.919	3.60E-07	0.46	-0.5766	5.132	0.9105	0.470	-12.8103	3.115	4.904e-05
SNP3	T	28.8279	5.208	3.11E-08	0.84	18.3474	9.618	0.05644	0.846	26.4515	4.580	**7.668e-09**
SNP4	T	-21.3664	3.957	6.66E-08	0.31	-12.3087	5.359	0.02163	0.305	-18.1708	3.183	**1.140e-08**
SNP5	C	25.3431	5.014	4.33E-07	0.16	24,9316	7.295	**0.000631**	0.160	25,2111	4.122	**1,054e-09**
SNP6	G	20.5386	3.733	3.76E-08	0.42	12.5946	4.984	0.01149	0.419	17.6833	2.988	**3.246e-09**
SNP7	T	26.7293	4.751	1.84E-08	0.80	11.1463	8.527	0.1911	0.789	23.0165	4.150	**2.840e-08**

Shown are FVC results for the leading SNPs, ordered by chromosome and position for each independent locus associated ($P < 5 \times 10^{-8}$) with FVC in a joint analysis of up to 77,093 individuals of European ancestry from the CHARGE-SpiroMeta GWAS (stage 1) and follow up (stage 2). Two-sided P values are given for stage 1, stage 2 and the joint meta-analysis of all stages. P values reaching genome-wide significance ($P < 5 \times 10^{-8}$) in the joint meta-analysis of all stages are indicated in bold. SNPs reaching independent replication in stage 2 ($P = 0.05/7 = 7.14 \times 10^{-3}$) are indicated with their stage 2 P value in bold. The joint meta-analysis includes data from stage 1 and stage 2. β values reflect effect-size estimates in milliliters (mL).

Since several of our sentinel SNPs were not genotyped in the CARe African Americans, we performed regional analyses (+/- 250 kb, $r^2 \geq 0.30$) around these. One SNP was significantly associated with FVC in African Americans, reaching a P value below the threshold for multiple testing (5.79×10^{-5}). This SNP was associated with a decrease in FVC of 64 ml per C allele ($P = 8.92 \times 10^{-6}$) in African Americans which is larger than the mean effect of the SNPs from the stage 1 and 2 meta-analysis.

Gene expression results

The mRNA expression profiles of genes from the six loci that were significant in the meta-analysis of stages 1 and 2, and the housekeeping gene GAPDH, were determined in human lung tissue and the primary cell samples (HBEC, HASM and PMBC) using RT-PCR. We detected transcripts for all six genes in lung tissue, HBEC and HASM. Transcripts for 5 of the 6 genes were present in PBMCs.

Expression Quantitative Trait Loci (eQTLs)

We investigated whether the top SNPs or their proxies ($r^2 \geq 0.7$) in the six new FVC loci were associated with gene expression using eQTL data as described above. Multiple

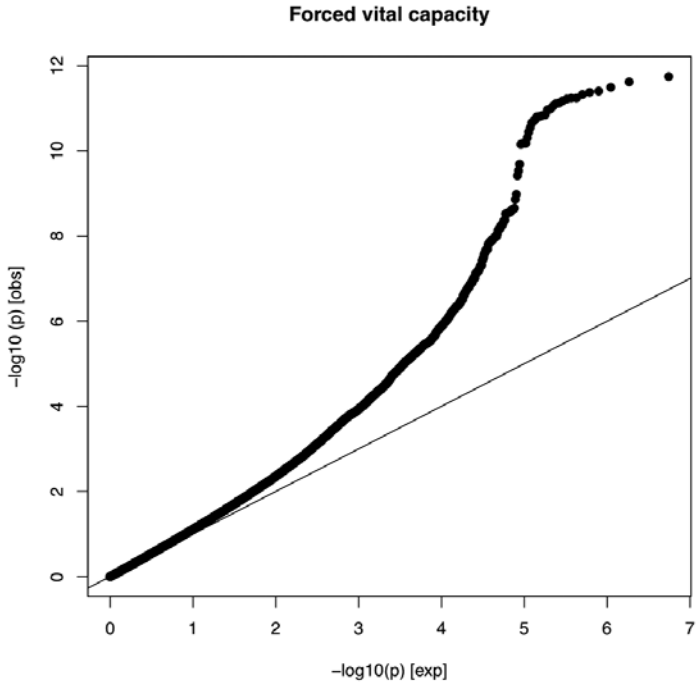


Figure 2. Association test statistic for FVC
Quantile-Quantile (QQ) plots show – log10(P) of observed genome-wide association results against expected association results for FVC. λgc before applying genomic control was 1.12 for FVC. The QQ-plot for all SNPs is shown in black. Previously unidentified SNPs in the GWAS of correlated pulmonary traits reach genome-wide significance.

SNPs in or near one gene showed significant cis-eQTL associations in peripheral blood, with the strongest association represented by SNP4_proxy (a proxy of SNP 4, $r^2 = 0.70$) at a P value of 8.4×10^{-81}. The sentinel SNP associated with FVC in this region (SNP 4) also exhibited a strong cis-effect on the gene ($P = 1.8 \times 10^{-35}$). We did not find statistically significant cis-eQTLs for the other FVC-associated variants and loci.

Putative regulatory variants

We queried the RegulomeDB (http://regulome.stanford.edu/) [32] database to assess whether any of the newly identified FVC-associated SNPs ($P < 1 \times 10^{-7}$, n = 150 SNPs) were located within known or predicted regulatory elements, including regions of DNAase hypersensitivity, binding sites of transcription factors, and promoter regions that have been biochemically characterized to regulate transcription. Five SNPs received high likelihood scores (based on the amount of supporting data) for mapping to regulatory regions and affecting gene expression

DISCUSSION

In a two- stage meta-analysis across 33 cohorts encompassing 77,093 individuals of European ancestry we found 6 novel loci associated with FVC. None of these genetic loci were identified in previous GWAS of spirometric measures of airflow obstruction (FEV1 or FEV1/FVC). The six new loci show consistent associations across studies and stages. The meta-analysis effect estimates range from 13 to 26 mL per allele, which is equivalent to the annual rate of decline of FVC ranges from 12 to 47 mL in the general population [33]. Expression analyses showed that all loci are expressed in lung tissue and primary lung cells (HBEC and HASM).

The six novel associations found in this analysis explain only a modest proportion of the additive polygenic variance of FVC (0.62%). Stage 2 effect size estimates were used to calculate the proportion of the variance explained by the six novel loci, to avoid the effect of winner's curse bias. When we take the other known loci for pulmonary function into account, the proportion of the additive polygenic variance explained is 1.66%, a finding that is comparable to many other complex traits [34]. The unexplained heritability has become a well-known phenomenon in genetic epidemiology [35] and possible explanations include multiple effects of common variants, rare variants, gene-by-environment interactions, gene-gene interactions and epigenetic regulation which are not captured by existing GWAS platforms.

We previously identified 3 regions associated at genome wide significance with FEV_1, which is a measure of airflow obstruction that is dependent on lung size, and 12 regions for the ratio of FEV_1 to FVC (FEV_1/FVC), which is a measure of airflow obstruction independent of lung size. Although FEV_1 and FVC are statistically correlated ($r = 0.83$ in the Rotterdam Study, adjusted for age, sex, height and height^2), these are clinically different entities. FVC is used for the evaluation of restrictive ventilatory defects and is a predictor of mortality independent of the FEV_1, standard risk factors, and even prior cardiovascular disease [5,6].

To test if our identified loci are associated with FVC across ethnicities, we looked up our top hits reaching genome-wide significance after stage 2 in a smaller dataset of African Americans. Only one of the SNPs (SNP3) was directly genotyped in the African Americans and was nominally significantly associated with FVC with a P value of 0.038. Three other SNPs were not represented on the platform so we used proxies. None of these three SNPs showed a significant association. Despite the smaller size of the African American dataset, one SNP (SNP1) reached the significance threshold of $P < 8.92 \times 10^{-6}$.

Interestingly, this SNP is monomorphic in individuals of European ancestry. These results support the involvement of this locus in lung function in both ethnic groups.

There are some limitations of our analysis. With our cross-sectional measures of FVC we cannot determine whether our signals are due to influence on lung growth or age-related lung function decline [31]. The SpiroMeta cohorts did not include adjustment for pack-years to avoid reduction in sample size from missing covariates. However, within the CHARGE cohorts, estimates from meta-analysis with and without adjustment for pack years were very similar (results not shown)

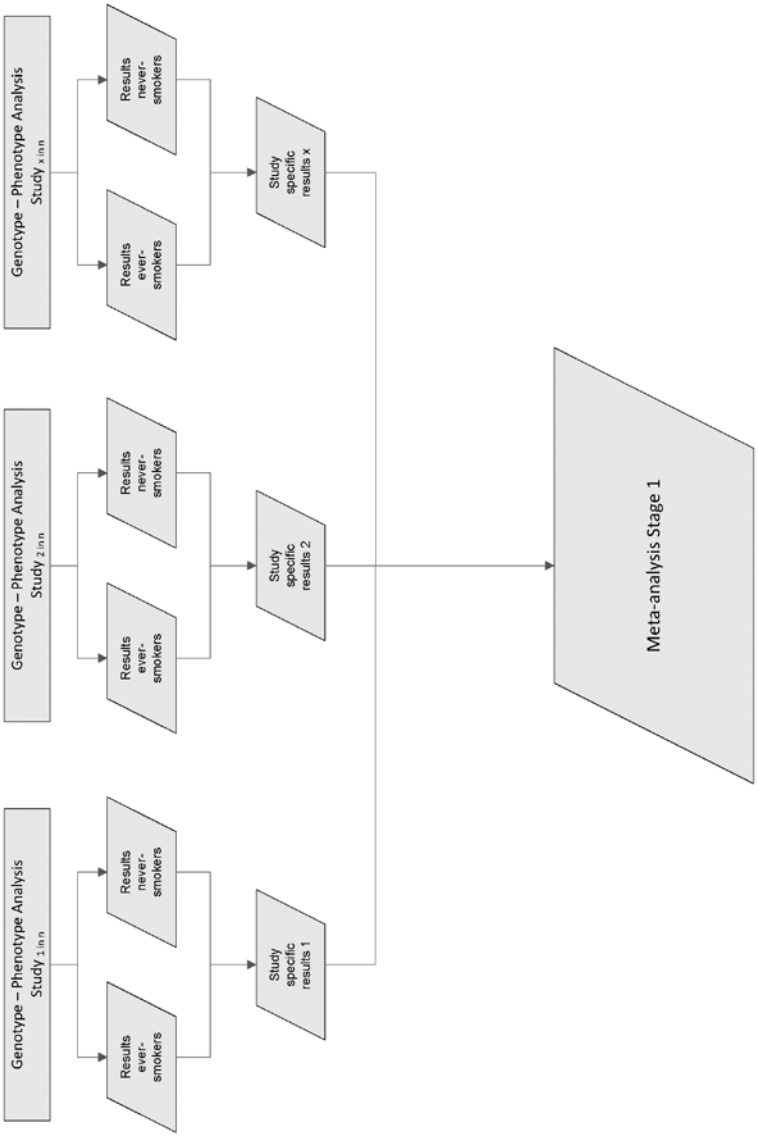
One of the strengths of our study is its considerable sample size. Our application of genomic control at the three stages is likely to be overly conservative because it has recently been shown that in large meta-analyses, test statistics are expected to be elevated under polygenic inheritance even when there is no population structure. Genomic inflation estimates increase with sample size, as has been shown for other traits [14,24,32,33]. Following the two staged meta-analysis, we were able to test the SNPs and their regions for tissue specific expression. Lastly, GWAS in general have been very successful in generating hypotheses for mechanistic follow-up, which can lead to a better understanding of complex diseases.

In conclusion, using a large-scale staged meta-analysis, we report six new loci associated with FVC which are all expressed in lung tissue and primary lung cells. Our findings point to previously unexplored pathways underlying lung function. Improvement of the understanding of the role that these genes play in normal lung development and disease pathogenesis could lead to novel therapeutic targets for lung diseases.

Supplementary Table 1. Baseline characteristics studies included in Stage 2

Participants from European ancestry										
Study	**N, total**	**N, males (%)**	**N, females (%)**	**Age(years) Mean (SD)**	**Height (cm) Mean (SD)**	**FVC (L) Mean (SD)**	**Weight (kg) Mean (SD)**	**N, never smokers (%)**	**N, ever smokers (%)**	**Pack-years in Smokers Mean (SD)**
BHS 1 & 2	4,094	1,795 (43.8)	2,299 (56.2)	52.9 (15.8)	168 (9)	3.84 (1.15)	74.3 (14.2)	2,124 (51.9)	1,970 (48.1)	NA*
CROA-TIA-Split	493	210 (42.6)	283 (57.4)	49.1 (14.6)	173 (9)	3.80 (1.06)	80.6 (16.3)	239 (48.5)	254 (51.5)	21.8 (24.0)
KORA F4	903	425 (47.1)	478 (52.9)	53.8 (4.5)	169 (9)	4.20 (0.97)	79.6 (16.9)	344 (38.1)	559 (61.9)	18.3 (18.6)
LBC1936	991	501 (50.6)	490 (49.4)	69.6 (0.8)	166 (9)	3.04 (0.87)	77.3 (14.5)	437 (44.1)	554 (55.9)	31.1 (27.9)
LifeLines	12,399	5,123 (41.3)	7,276 (58.7)	48.4 (11.2)	174 (9)	4.43 (1.03)	80.2 (14.9)	344 (38.1)	7,329 (59.1)	13.5 (11.5)
LLFS	3,899	1,734 (44.5)	2,165 (55.5)	68.8 (15.2)	166 (10)	3.20 (1.1)	75.3 (16.5)	2,203(56.7)	1,683(43.3)	9.1 (17.7)
Pivus	900	483 (48,7)	417 (51.3)	70.2 (0.2)	169 (9)	3.20 (0.9)	77.3 (14.4)	438 (48,7)	462 (51,3)	11.9 (15.3)
TwinsUK-II & III	1,161	0	1,161 (100)	54.1 (17)	162 (13)	3.21 (0.6)	68.1 (6.3)	761 (65.5)	400 (34.5)	4.9 (0.6)
African American participants										
Study	**N, total**	**N, males (%)**	**N, females (%)**	**Age(years) Mean (SD)**	**Height (cm) Mean (SD)**	**FVC (L) Mean (SD)**	**Weight (kg) Mean (SD)**	**N, never smokers (%)**	**N, ever smokers (%)**	**Pack-years in Smokers Mean (SD)**
CARe	5,497	2,121 (38.6)	3,376 (61.4)	51.3 (11.9)	168 (10)	3.31 (0.86)	85.9 (19.7)	2,501 (45.5)	2,996 (54.5)	8.6 (16.0)

* Not available. Sample population characteristics for each study. Characteristics are shown for studies analyzed in Stage 2 (GWAS meta-analysis): BHS 1 & 2, Busselton Health Study; Croatia-Split; KORA F4, Cooperative Health Research in the Region of Augsburg; LBC10936, Lothian Birth Cohort 1936; LIfeLines; LLFS, Long Life Family Study; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; TwinsUK-II & III; CARe, Candidate gene association resource; Definition of abbreviations: N, number; cm, centimeter; SD.; standard deviation; L, liters; FEV_1, Forced Expiratory Volume in 1 second; FVC, Forced Vital Capacity.



Supplementary Figure 1. Study Design
This figure shows the analysis approach for this study

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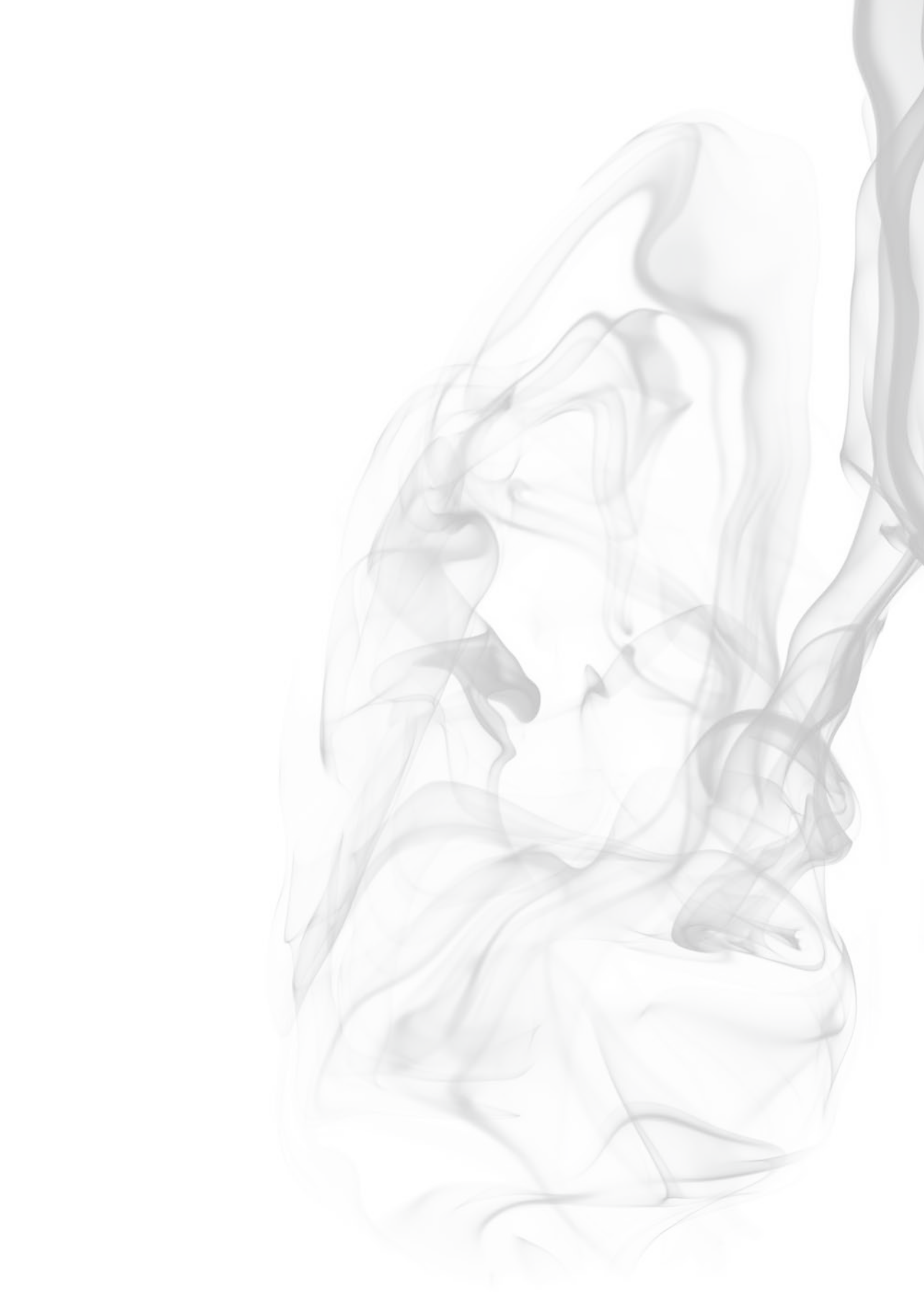

CHAPTER 3.3

Genome-Wide Joint Meta-Analysis of SNP and SNP-by-Smoking Interaction Identifies Novel Loci for Pulmonary Function

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Published in PLoS Genetics. 2012;8(12):e1003098.
doi: 10.1371/journal.pgen.1003098. Epub 2012 Dec 20.
PubMed PMID: 23284291

ABSTRACT

Genome-wide association studies have identified numerous genetic loci for spirometric measures of pulmonary function, forced expiratory volume in one second (FEV_1), and its ratio to forced vital capacity (FEV_1/FVC). Given that cigarette smoking adversely affects pulmonary function, we conducted genome-wide joint meta-analyses (JMA) of single nucleotide polymorphism (SNP) and SNP-by-smoking (ever-smoking or pack-years) associations on FEV_1 and FEV_1/FVC across 19 studies (total N = 50,047). We identified three novel loci not previously associated with pulmonary function. SNPs in or near *DNER* (smallest $P_{JMA} = 5.00\times10^{-11}$), *HLA-DQB1* and *HLA-DQA2* (smallest $P_{JMA} = 4.35\times10^{-9}$), and *KCNJ2* and *SOX9* (smallest $P_{JMA} = 1.28\times10^{-8}$) were associated with FEV_1/FVC or FEV_1 in meta-analysis models including SNP main effects, smoking main effects, and SNP-by-smoking (ever-smoking or pack-years) interaction. The HLA region has been widely implicated for autoimmune and lung phenotypes, unlike the other novel loci, which have not been widely implicated. We evaluated *DNER*, *KCNJ2*, and *SOX9* and found them to be expressed in human lung tissue. *DNER* and *SOX9* further showed evidence of differential expression in human airway epithelium in smokers compared to non-smokers. Our findings demonstrated that joint testing of SNP and SNP-by-environment interaction identified novel loci associated with complex traits that are missed when considering only the genetic main effects.

INTRODUCTION

Spirometric measures of pulmonary function, particularly forced expiratory volume in one second (FEV_1) and its ratio to forced vital capacity (FEV_1/FVC), are important clinical tools for diagnosing pulmonary disease, classifying its severity, and evaluating its progression over time. These measures also predict other morbidities and mortality in the general population [1-3]. Genetic factors likely play a prominent role in determining the maximal level of pulmonary function in early adulthood and its subsequent decline with age [4,5]. A relatively uncommon deficiency of α-1 antitrypsin, due to homozygous mutations of the *SERPINA1* gene, is a well-established genetic risk factor for accelerated decline in pulmonary function, but it accounts for little of the population variability in pulmonary function.

Genome-wide association studies (GWAS) have identified many common genetic variants underlying pulmonary function. The first GWAS of pulmonary function implicated *HHIP* for FEV_1/FVC [6,7]. GWAS meta-analyses for FEV_1/FVC and FEV_1 from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) and SpiroMeta Consortia have together identified 26 additional novel loci in or near the following genes: *ADAM19*, *AGER-PPT2*, *ARMC2*, *C10orf11*, *CCDC38*, *CDC123*, *CFDP1*, *FAM13A*, *GPR126*, *HDAC4*, *HTR4*, *INTS12-GSTCD-NPNT*, *KCNE2*, *LRP1*, *MECOM* (*EVI1*), *MFAP2*, *MMP15*, *NCR3*, *PID1*, *PTCH1*, *RARB*, *SPATA9*, *TGFB2*, *THSD4*, *TNS1*, and *ZKSCAN3* [8-10].

Inhaled pollutants, especially cigarette smoking, can have important adverse effects on pulmonary function. Candidate gene studies have not consistently identified interactions with cigarette smoking in relation to pulmonary function. Despite the importance of smoking and other environmental factors in the etiology of many complex human diseases and traits, few GWAS have incorporated gene-by-environment interactions [11-14]. Meta-analyses are generally necessary to provide sufficient sample size to detect moderate effects, and methods for joint testing of single nucleotide polymorphism (SNP) main effects and SNP-by-environment interactions in the meta-analysis setting have only recently been developed [15,16]. This strategy has the potential to identify novel loci that would not emerge from analyses based on the SNP main or interactive effects alone [15-17].

The well-documented and consistent deleterious effect of cigarette smoking on pulmonary function [18] makes it a good candidate for such an approach, since genetic factors may have heterogeneous effects on pulmonary function depending on smoking exposure. We conducted genome-wide joint meta-analyses (JMA) of SNP and SNP-by-smoking interaction (ever-smoking or pack-years) associations with cross-sectional pul-

monary function measures (FEV_1/FVC and FEV_1) in 50,047 study participants of European ancestry.

RESULTS

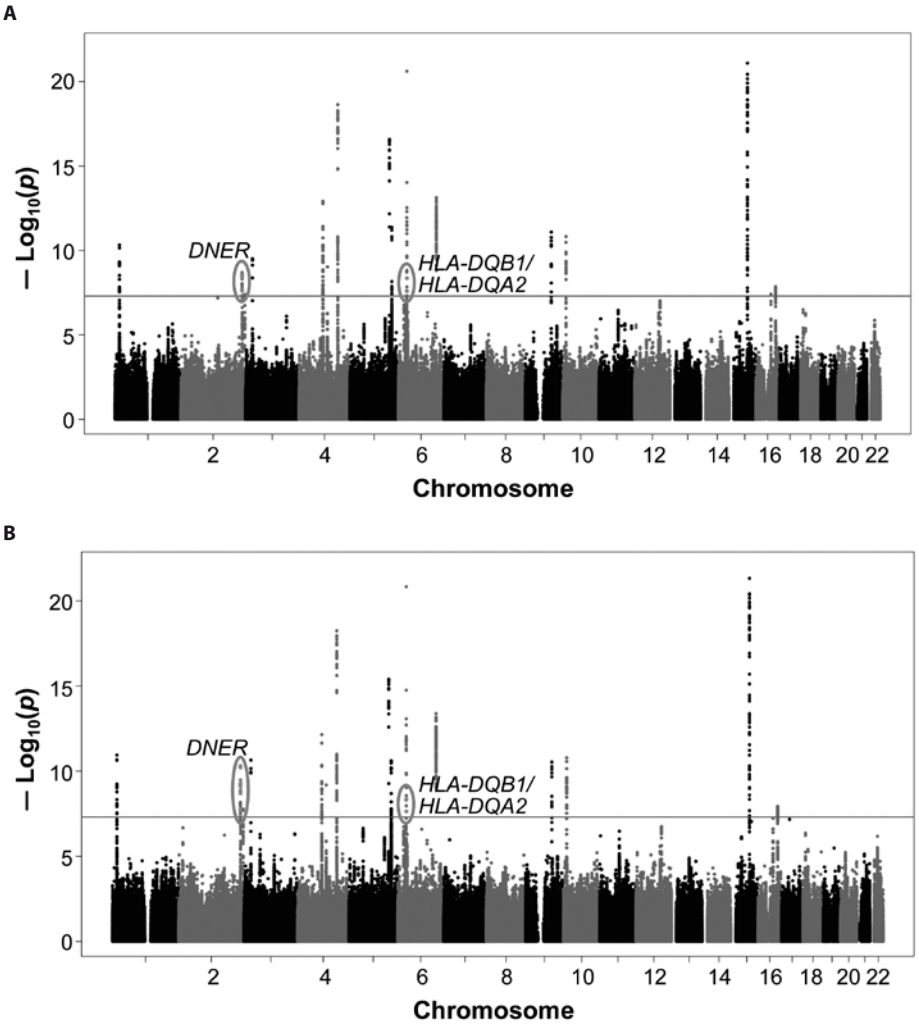
Table S1 presents characteristics of the 50,047 participants from 19 studies contributing to our analyses. As expected, mean FEV_1 and FVC values were lower in studies with the oldest participants. Standardized residuals of FEV_1 and FEV_1/FVC (see Methods) were used as the phenotypes for the JMA, in order to maximize comparability with our recent GWAS meta-analysis from the CHARGE and SpiroMeta Consortia [10]. Our original GWAS meta-analyses, conducted separately in CHARGE and SpiroMeta, showed that we were able to identify replicable genetic loci whether using actual pulmonary function measures [8] or their standardized residuals [9]. The standardized residual approach was similarly taken in GWAS of other complex quantitative traits, such as height and body mass index from the Genetic Investigation of ANthropometric Traits (GIANT) Consortium [19,20].

In each of the 19 studies, four regression models with differing SNP-by-smoking interaction terms were run: (1) SNP-by-ever-smoking for standardized FEV_1/FVC residuals, (2) SNP-by-pack-years for standardized FEV_1/FVC residuals, (3) SNP-by-ever-smoking for standardized FEV_1 residuals, and (4) SNP-by-pack-years for standardized FEV_1 residuals. Study-specific genomic inflation factors (λ_{gc}) were calculated for the 1 degree-of-freedom (d.f.) SNP-by-smoking interaction term, to ensure that there was no substantial inflation due to the main effect of smoking being misspecified [21]. All study-specific results had 1 d.f. $\lambda_{gc} \leq 1.09$ (Table S2), which is of comparable magnitude to other studies with large sample sizes [10,19,22,23].

The study-specific regression coefficients from each of the four models were then combined in JMA, and the resulting λ_{gc} values from the 2 d.f. JMA, calculated across all SNPs, ranged from 1.056 to 1.064. The quantile-quantile plots (Figure S1) show substantial deviation from expectation for SNPs having low P values from the JMA (P_{JMA}). The JMA results corresponding to the top SNP from each previously implicated locus [8-10] are presented in Table S3. To identify novel loci among the genome-wide significant loci implicated by our JMA models, the genomic regions surrounding the most significant SNP from each of the 27 previously implicated loci [8-10] (500 kb upstream to 500 kb downstream of each SNP) were removed from consideration (Table S3). Following the removal of all previously implicated loci [8-10], the quantile-quantile plots show that some deviation remained between observed and expected P values for high-signal SNPs suggesting the presence of novel signals.

In the JMA of SNP and SNP-by-smoking in relation to FEV_1/FVC, we observed two novel loci containing several significant SNP associations at the standard genome-wide Bonferroni-corrected threshold of $P_{JMA}<5\times10^{-8}$, when considering interaction with ever-smoking (Figure 1A) or pack-years (Figure 1B). The SNP associations from both loci also exceeded the more conservative genome-wide significance threshold of $P_{JMA}<1.25\times10^{-8}$, based on additional Bonferroni correction for the four JMA models.

The most statistically significant result was for rs7594321, an intronic SNP located in *DNER* (delta/notch-like EGF-related receptor) on chromosome 2, which gave $P_{JMA} = 2.64\times10^{-9}$ (corresponding $P_{INT} = 0.27$) in the ever-smoking model and $P_{JMA} = 5.00\times10^{-11}$



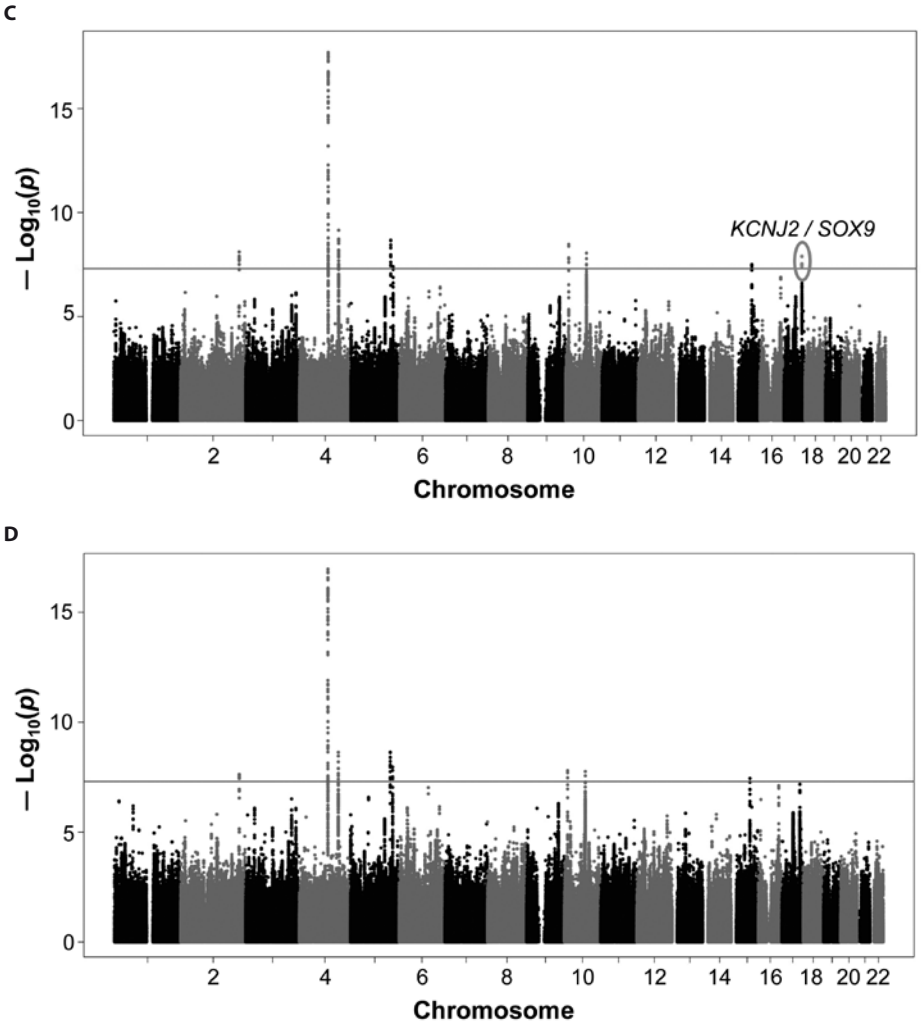


Figure 1. Genome-wide joint meta-analysis (JMA) of SNP and SNP-by-smoking interaction in relation to pulmonary function.

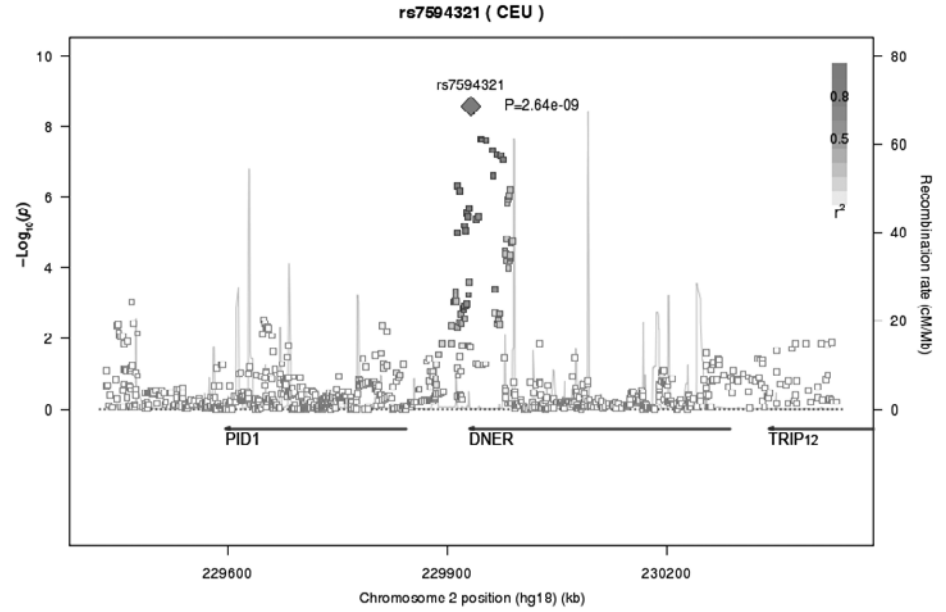
The Manhattan plots show the chromosomal position of SNPs in comparison to their 2log10 PJMA values. JMA results are shown for models with (A) SNP by-ever-smoking interaction term in relation to FEV_1/FVC, (B) SNP-by-pack-years interaction term in relation to FEV_1/FVC, (C) SNP-by-ever-smoking interaction term in relation to FEV_1, and (D) SNP-by-pack-years interaction term in relation to FEV_1. SNPs located within previously implicated loci are shown, but these loci were not considered when identifying novel loci from the joint modeling of SNP main effects and smoking interactive effects. Novel loci on chromosomes 2, 6, and 17 (shown in blue and circled) were identified as those having SNPs with genome-wide significant P values at the standard threshold ($P < 5 \times 10^{-8}$ as indicated by the solid red line). Names of the novel gene (or closest genes) are provided.

Table 1. Genome-wide significant SNPs from the joint meta-analysis (JMA) of SNP and SNP-by-smoking (ever-smoking or packyears) interaction in relation to pulmonary function.

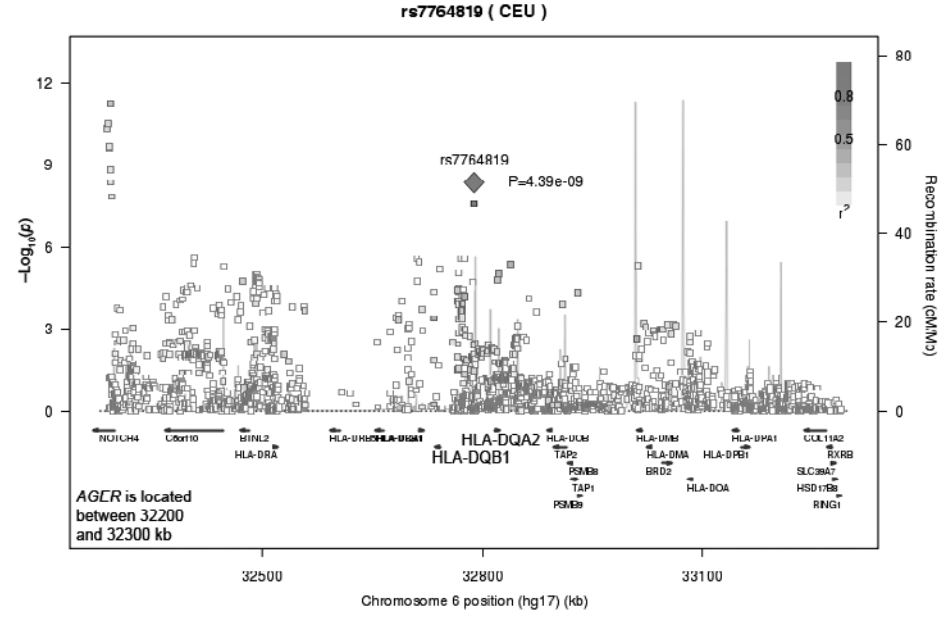
SNP (coded allele)	Chr	Gene/closest gene(s)	Coded allele frequency[1]	JMA results							
				Smoking metric	β_{SNP}	SE_{SNP}[2]	P_{SNP}	β_{INT}[3]	SE_{INT}	P_{INT}	P_{JMA}
SNPs implicated in relation to FEV_1/FVC											
rs7594321 (T)	2q36.3	*DNER*	0.35	Ever-smoking	0.049	0.0097	4.14×10^{-7}	-0.015	0.013	0.27	2.64×10^{-9}
				Pack-years	0.048	0.0070	7.03×10^{-12}	-0.00020	0.000074	6.88×10^{-3}	5.00×10^{-11}
rs7764819 (T)	6p21.32	*HLA-DQB1/ HLADQA2*	0.89	Ever-smoking	-0.060	0.015	6.32×10^{-5}	-0.0010	0.021	0.63	4.39×10^{-9}
				Pack-years	-0.064	0.011	5.95×10^{-9}	-0.000058	0.00010	0.56	4.35×10^{-9}
SNPs implicated in relation to FEV_1											
rs11654749 (T)	17q24.3	*KCNJ2/SOX9*	0.39	Ever-smoking	-0.028	0.0094	2.46×10^{-3}	-0.017	0.013	0.17	1.28×10^{-8}
				Pack-years	-0.038	0.006	2.29×10^{-8}	0.000047	0.000068	0.49	6.63×10^{-8}

After removing SNPs with known associations with FEV_1/FVC or FEV_1, three novel loci with genome-wide significant SNPs (standard threshold of $P < 5 \times 10^{-8}$) remained from the JMA testing in the current study. The most significant SNP from each locus is shown. FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; JMA, joint meta-analysis; SE, standard error ; SNP, single nucleotide polymorphism. [1]Weighted average coded allele frequency across the 19 studies. The coded allele refers to the effect allele. [2]β_{SNP}, per allele change in the FEV_1/FVC standardized residual due to the SNP main association. [3]β_{INT}, per allele change in the FEV_1/FVC standardized residual due to the interaction between SNP and smoking.

A


rs7594321 (CEU)
rs7594321
P=2.64e-09
0.8
0.5
r²
−Log10(p)
Recombination rate (cM/Mb)
PID1
DNER
TRIP12
229600
229900
230200
Chromosome 2 position (hg18) (kb)

B


rs7764819 (CEU)
rs7764819
P=4.39e-09
0.8
0.5
r²
−Log10(p)
Recombination rate (cM/Mb)
HLA-DQA2
HLA-DQB1
AGER is located
between 32200
and 32300 kb
32500
32800
33100
Chromosome 6 position (hg17) (kb)

C

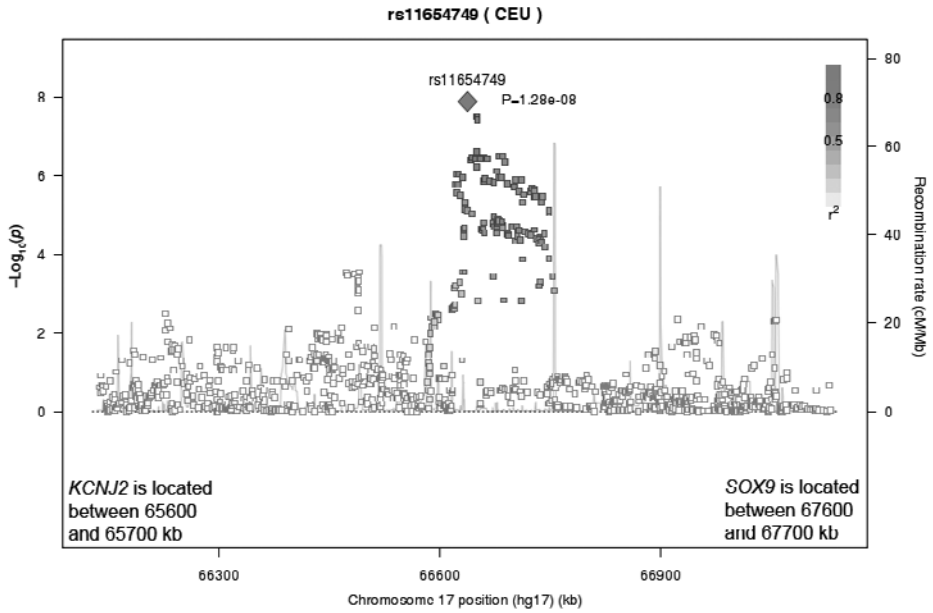


Figure 2. Regional association plots of novel loci implicated for pulmonary function
Three novel loci contained SNPs associated with FEV_1/FVC or FEV_1 at the standard genome-wide significance threshold ($P< 5 \times 10^{-8}$) in joint meta-analyses of SNP and SNP-by smoking interaction. SNPs are shown within 500 kb of the most significant SNPs on chromosomes (A) 2q36.3 associated with FEV1/FVC, (B) 6p21.32 associated with FEV_1/FVC, and (C) 17q24.3 associated with FEV_1. Pairwise r^2 values were based on the HapMap CEU population, and progressively darker shades of gray indicate higher r^2 values. Estimated recombination rates from HapMap are shown as background lines.

(corresponding P_{INT} = 0.0069) in the pack-years model (Table 1). For the ever/never-smoking interaction model, the observed level of significance for the JMA is plausible in the presence of a nominally significant SNP main effect and a nonsignificant interactive effect, as detailed in Text S1. The rs7594321 T allele had a positive β coefficient for the genetic main association and a negative β coefficient for the interaction (Table 1, Table S4 for study-specific results). The regression coefficients correspond to a per allele change of 0.049 (95% CI: 0.030, 0.068) in never-smokers and 0.035 (95% CI: 0.016, 0.053) in ever-smokers. A conserved binding site for the Zic1 transcription factor is located 115 base pairs away from rs7594321. Further, rs7594321 is located upstream of the previously implicated *PID1* gene (Figure 2A), but it is 713 kb away from the previously implicated SNP (rs1435867), which is located downstream of *PID1*. There is no linkage disequilibrium (LD) between rs7594321 and rs1435867 ($r^2 = 0$, $D' = 0$).

Our next most statistically significant SNP (rs7764819) is intergenic between two human leukocyte antigen (HLA) genes, *HLA-DQB1* and *HLA-DQA2*, on chromosome 6 (Figure 2B).

The HLA-DQ region is highly variable, and the association signal in this region is largely driven by two SNPs that are in high LD with one another (rs7764819 and rs7765379, $r^2 = 1$) but only low to moderate LD with all other genotyped and imputed SNPs. A GWAS meta-analysis of asthma implicating the HLA-DQ region similarly found highly significant associations with only a few SNPs [24]. Our top SNP rs7764819 gave $P_{JMA} = 4.39\times10^{-9}$ in the ever-smoking model and $P_{JMA} = 4.35\times10^{-9}$ in the pack-years model for FEV_1/FVC (Table 1). The corresponding P_{INT} values were >0.05 (see Text S1). The rs7764819 T allele had negative β coefficients for both the main association and interaction (Table 1, Table S5 for study-specific results), which correspond to a SNP effect of −0.060 (95% CI: −0.09, −0.031) in never-smokers and −0.070 (95% CI: −0.10, −0.042) in ever-smokers. Although rs7764819 is located 529 kb away from a previously implicated *AGER* SNP (rs2070600), there is some LD between the two SNPs ($r^2 = 0.29$, $D' = 0.81$). Conserved binding sites for two transcription factors, *HTF* and *Lmo2*, are located within 100 kb of rs7764819.

'Besides the *DNER* and *HLA-DQB1*/*HLA-DQA2* loci, SNPs from 12 other chromosomal regions having P_{JMA} values between 5×10^{-8} and 1×10^{-6} from either smoking model in relation to FEV_1/FVC are presented in Table S6. Secondary meta-analyses of the interaction product terms alone identified no SNP-by-smoking (ever-smoking or pack-years) interactions at genome-wide statistical significance with FEV_1/FVC. SNPs from two chromosomal regions had P_{INT} values between 5×10^{-8} and 1×10^{-6} in relation to FEV_1/FVC, as shown in Table S7.

For FEV_1, the JMA of SNP and SNP-by-smoking gave genome-wide significant associations ($P_{JMA}<5\times10^{-8}$) in the ever-smoking model for four SNPs on chromosome 17 (Figure 1C). However, these SNP associations did not exceed the more conservative significance threshold of $P_{JMA}<1.25\times10^{-8}$. No novel loci reached genome-wide significance level in the pack-years model in relation to FEV_1 (Figure 1D).

The most significant SNP (rs11654749) from both smoking models is intergenic between *KCNJ2* (a potassium inwardly-rectifying channel also known as KIR2.1) and *SOX9* (sex determining region Y-box 9) (Figure 2C). Conserved binding sites for four transcription factors (*HNF-1*, *CP2*, *Cdc5*, and *FOXF2*) are located within 100 kb upstream or downstream of rs11654749. The rs11654749 SNP gave $P_{JMA} = 1.28\times10^{-8}$ in the ever-smoking model and $P_{JMA} = 6.63\times10^{-8}$ in the pack-years model (Table 1). The corresponding P_{INT} values were >0.05 (see Text S1). The rs11654749 T allele had negative β coefficients for both the main association and interaction (Table 1, Table S8 for study-specific results). These estimates correspond to a SNP effect of −0.028 (95% CI: −0.047, −0.010) in never-smokers and −0.046 (95% CI: −0.063, −0.029) in ever-smokers. To better understand the magnitude of these β estimates, we compared our results with those observed in one of our previous GWAS meta-analyses of SNP main effects [9], where standardized residuals of the pulmonary function measures were similarly computed. For a SNP with MAF around

40%, an absolute β value of 0.028 would be equivalent to 19 mL per copy of the risk allele (comparable to a year of FEV_1 decline in healthy never-smokers), and an absolute β value of 0.046 would be equivalent to 31 mL per copy of the risk allele (comparable to a year and a half of FEV_1 decline in healthy never-smokers) [25].

Besides this *KCNJ2*/*SOX9* locus, SNPs from five other chromosomal regions have P_{JMA} values between 5×10^{-8} and 1×10^{-6} from either smoking model in relation to FEV_1 as shown in Table S6. In secondary meta-analyses of the interaction product terms, there were no SNP-by-smoking (ever-smoking or pack-years) interactions implicated at genome-wide statistical significance with FEV_1. SNPs from four chromosomal regions had P_{INT} values between 5×10^{-8} and 1×10^{-6} in relation to FEV_1, as shown in Table S7.

None of the most significant SNPs from the three novel loci we identified by the JMA were associated with FEV_1/FVC or FEV_1 at or near genome-wide significance in our previous GWAS meta-analysis of 48,201 participants from the CHARGE and SpiroMeta Consortia. In fact, the lowest P value observed for these SNPs was 1.04×10^{-5} (Table 2) [10].

Table 2. Look-up evaluation of SNP main associations with FEV_1/FVC and FEV_1 using data generated by our previous genome wide association study meta-analysis (N = 48,201), for the most significant SNP from each of the three novel loci implicated at genome-wide significance in the joint meta-analysis.

SNP (coded allele)	Gene/ closest gene(s)	FEV1/FVC			FEV1		
		β[1]	SE	*P*	β[1]	SE	*P*
rs7594321 (T)	*DNER*	0.032	0.0072	1.04×10^{-5}	0.0081	0.0074	0.27
rs7764819 (T)	*HLA-DQB1/ HLA-DQA2*	-0.044	0.011	8.79×10^{-5}	-0.0073	0.011	0.52
rs11654749 (T)	*KCNJ2/ SOX9*	-0.023	0.0071	0.0015	-0.031	0.0072	1.23×10^{-5}

FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; SE, standard error; SNP, single nucleotide polymorphism. [1]βSNP, per allele change in the FEV_1/FVC standardized residual due to the SNP main association.

To evaluate whether the three novel loci identified by the JMA were related to smoking, we evaluated their SNP associations with ever-smoking and cigarettes per day using GWAS meta-analysis results from the Oxford-GlaxoSmithKline (Ox-GSK) Consortium (N = 41,150) [26]. None of our implicated SNPs were associated with these smoking phenotypes at P<0.05 (Table S9), adding confidence that our JMA-implicated SNP associations were not simply reflective of smoking main effects.

Expression analyses

Three genes (*DNER*, *KCNJ2*, and *SOX9*) harboring or flanking novel genome-wide significant SNPs were selected for follow-up mRNA expression profiling in human lung tissue and a series of primary cells. Transcripts of all three genes were found in lung tissue, airway smooth muscle, and bronchial epithelial cells; *DNER* and *KCNJ2* transcripts were also found in peripheral blood cells (Table S10).

In a separate line of investigation, using the publically available Gene Expression Omnibus repository [27,28], we found that the expression profiling of *DNER* and *SOX9* showed differential expression in human airway epithelium of smokers compared to non-smokers (Figure S2A and S2B) [29]. Expression profiling of *KCNJ2* did not show statistically significant differential expression by smoking status (Figure S2C) [30]. We also identified novel genome-wide significant SNPs in the HLA-DQ region, but we did not examine HLA-DQ expression given the known expression of class II MHC antigens on a range of airway cell types [31], [32]. However, the lead SNP in this region (rs7764819) was associated with statistically significant effects on *HLA-DQB1* expression ($P = 1.2\times10^{-14}$), according to an eQTL analysis database of lymphoblastoid cell lines [33].

DISCUSSION

Few GWAS have accounted for potential interaction with environmental risk factors. To identify novel genetic risk factors that are missed when considering only genetic main effects [34], we used the newly available JMA method [15] to simultaneously summarize regression coefficients for the main SNP and SNP-by-smoking interactive effects in 50,047 participants from 19 studies, based on models that were fully saturated for the main effect of smoking. This study represents the most comprehensive analysis to date of gene-by-smoking interaction in relation to pulmonary function. We identified two novel loci (*DNER* and *HLA-DQB1*/*HLA-DQA2*) having highly significant evidence for association with FEV_1/FVC. A third novel locus (*KCNJ2*/*SOX9*) was associated with FEV_1. For the most significant SNPs at each of these three loci, there was no evidence for heterogeneity across the studies (smallest heterogeneity $P = 0.59$), indicating that the associations were not driven by one or a few studies and thus reflect accumulation of evidence across the studies. None of these three loci had previously been associated with pulmonary function.

The comparison of results with our prior GWAS meta-analysis of SNP main effects [10], using a comparable sample size, suggested that the SNP associations for our top SNPs were weaker in our previous analyses that examined only genetic main effects. However, our analyses and those of Manning et al. [14] suggest that some of the benefit of using the

joint test for some findings comes from the careful adjustment for the environmental main effect. Thus, future studies aimed at replicating these findings may wish to jointly test the SNP main and interactive effects [15,16,33] instead of implementing a standard test of only the SNP main effects. If there is no evidence for interaction at a given locus, the saturation of the main effect of the environmental factor may be important. The joint testing is applicable for both candidate gene [15] and genome-wide [14] approaches. Further, there was minimal overlap in the top SNPs associated with FEV_1/FVC and FEV_1, as similarly observed in our previous GWAS meta-analyses of SNP main effects [8-10]. Given that the biological underpinnings of these discrepant association findings remain unknown, future studies should evaluate these genetic loci in the context of the pulmonary function measure for which they were originally implicated.

Given that pulmonary function is a phenotype for which numerous genetic loci have been identified in GWAS and smoking is clearly associated with pulmonary function, it might seem surprising that none of the genome-wide significant SNPs implicated by the JMA demonstrated a substantial interaction per se. The lack of strong interactive effects does not negate the well-established harmful effects of cigarette smoking nor the need for broad public health campaigns to curb smoking. Instead, our findings demonstrate the value of applying the newly developed joint methods to uncover novel genetic risk factors that might shed light on the mechanisms leading to reduced pulmonary function.

Our pattern of SNP main and interactive results resemble the patterns seen in another recent application of the same JMA method to incorporate the interaction with body mass index (BMI) into GWAS of type 2 diabetes traits (fasting insulin and blood glucose) [14]. In that study with a sample size of 96,453, nearly double that of ours, the top JMA finding had a corresponding interaction P value of 1.6×10^{-4} [14]. In our study, the smallest interaction P value for our top JMA finding was 6.9×10^{-3}. In both our GWAS of smoking and pulmonary function and the recent GWAS of BMI and diabetes traits [14], the SNPs newly implicated by the JMA had marginally significant associations with the trait under study in models with no interaction term, but they became genome-wide significant when accounting for the environmental factor (cigarette smoking or BMI) and the SNP-by-environment interaction. Our JMA included careful modeling of the environmental factor to saturate the environmental main effects along with the interaction testing. In the GWAS of diabetes traits [14], the careful modeling of the environmental factor appeared to account for some of the novel findings from the JMA, consistent with the modest evidence for interaction [14]. Although our previous GWAS meta-analysis was conducted in ever/never-smoking strata, the regression models were not adjusted for smoking status or pack-years [10]. Some of our novel JMA findings compared with our

previous GWAS findings may reflect, in part, the saturated modeling of the smoking main effect rather than the interaction per se.

The current analysis of 50,047 participants included only 1,846 more participants than our previous GWAS meta-analysis of SNP main effects [10]. To evaluate the likelihood that this 3.8% increase in sample size above that in our previous meta-analysis of pulmonary function was sufficient to explain our identification of these three novel loci at genome-wide statistical significance in the current JMA, we calculated the statistical power to detect genetic main associations (QUANTO [35]) with minor allele frequency (MAF) and β estimates comparable to the three genome-wide significant SNPs presented in Table 1. The current study (total N = 50,047 participants) had only 0.7% to 4.2% more statistical power than our previous GWAS meta-analysis (total N = 48,201 participants) [10], suggesting that the JMA-implicated SNPs are not merely reflective of increased power to detect genetic main effects. Instead, our novel JMA findings demonstrate an advantage of the method used to jointly test the SNP and SNP-by-smoking interactive effects, including the benefit of the saturated modeling of the smoking main effect.

SNPs located in the *DNER* gene were significantly associated with FEV_1/FVC, even at the more conservative P value threshold of 1.25×10^{-8}. The JMA results for *DNER* SNPs were driven by both smoking-adjusted main effects and interaction with quantitative smoking history. The *DNER* protein product is a ligand of the Notch signaling pathway that has been implicated in neuronal differentiation and maturation [36,37], adipogenesis [38], and hair-cell development [39]. The Notch pathway is a critical controller of cellular differentiation in multiple organs including the lung [40,41]. Interestingly, the expression levels of many members of the Notch signaling cascade are significantly altered in airway epithelial cells of smokers [30]. We confirmed the expression of *DNER* transcripts in lung and peripheral cells, and by mining publicly available transcriptional profiling databases [29], we found that *DNER* is expressed in bronchial epithelial cells of non-smoking adults and, importantly, its expression is significantly higher in smokers (Figure S2A). Collectively, these results suggest that *DNER* plays a role in cigarette smoke-induced airflow obstruction and further corroborate the importance of the Notch signaling circuitry in the pathogenesis of obstructive lung disease.

Also in relation to FEV_1/FVC, intergenic SNPs between *HLA-DQB1* and *HLA-DQA2* exceeded the more conservative genome-wide significance threshold. The eQTL analyses indicated that the lead SNP is associated with expression of *HLA-DQB1* specifically. However, the major histocompatibility complex region is highly polymorphic with complex LD patterns, and a few specific functional SNPs might explain the observed associations [42]. Genetic variations within this region have been associated with several autoimmune

disorders [43] and asthma [24,44,45], and an interaction between HLA variants and cigarette smoking has been previously implicated [46]. We found little evidence for interaction with smoking at this locus, suggesting that the JMA results were primarily driven by smoking-adjusted genetic main effects. It is most likely that this locus was not identified in our previous GWAS meta-analysis, because the genetic main associations were not evaluated with careful adjustment for smoking status and pack-years. Adjustment for smoking in the current analysis may have removed residual variance in the outcome that is not attributable to genetic variation [14], thus making the identification of the newly associated SNPs possible.

Intergenic SNPs between *KCNJ2* and *SOX9* were significantly associated with FEV_1 at the standard P value threshold, but not the more conservative threshold. Similar to the HLA region, it appears that the JMA results for the *KCNJ2*/*SOX9* region were primarily driven by smoking-adjusted genetic main effects. This region is enriched for long-range regulatory elements for *SOX9*, although the possibility of this region containing *KCNJ2* regulatory elements cannot be discounted [47]. *KCNJ2* is a member of the inwardly-rectifying potassium channel family, which regulates membrane potential and cell excitability and is expressed in many tissues including myocardium, neurons, and vasculature. This potassium channel also affects human bronchial smooth muscle tone and airflow limitation [48]. Dominant negative mutations in *KCNJ2* cause the Andersen syndrome, characterized by ventricular arrhythmias, periodic paralysis, and a number of skeletal and cardiac abnormalities [49]. *SOX9* is a transcription factor that is essential for cartilage formation, [50] but it is also abundantly expressed in other tissues including the respiratory epithelium during development [51]. $Sox9^{-/-}$ and $Sox9^{+/-}$ mice have multiple skeletal anomalies and severe tracheal cartilage malformations and die prematurely from respiratory insufficiency [50,52]. Mutations in *SOX9* cause campomelic dysplasia characterized by skeletal defects and autosomal sex reversal [53]. These individuals develop respiratory distress due to chest wall abnormalities, narrowed airways resulting from tracheobronchial defects and hypoplastic lungs [54]. We confirmed that *KCNJ2* and *SOX9* transcripts were present in human lung tissue and peripheral cells. Using publicly available microarray data [29], we established that *SOX9* is expressed in human airway epithelial cells and its expression is significantly down-regulated in smokers relative to non-smoking adults (Figure S2B). Taken together, these results suggest that *SOX9* may be involved in cigarette smoke-induced airflow obstruction, but further investigation is required to elucidate putative mechanisms.

Most of the previously implicated SNPs had genome-wide significant (or nearly significant) associations with pulmonary function in the JMA, but some were associated with pulmonary function at P values that did not approach the genome-wide statistical

significance threshold in the JMA analysis. This pattern has two possible explanations. First, the identification of these SNPs at genome-wide statistical significance in our most recent analysis [10] required a sample size of nearly 95,000 individuals, which was obtained by combining discovery and replication cohorts, including additional genotyping on thousands of participants from studies without GWAS data. In the current analysis, the sample size is greatly reduced because of the need for detailed quantitative smoking data and because we were unable to perform additional genotyping in studies without GWAS data. Second, Manning et al.[15] showed that a meta-analysis of main SNP effects has slightly greater power than the JMA under the scenario of no interaction, so it is not surprising that a few of the prior SNP findings had varying levels of significance between our prior GWAS meta-analyses [8-10] and the current JMA study. While our sample size of over 50,000 study participants is large, and the study of Manning et al. [14] examining SNP-by-BMI interaction in relation to fasting insulin is nearly twice as large, identification of interactions is challenging from a statistical power perspective. Given the multiple testing issues in genome interaction testing, even larger sample sizes will likely be needed to identify gene-by-environment interactions with rare variants or with the modest effect sizes that we generally expect. Nonetheless, our findings exemplify the greater power achieved by using the joint methods, such as those reported by Manning et al. [15] and Kraft et al. [16,34], to incorporate interaction with a clearly associated environmental risk factor. The novel genetic loci identified here for pulmonary function would have remained unknown using standard GWAS approaches.

METHODS

Ethics statement

Nineteen independent studies contributed to our analyses. All study protocols were approved by the respective local Institutional Review Boards, and written informed consent for genetic studies was obtained from all participants included in our analyses.

Cohort studies

Of the 19 studies contributing to our analyses, 18 studies came from the CHARGE [8,55] or SpiroMeta [9] Consortium: Age, Gene, Environment, Susceptibility (AGES) – Reykjavik Study [56]; Atherosclerosis Risk in Communities (ARIC) Study [57]; British 1958 Birth Cohort (B58C) [58]; Coronary Artery Risk Development in Young Adults (CARDIA) [59,60]; Cardiovascular Health Study (CHS) [61]; European Community Respiratory Health Survey (ECRHS) [62]; European Prospective Investigation into Cancer and Nutrition (EPIC, obese cases and population-based subsets) [63]; Framingham Heart Study (FHS) [64,65]; Health, Aging, and Body Composition (Health ABC) Study [66]; Northern Finland Birth Cohort of 1966

(NFBC1966) [67,68]; Multi-Ethnic Study of Atherosclerosis (MESA) [69,70]; Rotterdam Study (RS-I, RS-II, and RS-III) [71]; Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) [72]; Study of Health in Pomerania (SHIP) [73]; and TwinsUK [74]. We reached out to other population-based studies with GWAS genotyping and data available on cigarette smoking and pulmonary function, resulting in the inclusion of LifeLines [75]. Given the greater power needed to detect novel genetic loci with subtle gene-environment interaction regardless of the statistical method used [16], we chose to maximize statistical power to discover novel genetic loci by combining all available participants and to use the regression coefficients across the many different component studies as evidence for consistency. This approach was similarly taken by another large-scale GWAS consortium for discovering SNP main effects [24].

Pulmonary function measurements and smoking information

All studies were included in our previous GWAS meta-analysis of pulmonary function or the follow-up replication analyses, wherein their pulmonary function testing protocols were described [10]. For studies with spirometry at a single visit (B58C, LifeLines, MESA, NFBC1966, SHIP, RS-I, RS-II, and RS-III), we analyzed FEV_1/FVC and FEV_1 measured at that visit. For studies with spirometry at more than one visit, we analyzed measurements from the baseline visit (AGES, ARIC, CARDIA, CHS, ECRHS, EPIC obese cases, EPIC population-based, Health ABC, and SAPALDIA) or the most recent examination with spirometry data (FHS and TwinsUK).

Smoking history (current-, past-, and never-smoking) was ascertained by questionnaire at the time of pulmonary function testing. Pack-years of smoking were calculated for current and past smokers by multiplying smoking amount (packs/day) and duration (years smoked). Table S11 presents the specific questions used to ascertain smoking history and pack-years in each of the 19 studies.

Genotyping, quality control, and imputation

Study participants were genotyped on various genotyping platforms, and standard quality control filters for call rate, Hardy-Weinberg equilibrium p-value, MAF, and other measures were applied to the genotyped SNPs (Table S12). To generate a common set of SNPs for meta-analysis, imputation was conducted with reference haplotype panels from HapMap phase II subjects of European ancestry (CEU) (Table S12) [76]. Imputed genotype dosage values (estimated reference allele count with a fractional value ranging from 0 to 2.0) were generated for approximately 2.5 million autosomal SNPs. Among participants with genome-wide SNP genotyping data, exclusions were made due to standard quality control metrics (call rate, discordance with prior genotyping, and genotypic and

phenotypic sex mismatch among others), missing pulmonary function data, or missing covariate data (Table S13).

Statistical analysis

Our analyses included 50,047 participants from 19 studies who passed their study-specific quality control and had complete data on pulmonary function and smoking. Each study transformed the pulmonary function measures to residuals using linear regression of FEV_1/FVC (%) and FEV_1 (mL) on age, age^2, sex, and standing height as predictors. Principal component eigenvectors and recruitment site were also included as covariates to adjust for population stratification (if applicable). The residuals were converted to z scores (henceforth referred to as standardized residuals). We confirmed that smoking was inversely associated with the FEV_1/FVC and FEV_1 standardized residuals in all 19 studies (meta-analysis $\beta = -0.0030$ and corresponding $P<1\times10^{-6}$ for pack-years of smoking).

The FEV_1/FVC and FEV_1 standardized residuals were used as the phenotypes for genome-wide association testing with linear regression models, which included the following predictor variables: imputed SNP genotype dosages, smoking history (dichotomous variable, 0 = never-smokers and 1 = ever-smokers), smoking status (dichotomous variable, 0 = never- and past-smokers and 1 = current-smokers), pack-years of smoking (continuous variable), and a SNP-by-smoking interaction product term. Two of the 19 studies (FHS and TwinsUK) had much relatedness among participants, and we took appropriate account of relatedness in the association testing (Table S12). Four regression models with interaction terms for ever-smoking or pack-years were specified in relation to standardized residuals for FEV_1/FVC or FEV_1. As it has long been advised in studying interactions, the regression models were designed to fully saturate the main smoking effect on pulmonary function, so that the interaction terms do not capture residual main effects [77]. In each of the 19 studies, the genome-wide analyses were implemented with robust variance estimation using the software packages indicated in Table S12.

Our analyses were aimed at finding novel loci associated with pulmonary function when considering an interaction with cigarette smoking, so we chose to implement JMA of SNP main and interactive SNP-by-smoking effects (two d.f. test of the null hypothesis $\beta_{SNP} = 0$ and $\beta_{INT} = 0$) [15]. Manning et al. previously compared the joint methods, such as JMA, with other methods that incorporate gene-environment interaction (such as screening by main effects [78] or conducting a 1 d.f. meta-analysis of the interaction product term), and they found that the joint methods offer optimal statistical power over a range of scenarios for SNP main and interactive effects [15,34]. Therefore, our analyses centered on the JMA method, which simultaneously estimates regression coefficients for the SNP and

SNP-by-smoking interaction terms, while accounting for their covariance, to generate a joint test of significance [15]. It also accounts for the unequal variances from studies of different sample sizes. Secondarily, we implemented meta-analyses of just the β coefficient from the interaction term for comparison with the JMA results. Of note, the two-step gene-environment interaction study designs by Murcray et al. [79,80] and Gauderman et al. [81] are applicable to case-control or case-parent trio studies, respectively, and were thus not considered for our population-based studies of continuous traits.

The JMA was conducted with fixed effects on approximately 2.5 million SNPs using METAL software (version 2010-02-08) [82] and patch source code provided by Manning et al. [82]. Genomic control correction was applied by computing λ_{gc} as the ratio of the observed and expected (2 d.f.) median chi-square statistics and dividing the observed chi-square statistics by λ_{gc}. SNPs having $P_{JMA}<5\times10^{-8}$ (the standard Bonferroni-adjusted P value) were considered statistically significant [83]. Further correction for the four different (albeit related) JMA models yielded a conservative P_{JMA} threshold of 1.25×10^{-8}. In addition to reporting the P_{JMA} for the most significant SNP from each novel locus, we used the β and standard error (SE) estimates from the JMA results to calculate the P values corresponding to the SNP main association (P_{SNP}) and the SNP-by-ever-smoking interaction (P_{INT}) [15].

Bioinformatics analysis

Gene annotation was performed using the gene prediction tracks "UCSC Genes" and "RefSeq Genes" in the UCSC browser (http://genome.ucsc.edu). The "sno/miRNA" track from the USCS browser was used to search for any microRNA within 100 kb upstream or downstream of each SNP, and the "TFBS Conserved" track was used to search for conserved transcription factor binding sites (TFBSs) at or near the most significant SNPs. The SNAP program [84] was used to infer LD patterns, based on the HapMap phase II CEU population.

Expression analyses

We used separate types of expression analyses to confirm the biologic plausibility of our findings. First, we carried out mRNA expression profiling to show whether or not the implicated genes are expressed in human tissues relevant to pulmonary function. The mRNA expression profiles of implicated genes were determined using reverse transcription polymerase chain reaction (RT-PCR). RNA was sourced from lung (Ambion/ABI), human bronchial epithelial cells (Clonetics) [85], and peripheral blood mononuclear cells (3H Biomedica). RNA from human airway smooth muscle cells, cultured as previously described from tissue obtained at thoracotomy [86], was extracted using a commercially available kit (Qiagen). Ethical approval for the use of primary cells was obtained from

the local ethics committees. cDNA was generated using 1 µg of RNA template using random hexamers and a SuperScript kit (Invitrogen) as directed by the manufacturer. PCR assays were designed to cross intron-exon boundaries, where possible and where splice variation was known, in order to detect all variants. The GAPDH gene was used as a positive control for the cDNA quality, and water was used as a negative control. Primer sequences for the genes of interest are given in Table S14. All PCR were done using Platinum Taq High Fidelity (Invitrogen) with 100 ng of cDNA template in a 25 µL reaction. Cycling conditions were as follows: 94°C for 2 minutes, 35 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 68°C for 90 seconds. Following PCR, gel bands were directly sequenced to confirm the presence of the gene's transcript.

Second, we used another publically available data repository to investigate whether any of the implicated genes showed evidence for differential expression depending on smoking history. The gene expression profiles of human airway epithelium from healthy smokers (N = 10) and nonsmokers (N = 12) were obtained from the Gene Expression Omnibus site (http://www.ncbi.nlm.nih.gov/geo/) [27,28], based on robust multichip average processing of probe intensities from Affymetrix HG-U133 Plus 2.0 microarrays (GEO dataset number GSE4498) [29]. Mean expression levels of genes around our genome-wide significant findings from the JMA were compared between smokers versus nonsmokers. The P value for the difference in means between smokers and nonsmokers was calculated using the nonparametric Mann-Whitney test.

Third, our genome-wide significant SNPs from novel loci were searched against an expression quantitative trait loci (eQTL) data repository based on lymphoblastoid cell lines [33], to investigate whether any of the implicated SNP variants might influence the expression of the nearby genes. $P<5\times10^{-8}$ was used to designate statistically significant eQTL associations.

All supplementary files and figures can be found via:
http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003098

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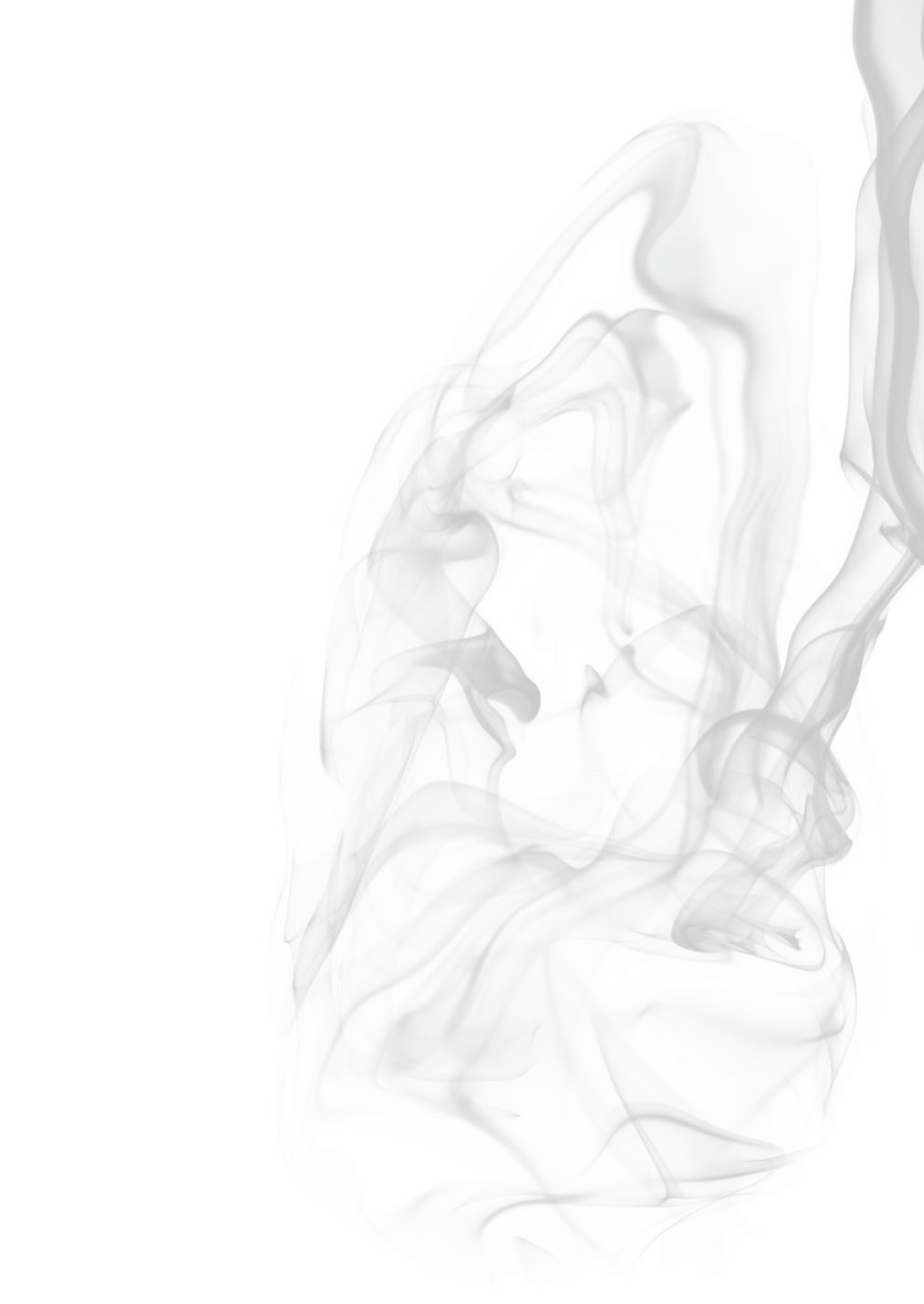

CHAPTER 3.4

Genome-Wide Association Studies of Longitudinal Change in Adult Lung Function

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Manuscript submitted

ABSTRACT

Background: Genome-wide association studies (GWAS) have identified numerous loci influencing cross-sectional lung function, but less is known about genes influencing longitudinal change in lung function.

Methods: We performed GWAS of the rate of change in forced expiratory volume in the first second (FEV_1) in 14 longitudinal, population-based cohort studies comprising 27249 adults of European ancestry using linear mixed effects model and combined cohort-specific results using fixed effect meta-analysis to identify novel genetic loci associated with longitudinal change in lung function. As a secondary aim, we estimated the rate of decline in FEV_1 by smoking pattern across these 14 studies using meta-analysis.

Results: The overall meta-analysis produced suggestive evidence for association at the novel *IL16*/*STARD5*/*TMC3* locus on chromosome 15 ($P = 5.71 \times 10^{-7}$). In addition, meta-analysis using the five cohorts with ≥3 FEV_1 measurements per participant identified the novel *ME3* locus on chromosome 11 ($P = 2.18 \times 10^{-8}$) at genome-wide significance. Neither locus was associated with FEV_1 decline in two additional cohort studies. We confirmed gene expression of *IL16*, *STARD5*, and *ME3* in multiple lung tissues. Publicly available microarray data confirmed differential expression of all three genes in lung samples from COPD patients compared with controls. The combined estimate for FEV_1 decline was 26.9, 29.2 and 35.7 mL/year in never, former, and persistent smokers, respectively.

Conclusions: In this large-scale GWAS, we identified two novel genetic loci in association with the rate of change in FEV_1 that harbor candidate genes with biologically plausible functional links to lung function.

INTRODUCTION

Forced expiratory volume in the first second (FEV_1) is a reliable spirometric parameter that reflects the physiological state of the lungs and airways. Reduced FEV_1 relative to forced vital capacity (FVC), is a defining feature of chronic obstructive pulmonary disease (COPD), a leading cause of death globally.[1] FEV_1 is also a predictor of morbidity and mortality in the general population.[2,3] Lung function reaches its peak in early adulthood, followed by a plateau, and then subsequently declines. As first reported by Fletcher and Peto,[4] decline in lung function is accelerated in smokers, leading to increased risks of COPD and premature death. While cigarette smoking is a key risk factor for accelerated loss of lung function, genetic variation is hypothesized to also play an important role.[5,6] Family and twin studies of the longitudinal change in lung function report heritability estimates between 10 and 39%.[7,8]

Recent large-scale genome-wide association studies (GWAS) identified 26 novel loci for cross-sectional lung function,[9-11] demonstrating the power of GWAS with large sample size to identify common genetic variants with modest effect sizes. However, cross-sectional measurements in adults reflect the combination of maximal attained lung growth and subsequent decline. GWAS that specifically study the longitudinal change in lung function are needed to distinguish the genetic contributions to age-related decline. To date, only one population-based GWAS meta-analysis of longitudinal change in lung function has been reported.[12] Separate analyses were conducted in 1441 asthmatic and 2667 non-asthmatic participants; association was found at one novel locus in each analysis, though only the locus in non-asthmatics replicated.

In this study, we conducted primary GWAS of the rate of change in FEV_1 in each of 14 population-based cohort studies from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) and SpiroMeta consortia, comprising 27249 adult participants of European ancestry and 62130 FEV_1 measurements. We then performed meta-analysis of the cohort-specific results, followed up our most statistically significant associations in the AGES-Reykjavík cohort study and the Lung Health Study (LHS) for corroborative evidence, and explored the biological basis for identified associations using cell-specific gene expression studies, and expression quantitative trait loci (eQTL) look-up.

METHODS

All 14 cohort studies are members of the CHARGE or SpiroMeta Consortium (Table 1). The respective local Institutional Review Boards approved all study protocols, and written informed consent for genetic studies was obtained from all participants.

Spirometry tests were performed at baseline and at least one follow-up time point by trained technicians and in accordance with the American Thoracic Society or European Respiratory Society recommendations (details in online supplement).[13] FEV_1 measurements meeting acceptability criteria were included in the current study.

Studies performed genotyping following standard quality control measures; imputation was conducted based on the HapMap CEU reference panel to generate genotype dosages for ~ 2.5 million autosomal single nucleotide polymorphisms (SNPs) (Table E1).

Each cohort study performed the GWAS using a linear mixed effects model. The model included a random intercept and a random slope, and fixed effects for time (a continuous variable quantifying the time distance between each FEV_1 measurement and baseline), SNP and its interaction with time (SNP-by-time), baseline age, gender, standing height, smoking pattern during follow-up and its interaction with time (smoking-by-time), baseline smoking pack-years, study site, and principal components for genetic ancestry (as needed). Cohort-specific results for the SNP-by-time interaction term, which estimates the effect of genotype on the rate of change in FEV_1, were shared, and two meta-analyses, one using all 14 studies and the other using the five studies with ≥3 FEV_1 measurements per participant, were performed using METAL software with inverse variance weighting to combine effect estimates after applying genomic control correction.[14]

We sought corroborative evidence for SNPs with $P < 1 \times 10^{-5}$ in the AGES-Reykjavík cohort study (n = 1494), and in LHS (n = 4048), a clinical cohort study of smokers with mild COPD, in which a longitudinal GWAS was recently reported.[15]

Expression profiles of genes at the novel loci were evaluated in human lung tissues and primary cell samples using RT-PCR (Table E7). Using publicly available data from the Lung Genomics Research Consortium (LGRC), expression profiles of these genes were compared in lung specimens of 219 COPD patients and 137 controls, and sentinel (most associated) SNPs at the novel loci were also searched against an eQTL database of lymphoblastoid cell lines.[16]

Table 1. Baseline characteristics of cohort studies included in the meta-analysis*

Cohort:	ARIC	B58C	BHS	CARDIA	CHS	FHS	Health ABC
No. of participants	8,242	827	1,009	1,492	3,159	3,230	1,586
No. of FEV_1 measurements	15,582	1,653	3,073	6,140	7,140	11,275	4,426
No. of FEV_1 per person	2	2	7	5	3	5	4
Follow-up duration, yr	5.6	10	29	20.1	7.9	14.7	9.5
Males, %	46.5	48.6	41.6	46.9	39	47	52.7
Baseline age, yr	54.6 (5.7)	35.0 (0.2)	37.5 (12.8)	27.5 (2.3)	72.3 (5.4)	50.9 (10.3)	73.8 (2.8)
Baseline height, cm	168.7 (9.4)	170.1 (9.5)	168.1 (8.9)	171.2 (9.3)	164.6 (9.4)	168.4 (9.3)	166.8 (9.3)
Current smokers, %	20.2	27.1	20.9	24.8	10.8	24.6	6.4
Former smokers, %	32.6	41.5	16.5	17.3	35.7	39.8	49.9
Baseline pack-years†	25.9 (21.7)	7.5 (11.4)	8.2 (17.8)	6.0 (6.5)	33.2 (27.0)	25.4 (21.3)	36.8 (32.2)
Baseline FEV_1, mL	2972 (758)	3631 (744)	3230 (927)	3818 (781)	2123 (652)	2989 (806)	2308 (649)
Baseline FEV_1/FVC, %	74.1 (7.1)	80.6 (5.8)	78.2 (9.2)	81.6 (6.5)	70.5 (10.5)	75.7 (8.0)	74.7 (7.8)

Cohort:	KORA	LBC1921	LBC1936	PIVUS	RS	SAPALDIA	SHIP
No. of participants	890	512	1,002	818	1,321	1,401	1,760
No. of FEV_1 measurements	1,597	706	1,790	1,469	2,016	2,692	2,571
No. of FEV_1 per person	2	2	2	2	2	2	2
Follow-up duration, yr	3.2	8.9	4.8	5.8	8.3	10.9	7.9
Males, %	47.2	41.4	50.8	49.9	45.1	48	49.4
Baseline age, yr	53.8 (4.5)	79.1 (0.6)	69.6 (0.8)	70.2 (0.2)	74.4 (5.6)	41.1 (11.2)	52.4 (13.6)
Baseline height, cm	169.3 (9.3)	163.2 (9.4)	166.5 (8.9)	169.0 (9.3)	167.3 (9.1)	169.4 (9.1)	169.5 (9.7)
Current smokers, %	20.5	7.0	12.9	10.2	11.1	26.9	32.8
Former smokers, %	40.9	50.4	42.6	39.6	56.7	25.8	23.8
Baseline pack-years†	11.2 (17.1)	15.3 (22.3)	16.9 (25.8)	14.3 (15.8)	25.7 (21.3)	17.4 (18.0)	11.3 (11.9)
Baseline FEV_1, mL	3280 (792)	1887 (625)	2371 (687)	2452 (682)	2215 (652)	3516 (861)	3238 (876)
Baseline FEV_1/FVC, %	77.5 (6.2)	79.0 (11.8)	78.3 (10.2)	76.0 (10.0)	74.8 (7.9)	78.5 (8.2)	83.1 (6.6)

Definition of abbreviations: ARIC = Atherosclerosis Risk in Communities; B58C = British 1958 Birth Cohort; BHS = Busselton Health Study; CARDIA = Coronary Artery Risk Development in Young Adults; CHS = Cardiovascular Health Study = FHS, Framingham Heart Study; Health ABC = Health, Aging, and Body Composition; KORA = Cooperative Health Research in the Region of Augsburg; LBC1921 = Lothian Birth Cohort 1921; LBC1936 = Lothian Birth Cohort 1936; PIVUS = Prospective Investigation of the Vasculature in Uppsala Seniors; RS = Rotterdam Study; SAPALDIA = Swiss Study on Air Pollution and Lung Diseases in Adults; SD = standard deviation; SHIP = Study of Health in Pomerania. * Data are presented as mean (SD) unless otherwise indicated; total no. participants = 27249, total no. FEV_1 measurements = 62130. † Pack-years are calculated among current and former smokers at study baseline.

RESULTS

The majority of the 14 cohort studies had FEV_1 at two times, but five studies (BHS, CARDIA, CHS, FHS, Health ABC) had ≥3 FEV_1 measurements per participant. The maximum length of follow-up ranged from 4 to 29 years. Studies with older participants generally had fewer current smokers and more former smokers, and had lower mean baseline FEV_1.

All 14 studies implemented a preliminary mixed model adjusted for all specified variables except the SNP terms and reported the estimated rate of change in FEV_1 by smok-

Table 2. Model estimates for the rate of change in FEV_1 in never smokers and effects of other smoking patterns (compared with never smokers) on the rate of change in FEV_1 (mL/year)*

Study	Annual FEV_1 change in never smokers (referent group)		Additional Effect† of smoking patterns on annual FEV_1 change					
			Persistent smokers		Intermittent smokers		Former smokers	
	β	SE	β	SE	β	SE	β	SE
ARIC	-14.0	1.3	-12.4	1.7	-5.5	2.1	-5.3	1.4
B58C	-29.6	1.5	-9.4	2.8	-2.2	3.4	-3.0	3.0
BHS	-23.0	1.0	-20.0	3.0	-8.0	2.0	-9.0	2.0
CARDIA	-26.4	0.5	-6.7	1.3	-0.2	1.0	1.0	1.2
CHS	-35.0	1.1	-2.2	3.3	-4.6	2.2	-2.4	1.7
FHS	-26.0	0.6	-8.1	1.3	-2.9	1.0	-1.1	0.8
Health ABC	-39.7	1.3	-12.9	6.1	-6.8	4.4	-2.6	1.7
KORA	-22.1	3.7	2.2	7.2	-10.4	9.3	2.8	5.2
LBC1921	-10.0	3.6	-11.6	15.7	2.8	14.4	-18.8	4.9
LBC1936	-32.3	3.6	-19.0	9.9	40.1	16.8	4.3	5.3
PIVUS	-21.1	2.5	-15.9	8.2	-21.7	13.4	-3.9	3.9
RS	-27.5	3.7	-1.8	9.0	9.3	8.6	-4.6	4.5
SAPALDIA	-29.7	1.2	-7.4	2.3	-2.0	2.6	-2.8	2.1
SHIP	-31.8	2.8	-0.4	10.9	-0.1	3.9	-15.0	7.3
14-cohort meta-analyzed estimate	-26.9	0.3	-8.8	0.7	-2.6	0.6	-2.3	0.5

Data shown are the effect estimates (β and SE) of the time and smoking-by-time interaction terms in the preliminary mixed effects model fully adjusted for all specified variables except the SNP terms.
Time represents the rate of change in FEV_1 in never smokers and the smoking-by-time interaction term represents the effects of the other three smoking patterns on the rate of change in FEV_1, compared with never smokers. Smoking categories are defined as persistent (smoke throughout follow-up), intermittent (stop and/or start smoking during follow-up) and former (smoke only prior to start of follow-up).
† Effect estimates in smoking categories are added to estimate in never smokers to compute the actual rate of change in each group (for example, in ARIC, the point estimate of the rate of change in FEV_1 in persistent smokers was -14.0 - 12.4 = -26.4 mL/year).

ing pattern (Table 2). The rate of decline in FEV_1 in never smokers ranged from 10.0 to 39.7 mL/year, and was generally steeper in studies with older participants, as expected.[4] Across all 14 studies, the meta-analyzed rate of change in FEV_1 was a decline of 26.9±0.3 mL/year in never smokers, and was 8.8±0.7, 2.6±0.6, and 2.3±0.5 mL/year steeper in persistent, intermittent, and former smokers, respectively (Table 2). We repeated the meta-analyses in the five cohort studies with ≥3 FEV_1 measurements per participant, and found similar, although less statistically significant results.

Study-specific genomic inflation factors (λ_{gc}) were calculated for the SNP-by-time interaction term and used for study-level genomic control prior to the meta-analyses. Study-specific λ_{gc} values ranged from 0.96 to 1.11 (Table E1) and the meta-analysis λ_{gc} was 1.01 for both the 14-study and five-study meta-analyses. Figures E1 and E2 (online supplement) present the Manhattan and quantile-quantile (QQ) plots.

In the meta-analysis including all 14 cohort studies, 15 SNPs at nine independent loci were associated with the rate of change in FEV_1 at $P < 1 \times 10^{-5}$, and none reached the genome-wide significance threshold of $P < 5 \times 10^{-8}$. The association results for the sentinel SNPs at these nine loci are presented in Table 3, and more detailed results for all 15 SNPs are included in Table E2 (online supplement). The most statistically significant association, and the only one that reached $P < 1 \times 10^{-6}$, was for rs4077833, an intronic SNP located in the novel *IL16/STARD5/TMC3* gene region on chromosome 15 ($P = 5.71 \times$

Table 3. Association of the most statistically significant SNPs with the rate of change in FEV_1 (mL/year) in the meta-analysis of 14 cohort studies (n = 27249)*

SNP	Chr	Position	Closest Gene(s)	Coded Allele	Frequency	β	SE	*P* Value
rs12137475	1	44059735	*ST3GAL3*	T	0.11	-3.5	0.8	3.90×10^{-6}
rs766488	1	61583103	*NFIA*	A	0.31	1.4	0.3	6.60×10^{-6}
rs17698444	1	215483178	*ESRRG/GPATCH2*	C	0.89	-2.2	0.5	2.62×10^{-6}
rs12692550	2	159958017	*BAZ2B*	T	0.17	-1.7	0.4	5.16×10^{-6}
rs2260722	13	113236292	*TMCO3*	A	0.72	-1.5	0.3	1.83×10^{-6}
rs4077833	15	79419738	*IL16/STARD5/TMC3*	C	0.10	2.3	0.5	5.71×10^{-7}
rs8027498	15	89595638	*SV2B*	A	0.25	1.4	0.3	9.41×10^{-6}
rs8051319	16	15794449	*MYH11*	T	0.60	1.7	0.3	5.12×10^{-6}
rs740557	17	62451139	*CACNG4*	C	0.85	-2.3	0.5	3.59×10^{-6}

Definition of abbreviations: Chr = chromosome; SE = standard error; SNP = single-nucleotide polymorphism.

* Data reported are the meta-analysis results of the SNP-by-time interaction term from the GWAS mixed effects model. A positive β-coefficient indicates an attenuation of FEV_1 decline and a negative β-coefficient an acceleration of FEV_1 decline.

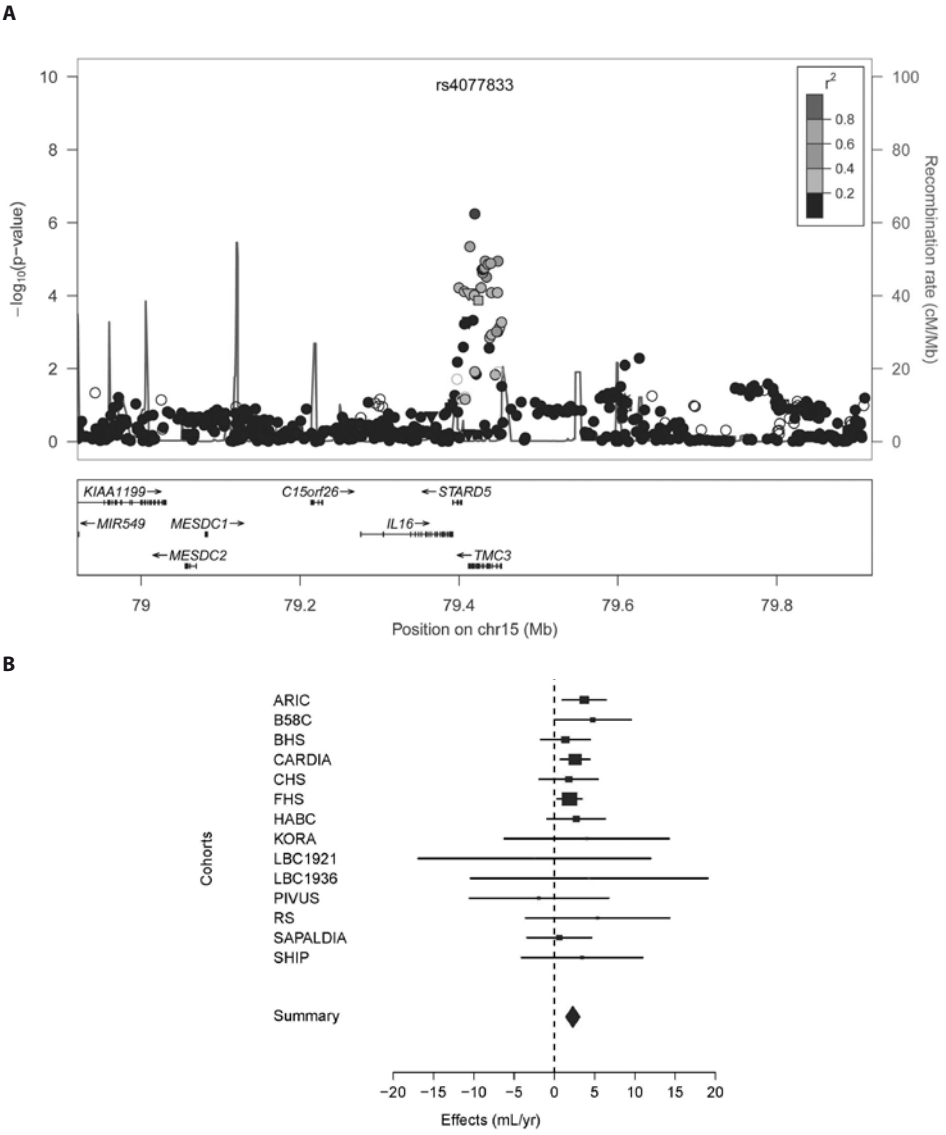

Figure 1. Association of the chromosome 15 locus with the rate of change in FEV_1 in the meta-analysis of 14 cohort studies

A) Regional association plot, where the X-axis is Megabase (Mb) position and Y-axes are the negative log of the P value on the left and recombination rate on the right. The sentinel SNP is colored in purple and linkage disequilibrium to the sentinel SNP is depicted by degree of color according to the legend.

B) Forest plot for rs4077833, where the size of the square for each study represents its contributing weight to the meta-analysis.

10^{-7}; Figure 1). The C allele of rs4077833, with a frequency of 10%, was associated with an attenuation of the rate of decline in FEV_1 by 2.3 mL/year.

For estimation of longitudinal trajectory in lung function, having more than two measurements over time provides greater precision.[4] We performed a further meta-analysis with the five cohort studies (BHS, CHS, CARDIA, FHS, Health ABC) having ≥3 FEV_1 measurements per participant, with a combined sample size of 10476 participants and 32054 FEV_1 measurements (online supplement for details). A novel region on chromosome 11 had a genome-wide significant association ($P < 5 \times 10^{-8}$) with the rate of change in FEV_1 (Table 4). The most statistically significant finding at this locus was for rs507211, an intronic SNP located in *ME3* (Figure 2). Six other SNPs, which are in linkage disequilibrium (LD) with rs507211 and are located in *ME3*, were identified at $P < 1 \times 10^{-6}$ (Table E3). The rs507211 A allele, with a frequency of 25%, was associated with an attenuation of the rate of decline in FEV_1 by 2.09 mL/year ($P = 2.18 \times 10^{-8}$). Besides the *ME3* locus, 17 SNPs from four other chromosomal regions had P values between 5×10^{-8} and 1×10^{-5} for associations with the rate of change in FEV_1 (Tables 4 and E3).

Corroborative evidence was sought for the sentinel SNP at each of the 14 loci associated at $P < 1 \times 10^{-5}$ (from both the 14-study and five-study meta-analyses) in 1494 adults from the AGES-Reykjavík population-based cohort study (Table E4). A P value of 0.004, representing the Bonferroni correction for 14 tests at the $\alpha = 0.05$ level, was selected a priori as the threshold for statistical significance. No SNPs achieved this threshold. The lowest P value was for rs740577 in *CACNG4* ($P = 0.08$), which showed consistent effect direction and magnitude with the original meta-analysis.

These same 14 SNPs were further examined in LHS, a clinical cohort study of 4048 smokers with mild COPD for evidence of consistent association between healthy and

Table 4. Association of the most statistically significant SNPs with the rate of change in FEV_1 (mL/year) in the meta-analysis of the five cohort studies with ≥3 FEV_1 measurements per participant (n = 10476)

SNP	Chr	Position	Closest Gene(s)	Coded Allele	Frequency	β	SE	*P* Value
rs10209501	2	28536881	*FOSL2/PLB1*	A	0.33	1.6	0.4	7.09×10^{-6}
rs12692550	2	159958017	*BAZ2B*	T	0.18	-2.0	0.4	2.02×10^{-6}
rs1729588	3	110790025	*FLJ25363/MIR4445*	A	0.30	1.6	0.4	8.38×10^{-6}
rs10764053	10	19863644	*C10orf112*	T	0.47	1.5	0.3	4.15×10^{-6}
rs507211	11	86054387	*ME3*	A	0.25	2.1	0.4	2.18×10^{-8}

Definition of abbreviations: Chr = chromosome; SE = standard error; SNP = single-nucleotide polymorphism.

* Data reported are the meta-analysis results of the SNP-by-time interaction term from the GWAS mixed effects model. A positive β-coefficient indicates an attenuation of FEV_1 decline and a negative β-coefficient an acceleration of FEV_1 decline.

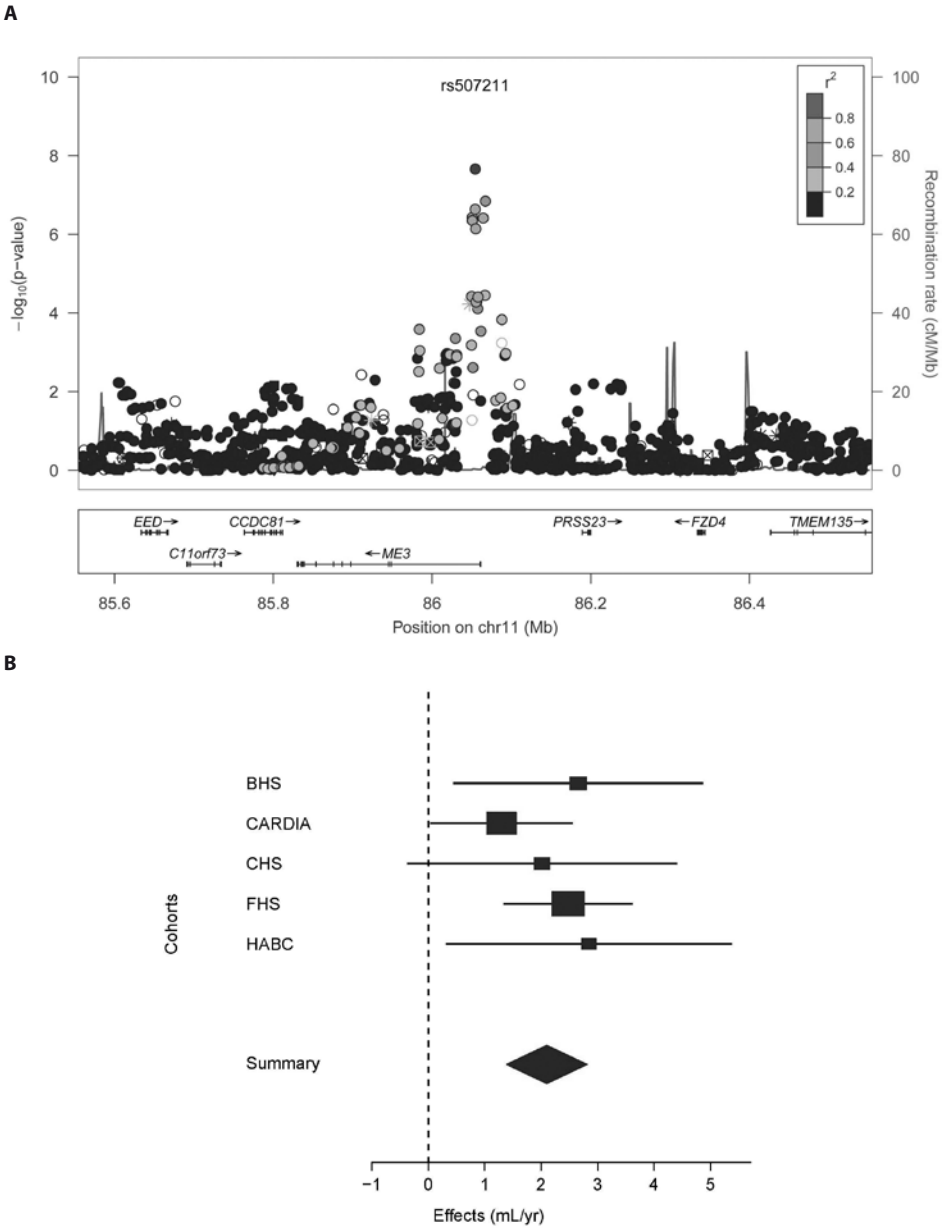

Figure 2. Association of the chromosome 11 locus with the rate of change in FEV_1 in the meta-analysis of the five cohort studies with ≥3 FEV_1 measurements per participant.
A) Regional association plot, where the X-axis is Megabase (Mb) position, and the Y-axes are the negative log of the P value on the left and recombination rate on the right. The sentinel SNP is colored in purple and linkage disequilibrium to the sentinel SNP is depicted by degree of color according to the legend.
B) Forest plot for rs507211, where the size of the square for each study represents its contributing weight to the meta-analysis.

diseased individuals.[17] None of the 14 SNPs were associated with the rate of change in FEV_1 in LHS at $P < 0.004$ (Table E4).

Previous meta-analyses in the CHARGE and SpiroMeta consortia identified 26 novel loci associated with cross-sectional FEV_1 and/or FEV_1/FVC at genome-wide significance.[9-11] We examined the sentinel SNPs from these loci in the meta-analysis of the 14 cohort studies for association with the rate of change in FEV_1 (Table E5). Given the a priori association with cross-sectional lung function, a P value threshold of 0.05 was used. Sentinel SNPs in *PID1*, *HHIP*, *GPR126*, and *CFDP1* showed association with the rate of change in FEV_1 ($0.005 \leq P \leq 0.048$).

Three genes (*IL16*, *STARD5*, and *TMC3*) at the novel chromosome 15 locus and *ME3* at the novel chromosome 11 locus were selected for follow-up mRNA expression profiling in human lung tissue, and primary cultures of human bronchial epithelial and airway smooth muscle cells, together with control tissues (peripheral blood mononuclear cells and brain). Transcripts of *STARD5* and *ME3* were found in all lung-derived tissues, transcripts of *IL16* were found in lung tissue and smooth muscle cells, but not in epithelial cells, and *TMC3* was not expressed in any of the lung-derived tissues (Table E6).

Using the public LGRC data repository, we found that the expression profiles of *IL16*, *STARD5*, and *ME3* in human lung samples showed statistically significant differences ($P < 0.05$) between COPD patients and controls (Figure E3). Lower levels of *IL16* ($P = 0.004$) were observed in COPD patients compared with controls, whereas higher levels of *STARD5* ($P = 3.22 \times 10^{-9}$) and *ME3* ($P = 0.044$) were observed in COPD patients compared with controls. Data on *TMC3* expression were not available.

We performed additional follow-up analysis of the sentinel SNPs at the two novel loci using an eQTL database of lymphoblastoid cell lines (Table E8). Trans-eQTL associations were observed between rs4077833 at the *IL16*/*STARD5*/*TMC3* locus and a nuclear receptor, NR1I2 (chromosome 3; $P = 6.84 \times 10^{-4}$) and between rs507211 at the *ME3* locus and *KIAA1109* (chromosome 4; $P = 5.20 \times 10^{-4}$), which is part of a gene cluster (*KIAA1109-TENR-IL2-IL21*) that encodes two interleukins (*IL2* and *IL21*).[18]

DISCUSSION

Although the genetic contribution to cross-sectional lung function phenotypes has been addressed by large-scale GWAS, much less information is available for longitudinal lung function phenotypes. To identify novel loci that specifically affect lung function

change over time, we performed a large-scale GWAS of the rate of change in FEV_1 in 27249 participants from 14 population-based cohort studies. We identified a novel locus (*IL16*/*STARD5*/*TMC3*) on chromosome 15 with suggestive evidence for association with the rate of change in FEV_1. Given the greater precision to estimate longitudinal trends with more measurements, a meta-analysis of the five cohort studies with ≥3 FEV_1 measurements per participant was performed, and it identified a second novel locus (*ME3*) on chromosome 11 at genome-wide statistical significance. For both loci, the minor allele was protective, and the magnitude of the association with the rate of change in FEV_1 was similar to that of being an intermittent or former smoker versus a never-smoker.

The sentinel SNP at the novel chromosome 15 locus is located in *TMC3*, although two neighboring genes, *IL16* and *STARD5* both harbor SNPs that are in modest LD with the sentinel SNP (Figure 1). *TMC3*, a member of the transmembrane channel-like gene family, likely functions as an ion channel, transporter, or modifier,[19] and has been associated with deafness and skin cancer.[20,21] *IL16* is a pleiotropic immunomodulatory cytokine that acts as a chemoattractant for $CD4^+$ cells and contributes to their recruitment and activation in response to inflammation.[22] Notably, asthma was the first disease where increased *IL16* expression was observed.[23] Subsequent studies confirmed that in the non-diseased state *IL16* is almost exclusively expressed by T lymphocytes in lymphatic tissue, whereas in asthmatic patients *IL16* is also synthesized by airway epithelial cells to inhibit airway inflammation.[24-26] A promoter polymorphism (T-295C) in *IL16* was associated with asthma in a Caucasian population in England,[27] although this finding was not confirmed in an Australian study.[28] *STARD5* belongs to the steroidogenic acute regulatory lipid transfer domain protein superfamily, and is involved in the trafficking of cholesterol and other lipids between intracellular membranes.[29] Recent in vitro studies showed increased *STARD5* expression and protein redistribution as a protective mechanism in response to induced endoplasmic reticulum (ER) stress and consequent overaccumulation of intracellular free cholesterol.[30] We confirmed the expression of *STARD5* in all human lung tissues examined and of *IL16* in human lung smooth muscle cells, but not epithelial cells, in line with previous observations. In contrast, no expression of *TMC3* was detected in any of the tested human lung tissues. We also found significantly lower levels of *IL16* in whole lung samples from COPD patients compared with controls, in contrast to its increased expression in asthma, and significantly higher levels of *STARD5* in COPD patients compared with controls. Taken together, these results suggest *IL16* as the most likely candidate accounting for the observed association, but further investigation is needed to elucidate underlying mechanisms.

The sentinel SNP at the novel chromosome 11 locus is located in *ME3*, whose protein product is a mitochondrial NADP(+)-dependent malic enzyme that catalyzes the oxida-

tive decarboxylation of malate to pyruvate using NADP+ as a cofactor.[31] Mitochondrial malic enzymes play a role in the energy metabolism in tumors, and are considered potential therapeutic targets in cancer.[32,33] We performed independent expression profiling of *ME3* and confirmed its expression in all human lung tissues examined, and found significantly higher levels of *ME3* in lung samples from COPD patients compared with controls. In addition, we looked up the sentinel SNP in *ME3* in a recent GWAS of airway obstruction and found a P value of 0.049.[34] Taken together, these results support *ME3* as a biologically plausible candidate in the regulation of lung function and pathogenesis of COPD.

The identification of trans-eQTL associations for the sentinel SNPs at both the *IL16/STARD5/TMC3* and *ME3* loci is interesting, and while the interpretation of trans-eQTL associations is ambiguous,[35] the regions these SNPs regulate merit further study.

Besides the GWAS meta-analyses, the assembly of 14 longitudinal cohort studies allowed us to meta-analyze the association of cumulative smoking patterns with the rate of change in FEV_1 in the general population. The meta-analyzed estimate for the rate of decline in FEV_1 in never smokers was 26.9 mL/year, and the annual decline was steeper in persistent, intermittent, and former smokers by 8.8, 2.6, and 2.3 mL/year, respectively. These findings provide a reference point for the effect of cigarette smoking on longitudinal lung function change in the general population.

There is phenotypic variation among the 14 cohort studies in aspects such as baseline age and cigarette smoking, and in factors that are of special importance to this longitudinal GWAS, such as the number of FEV_1 measurements per participant and follow-up duration. Phenotypic heterogeneity represents a general challenge in genetic epidemiology, particularly in the investigation of longitudinal phenotypes. Thus, we performed a meta-analysis using the subset of cohort studies with ≥3 FEV_1 measurements per participant, given that longitudinal trajectories are best estimated over longer time periods and with more measurements. There was little overlap between the top loci identified in the two meta-analyses at $P < 1 \times 10^{-5}$, suggesting that phenotypic heterogeneity affected the association results. Future meta-studies of lung function decline should aim to increase sample size while maintaining high phenotypic comparability among participating studies.

We sought corroborative evidence in a single cohort study of 1494 participants. This sample size is much smaller and arguably insufficient compared with replications applied to previous studies of cross-sectional lung function phenotypes. Thus, despite the lack of corroboration for the two novel loci identified in the meta-analyses, results from

the complementary gene expression analyses provide compelling evidence for biologically plausible roles of the implicated genes in the longitudinal change in lung function.

None of the 14 sentinel SNPs were associated with the rate of change in FEV_1 in the COPD patient-based LHS cohort. Similarly, a previous population-based GWAS of lung function decline noted a high degree of heterogeneity in findings when analyses were stratified by presence/absence of asthma.[12] The observed discrepancy of association results suggests that the genetic determination of lung function decline may be different in healthy individuals compared with COPD patients, may contribute differentially in a pre-diseased vs. post-diseased state in which medications may influence the rates of decline, or that LHS was underpowered for confirming our findings.

In summary, we performed GWAS of the longitudinal change in lung function and subsequent meta-analyses, using harmonized data from more than 27000 participants of European ancestry to identify genetic loci influencing the rate of change in FEV_1. We identified the novel *ME3* locus on chromosome 11 at genome-wide significance and found suggestive evidence for association at the novel *IL16/STARD5/TMC3* locus on chromosome 15. Additional expression analyses confirmed the expression of *ME3*, *IL16*, and *STARD5* in multiple lung tissues, and found differential expression profiles of these three genes in the lungs of COPD patients compared to non-COPD controls. These results support the involvement of these implicated genes in the longitudinal change in lung function in the general population.

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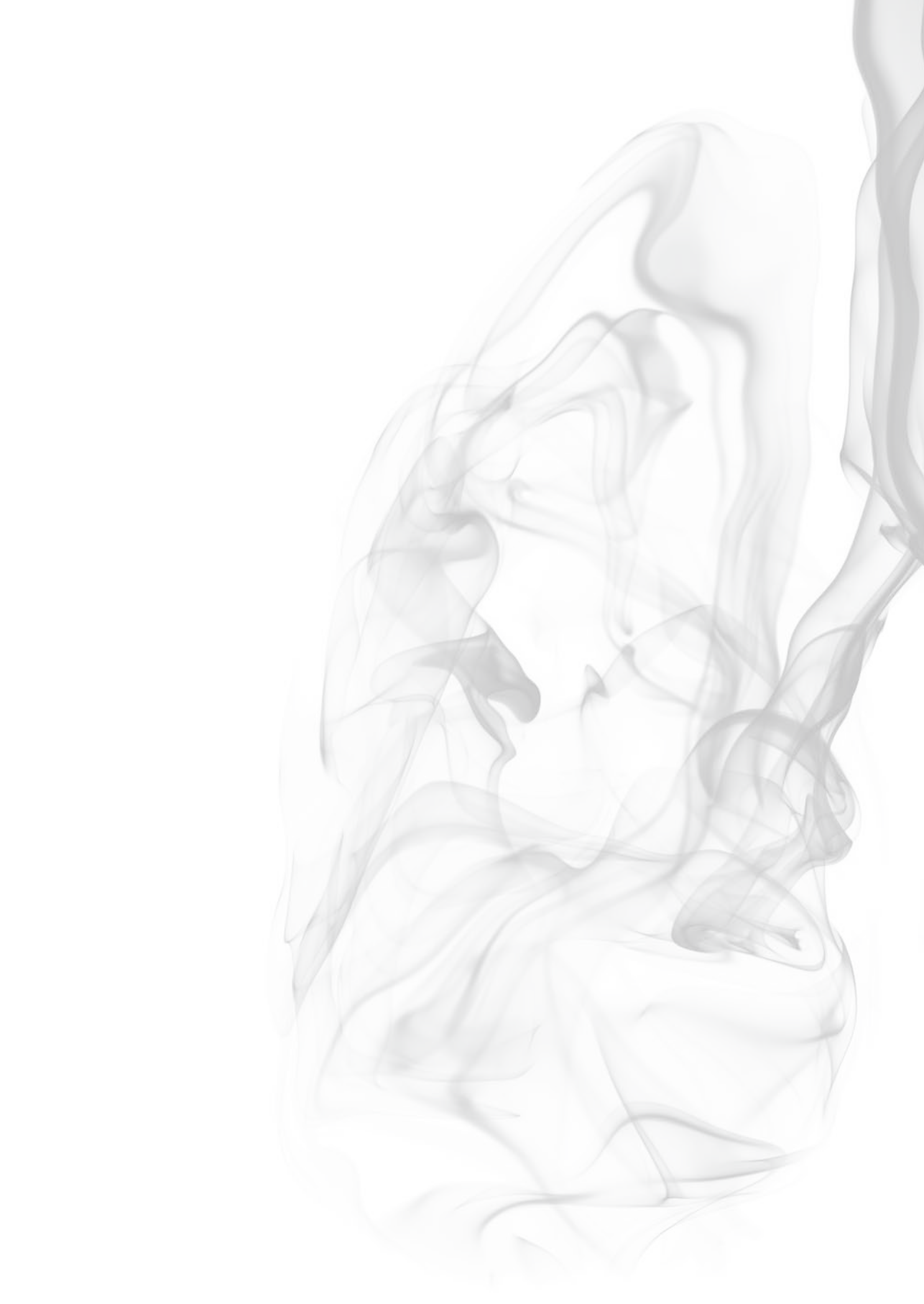

CHAPTER 3.5

Genome-Wide Association Studies Identify *CHRNA5*/3 and *HTR4* in the Development of Airflow Obstruction

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Published in American Journal of Respiratory and Critical Care Medicine, 2012 Oct 1;186(7):622-32.
doi: 10.1164/rccm.201202-0366OC. Epub 2012 Jul 26. PubMed PMID: 22837378; PubMed Central PMCID: PMC3480517.

ABSTRACT

Introduction Rationale Genome-wide association studies (GWAS) have identified loci influencing lung function, but fewer genes influencing chronic obstructive pulmonary disease (COPD) are known.

Objectives Perform meta-analyses of GWAS for airflow obstruction, a key pathophysiologic characteristic of COPD assessed by spirometry, in population-based cohorts examining all participants, ever smokers, never smokers, asthma-free participants, and more severe cases.

Methods Fifteen cohorts were studied for discovery (3,368 affected; 29,507 unaffected), and a population-based family study and a meta-analysis of case-control studies were used for replication and regional follow-up (3,837 cases; 4,479 control subjects). Airflow obstruction was defined as FEV_1 and its ratio to FVC (FEV_1/FVC) both less than their respective lower limits of normal as determined by published reference equations.

Measurements and Main Results The discovery meta-analyses identified one region on chromosome 15q25.1 meeting genome-wide significance in ever smokers that includes AGPHD1, *IREB2*, and *CHRNA5*/*CHRNA3* genes. The region was also modestly associated among never smokers. Gene expression studies confirmed the presence of *CHRNA5*/3 in lung, airway smooth muscle, and bronchial epithelial cells. A single-nucleotide polymorphism in *HTR4*, a gene previously related to FEV_1/FVC, achieved genome-wide statistical significance in combined meta-analysis. Top single-nucleotide polymorphisms in *ADAM19*, *RARB*, *PPAP2B*, and *ADAMTS19* were nominally replicated in the COPD meta-analysis.

Conclusions These results suggest an important role for the *CHRNA5*/3 region as a genetic risk factor for airflow obstruction that may be independent of smoking and implicate the *HTR4* gene in the etiology of airflow obstruction.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death worldwide, and cigarette smoking is the most widely recognized risk factor for this disease. COPD is defined based on spirometry as airflow obstruction that is not fully reversible after administration of a bronchodilator. Airflow obstruction is a key pathophysiologic characteristic of COPD that is assessed by spirometry. Both COPD and spirometry measures of lung function have been demonstrated to have a genetic component. Family studies have reported an increased risk for COPD in relatives of a COPD proband [1] as well as significant heritability of pulmonary function measured by spirometry in population-based cohorts [2]. The α1-antitrypsin gene (*SERPINA1*/A1AT) is known to be associated with COPD and leads to increased risk for early-onset disease among individuals carrying the susceptibility alleles, but few other genes have such a conclusive relationship to COPD.

Recent genome-wide association studies (GWAS) have examined two spirometry measures of lung function, FEV_1 and its ratio to FVC (FEV_1/FVC). Two large-scale GWAS meta-analyses identified a total of 11 loci related to FEV_1 or FEV_1/FVC [3,4], and a larger meta-analysis incorporating these studies along with new studies identified an additional 16 loci [5]. Two genetic loci identified by the above studies, *HHIP* and *FAM13A*, have been demonstrated to influence risk of COPD at genome-wide levels of statistical significance [6-9]. GWAS of COPD have also identified associations with SNPs in a region on chromosome 15q25.1 that includes cholinergic nicotinic receptor genes (*CHRNA5-CHRNA3-CHRNB4*) and the iron-responsive element binding protein 2 (*IREB2*) [7], but some questions remain as to the underlying genetic signal because of substantial linkage disequilibrium in the region. This region has also been associated with lung cancer [10,11] and nicotine dependence [12-15], leading to the hypothesis that the association with the various disease endpoints may be mediated through the nicotinic receptor genes and thus smoking, smoking intensity, and cessation [16]. In a meta-analysis of lung cancer among never smokers, no association to the CHRNA genes was observed, supporting the hypothesis that association was mediated through smoking behavior [17]. However, the observation of increased *IREB2* protein and mRNA expression in COPD lung tissue compared with controls supports its potential involvement as well [18].

The standard definition of COPD is based on the presence of airflow obstruction that persists after administration of bronchodilator [19]. In large population-based cohorts, post-bronchodilator spirometry is not generally available, so we have studied prebronchodilator airflow obstruction as a proxy for COPD. In this study, we performed GWAS using a standardized definition of airflow obstruction and control subjects across 15

population-based cohort studies and conducted a meta-analysis. We then sought replication of our top single-nucleotide polymorphisms (SNPs) and regions in a set of four COPD case-control studies previously included in a meta-analysis and in a population-based family study that used the same airflow obstruction phenotype definitions used in the discovery analyses.

METHODS

Discovery Phase

Most of the cohorts used in the discovery phase of this meta-analysis were included in meta-analyses of cross-sectional quantitative pulmonary function measures in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium[3], the SpiroMeta consortium [4], and/or their joint analysis [5]. Cohorts not included in previous GWAS discovery sets for pulmonary function include Rotterdam Study III (RS3), Swiss Study on Air Pollution and Lung and Heart Disease in Adults (SAPALDIA), Lothian Birth Cohort (LBC1936), Multi-Ethnic Study of Atherosclerosis (MESA), and COPD Pathology: Addressing Critical gaps, Early Treatment and diagnosis, and Innovative Concepts (COPACETIC). All of the included participants are white and of European descent.

Standardized definitions of airflow obstruction based on the lower limit of normal of FEV_1 and FEV_1/FVC from the National Health and Nutrition Examination Survey III prediction equations [20] were used across all cohorts. The presence of airflow obstruction was defined as an FEV_1 and FEV_1/FVC both less than the lower limit of normal [21] based on prediction equations that include age, age^2, and height^2 calculated separately by sex. Unaffected participants were defined by FEV_1, FVC, and FEV_1/FVC all above the lower limit of normal. Individuals below the lower limit of normal for FEV_1 or FEV_1/FVC but not both were excluded from these analyses. Logistic regression models were adjusted for current and former smoking dummy variables, pack-years of smoking, age, sex, standing height, center/cohort as needed, and principal components for genetic ancestry as needed.

Genome-wide imputation and analyses were performed by the cohort investigators, and results were shared for meta-analysis. Details of individual cohorts' imputation and GWAS methods are provided in the online supplement text and Table E1 in the online supplement. Genome-wide and regional meta-analyses were performed using METAL software [22] with inverse variance weighting to combine effect size estimates after applying a genomic control correction [23].

Five discovery analyses were performed. GWAS were performed in (1) all cohorts with both ever and never smokers, (2) ever smokers, (3) never smokers, (4) asthma-free participants, and (5) the subset of more severe airflow obstruction with FEV_1 less than 65% predicted (excluding milder cases from analysis). Never smoking GWAS were performed in eight cohorts. In the 10 cohorts that collected self-reported asthma data, an analysis was performed excluding all participants reporting a history of asthma with diagnosis before age 40 years or missing onset age.

Regional meta-analysis and replication

Two strategies were implemented for follow-up of top results. In two regions with association signals spanning multiple genes in discovery meta-analyses, results across the whole region were requested from the replication studies, and combined meta-analyses were performed to refine the association signal. These regions were located on chromosome 6 (27,599,278–32,787,304 bp) and chromosome 15 (76,499,754–76,711,042 bp). In addition, 60 SNPs with P values less than or equal to 1×10^{-5} in any of the five discovery meta-analyses were selected for replication. Combined meta-analysis was performed with the Family Heart Study (FamHS), which evaluated the same airflow obstruction phenotype as used in the discovery phase (331 affected and 2,550 unaffected). Replication was further evaluated in a meta-analysis of studies with clinically ascertained COPD (3,499 cases and 1,922 control subjects) [24]. Gene expression in lung tissues was evaluated for two genes on chromosome 15. Additional details are included in the online supplement: http://www.atsjournals.org/doi/suppl/10.1164/rccm.201202-0366OC/suppl_file/Wilk_ODS.pdf

RESULTS

Descriptive characteristics of the 15 discovery cohorts are provided in Tables 1 and 2. The mean FEV_1 % predicted for participants with airflow obstruction ranged from 48.9 to 68.7% across cohorts, and for unaffected participants the means were generally around 100%. The mean FEV_1/FVC ratio ranged from 49.5 to 62.5% among affected participants and 74.1 to 81% among unaffected participants across the cohorts. The mean ages at measurement of spirometry across the cohorts ranged from 45 to 76 years. The number of participants contributing to each of the five discovery GWAS meta-analyses are provided in Table 3. The genomic control (λ_{GC}) values ranged from 0.946 to 1.045 for each cohort's GWAS and from 1.011 to 1.060 in the meta-analysis (Table E2). Figures E1 to E5 present the Manhattan and quantile-quantile (QQ) plots for the five discovery meta-analyses.

Table 1. Descriptive characteristics of cohorts included in the discovery meta-analysis

	ARIC	FHS	CHS	COPACETIC	B58C	EPIC	MESA
No. affected	914	571	402	312	264	127	104
No. Unaffected	6,602	5,866	2,183	996	4,374	1,023	979
Age, yr	54.3 (5.7)	51.6 (14.6)	72.3 (5.3)	60.2 (5.6)	45.2 (0.39)	58.2 (9.0)	66.1 (9.8)
Male, %	47.2	46.4	39.4	39.4	49.6	46.8	49.6
Height, cm	169 (9)	168 (10)	165 (9)	165 (9)	169 (9)	167 (9)	169 (10)
BMI, kg/m²	27.0 (4.7)	27.2 (5.2)	26.2 (4.3)	-	27.4 (4.9)	26.4 (3.9)	28.0 (5.2)
Current Smoker, %	21.8	14.2	9.3	9.3	21.3	10.1	7.9
Former Smoker, %	35.8	38.1	39.7	39.7	49.1	44.6	50.8
Pack-years smoking *	27.5 (21.4)	21.9 (21.2)	32.2 (26.7)	39.6 (17.0)	14.7 (11.7)	17.6 (16.0)	29.4 (28.5)
FEV_1/FVC							
Affected	58.1 (8.6)	58.6 (8.3)	52.0 (10.7)	51.0 (9.1)	62.5 (7.0)	57.0 (8.5)	56.6 (9.2)
Unaffected	76.6 (4.4)	77.6 (5.3)	74.1 (5.6	75.5 (4.8)	80.9 (5.6)	81.0 (6.4)	75.5 (5.8)
FEV_1 % predicted							
Affected	62.2 (13.4	63.2 (12.8)	52.1 (15.5)	58.9 (11.1)	68.7 (9.5)	57.1 (13.8)	61.0 (12.7)
Unaffected	99.5 (11.1)	100.4 (11.8)	98.4 (13.5)	105.8 (12.8)	100.7 (10.7)	96.6 (9.9)	98.2 (12.0)

Definition of abbreviations: ARIC = Atherosclerosis Risk in Communities; B58C = British 1958 Birth Cohort; CHS = Cardiovascular Health Study; COPACETIC = COPD Pathology: Addressing Critical gaps, Early Treatment and diagnosis, and Innovative Concepts; EPIC = European Prospective Investigation into Cancer and Nutrition; FHS = Framingham Heart Study; MESA = Multi-Ethnic Study of Atherosclerosis. Data are presented as mean (SD) unless otherwise indicated. * Pack-years calculated among current and former smokers.

Discovery meta-analysis

One region on chromosome 15 had 11 SNPs with genome-wide significant results (P values $< 5 \times 10^{-8}$) in discovery meta-analysis of ever smokers (Table 4). An SNP in the *AGPHD1* gene between the *IREB2* gene and CHRN gene cluster was the top association with airflow obstruction among ever smokers (rs8031948, P value = 2.8×10^{-9}) with the minor allele conferring a 22% higher risk of airflow obstruction. Among 14 cohorts with both smoking and never-smoking participants, the top SNP results for all subjects combined were found in the same chromosome 15 region but localized to the *CHRNA5* gene (rs17486278, P value = 1.9×10^{-7}). For comparison, results among never smokers (504 affected, 10,690 unaffected from eight cohorts) are included in Table 4, and the smallest P value in the region (8.4×10^{-5}) occurs at a synonymous SNP (rs1051730) in *CHRNA3*. The odds ratios (OR) shown in Table 4 demonstrate consistency in the effect size for the tested allele across the analyses of all cohorts with both ever and never smoking participants (14 cohorts), ever smokers (15 cohorts), and never smokers (8 cohorts). The results

Table 2. Descriptive characteristics of additional cohorts included in the discovery meta-analysis

	AGES	Health ABC	RS1	Sapaldia	BHS	RS3	LBC1936	RS2
No. affected	109	108	99	98	89	70	61	40
No. Unaffected	1,562	1,129	1,003	833	661	1,001	627	668
Age, yr	76.2 (5.6)	73.8 (2.8)	74.4 (5.7)	51.0 (11.1)	54.6 (16.3)	56.6 (5.5)	69.6 (0.9)	67.1 (6.3)
Male, %	40.6	52.1	42.9	48.1	44.0	42.3	49.4	43.8
Height, cm	167(9)	167 (9)	167 (9)	169 (9)	168 (9)	171 (9)	166 (9)	168 (9)
BMI, kg/m²	27.1 (4.5)	26.5 (4.1)	27.4 (4.0)	25.7 (4.3)	25.8 (4.0)	27.4 (4.6)	27.4 (4.1)	27.6 (4.0)
Current Smoker, %	9.7	6.6	10.7	20.4	16.4	19..0	11.1	13.4
Former Smoker, %	42.2	49.0	56.2	32.9	26.8	47.0	40.7	51.7
Pack-years smoking *	24.5 (22.0)	36.0 (32.4)	25.8 (23.0)	21.5 (23.0)	20.1 (19.6)	18.5 (18.0)	34.3 (23.1)	23.8 (22.9)
FEV_1/FVC								
Affected	49.5 (18.1)	56.3 (6.1)	56.0 (7.2)	59.2 (7.3)	58.2 (9.8)	59.2 (8.4)	54.6 (8.6)	56.7 (6.9)
Unaffected	75.6 (7.2)	76.0 (5.1)	75.6 (5.4)	76.8 (5.0)	78.4 (5.4)	80.2 (5.8)	80.4 (6.7)	78.2 (5.9
FEV_1 % predicted								
Affected	48.9 (20.2)	55.3 (11.6)	56.9 (11.0)	67.6(10.4)	57.3 (16)	60.4 (13.3)	50.8 (10)	58.8 (10.5)
Unaffected	93.2 (18.3)	100.2 (14.0)	102.8 (18.7)	103.5 (11.3)	98.9 (11.7)	104.6 (13.1)	100.4 (12.2)	105.5 (17.3)

Definition of abbreviations: AGES = Age, Gene, Environment Susceptibility; BHS = Busselton Health Study; Health ABC = Health, Aging and Body Composition; LBC1936 = Lothian Birth Cohort 1936; RS1 = Rotterdam Study I; RS2 = Rotterdam Study II; RS3 = Rotterdam Study III; SAPALDIA = Swiss Study on Air Pollution and Lung and Heart Disease in Adults. Data are presented as mean (SD) unless otherwise indicated. * Pack-years calculated among current and former smokers.

in Table 4 were based on meta-analyses that included different cohorts as presented in Table 3, and thus the ever and never smoker results do not reflect a straightforward stratified analysis of all participants. The inclusion of the COPACETIC study in the ever smokers meta-analysis contributed to the improved association signal in the region. In the GWAS of a more severe airflow obstruction phenotype defined by FEV_1 less than 65% predicted, a missense SNP in the *CHRNA5* gene (rs16969968, Asp398Asn) had the third ranked P value (5×10^{-7}) with an OR of 1.22. No other genome-wide significant associations were identified among discovery meta-analyses. In the meta-analysis of never smokers (504 affected and 10,690 control subjects), several top SNPs were observed 570 kb away from *ADARB2* (the closest gene), and results from the FEV_1 less than 65% meta-analysis also implicated this locus. Among never smokers, the chromosome

Table 3. Sample Sizes Contributed by each cohort for the five discovery meta-analyses of airflow obstruction

	All Participants		Ever Smokers		Never Smokers		Asthma Free*		FEV_1 < 65 %†	
	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected
ARIC	914	6,602	821	3,510	93	3,092	814	6,355	452	6,602
FHS	571	5,866	457	2,909	114	2,957	391	5,210	274	5,866
CHS	402	2,183	317	950	85	1,233	363	2,135	292	2,183
RS1	99	1,003	87	650	12‡	353‡	97§	967§	68	1,003
RS2	40	668	37	424	3‡	244‡	NA	NA	29	668
RS3	70	1,001	57	650	13‡	351‡	NA	NA	39	1,001
Health ABC	108	1,129	94	593	14‡	536‡	70	1,077	80	1,129
AGES	109	1,562	81	787	28	775	NA	NA	34	1,562
EPIC	127	1,023	79	490	48	533	110	992	88	1,023
BHS	89	661	46	278	43	383	20	421	53	661
SAPALDIA	98	833	59	437	39	396	42	620	38	833
LBC1936	61	627	50	306	11‡	321‡	60	622	56	627
B58C	264	4,374	210	3,053	54	1321	183	4,036	75	4,374
COPACETIC	0	0	312	996	NA	NA	NA	NA	142	996
MESA	104	979	89	533	15‡	531‡	85	923	54	979
Total	3,056	28,511	2,796	16,566	504	10,690	2,138	22,391	1,774	29,507

Definition of abbreviations: Definition of abbreviations: AGES = Age, Gene, Environment Susceptibility; ARIC = Atherosclerosis Risk in Communities; B58C = British 1958 Birth Cohort; BHS = Busselton Health Study; CHS = Cardiovascular Health Study; COPACETIC = COPD Pathology: Addressing Critical gaps, Early Treatment and diagnosis, and Innovative Concepts; EPIC = European Prospective Investigation into Cancer and Nutrition; FHS = Framingham Heart Study; Health ABC = Health, Aging and Body Composition; LBC1936 = Lothian Birth Cohort 1936; MESA = Multi-Ethnic Study of Atherosclerosis.; RS1 = Rotterdam Study I; RS2 = Rotterdam Study II; RS3 = Rotterdam Study III; SAPALDIA = Swiss Study on Air Pollution and Lung and Heart Disease in Adults. *Asthma-free: no history of an asthma diagnosis before age 40 years; participants reporting asthma with missing data on age at diagnosis were also excluded. † FEV_1<65%: cases were restricted to those with FEV_1 < 65% and FEV_1/FVC less than the lower limit of normal; the definition of control subjects was the same as used for all participants. NA = Not analyzed due to low number of cases. §Results were not available for discovery meta-analysis.

6 major histocompatability locus (MHC) region was among top results. The discovery meta-analysis results for the 60 SNPs selected for replication are included in Table 5. Genome-wide results for all five definitions of airflow obstruction are available in the online supplement.

Meta-analysis of Chromosome 6 and 15 regions with replication studies

Regional meta-analyses were performed to further evaluate the regions on chromosomes 6 and 15 with the additional two replication studies. In discovery analysis of all

Table 4. Genome-wide significant results on chromosome 15 from discovery meta-analysis of airflow obstruction

				Coded allele		All* 14 cohorts		Never-smokers 8 cohorts		Ever-smokers 15 cohorts	
SNP	**position**	**gene**	**function**	**allele**	**frequency**	**OR**	**P_value**	**OR**	**P-value**	**OR**	**P-value**
rs8031948	76603112	*AGPHD1*	Intronic	t	0.35	1.17	4.76E-07	1.15	1.53E-03	1.22	2.78E-09
rs931794	76613235	*AGPHD1*	Intronic	a	0.65	0.86	6.18E-07	0.87	1.46E-03	0.82	4.69E-09
rs10519203	76601101	*AGPHD1*	Intronic	a	0.65	0.86	7.16E-07	0.86	1.27E-03	0.82	5.67E-09
rs9788721	76589924	*AGPHD1*	Intronic	t	0.65	0.86	1.04E-06	0.86	1.17E-03	0.82	9.76E-09
rs2036527	76638670			a	0.34	1.18	3.51E-07	1.18	2.85E-04	1.22	1.72E-08
rs17486278	76654537	*CHRNA5*	Intronic	a	0.66	0.85	1.91E-07	0.84	1.06E-04	0.83	2.43E-08
rs7180002	76661048	*CHRNA5*	Intronic	a	0.66	0.85	2.23E-07	0.84	1.20E-04	0.83	2.68E-08
rs1051730	76681394	*CHRNA3*	synonymous	a	0.33	1.17	3.29E-07	1.20	8.36E-05	1.21	3.36E-08
rs16969968	76669980	*CHRNA5*	missense	a	0.34	1.17	3.46E-07	1.19	1.25E-04	1.20	3.47E-08
rs951266	76665596	*CHRNA5*	Intronic	a	0.34	1.17	3.64E-07	1.19	1.41E-04	1.21	3.47E-08
rs1317286	76683184	*CHRNA3*	Intronic	a	0.66	0.85	3.93E-07	0.84	1.18E-04	0.83	4.74E-08

Definition of abbreviation: OR = Odds Ratio. *All cohorts with both ever and never smoking participants

Table 5. Odds ratios (OR) and p-values for the 60 SNPs identified in the discovery meta-analysis and selected for replication and combined meta-analysis with the Family Heart Study. SNPs are ordered by the combined meta-analysis p-value.

	Coded Allele					Discovery meta-analysis			Family Heart Study*		Combined meta-analysis	
SNP	**Allele**	**Freq**	**Chr**	**position**	**Closest gene**	**OR**	**P-value**	**analysis**	**OR**	**P-value**	**OR**	**P-value**
rs7733088	A	0.38	5	147836526	*HTR4*	0.82	6.53E-08	smoker	0.75	0.015	**0.81**	**4.09E-09**
rs2044029	A	0.4	15	69467013	*THSD4*	1.16	4.95E-07	all	1.20	0.078	1.17	1.06E-07

Table 5. (Continued)

	Coded Allele					Discovery meta-analysis			Family Heart Study*		Combined meta-analysis	
SNP	Allele	Freq	Chr	position	Closest gene	OR	P-value	analysis	OR	P-value	OR	P-value
rs181654	A	0.28	10	119369646	*EMX2*	0.82	1.24E-06	no asthma	0.76	0.030	0.81	1.31E-07
rs4597955	A	0.59	5	147827466	*HTR4*	0.84	3.12E-06	smoker	0.76	0.011	0.83	1.57E-07
rs12905014	T	0.95	15	90684844	*ST8SIA2*	0.58	5.24E-06	FEV_1 65%	0.47	0.020	0.57	3.83E-07
rs11744671	T	0.92	5	156853809	*ADAM19*	0.72	8.25E-07	no asthma	0.77	0.264	0.72	4.51E-07
rs8033889	T	0.21	15	69467134	*THSD4*	1.18	2.49E-06	all	1.23	0.066	1.19	4.67E-07
rs6684428	A	0.16	1	56132401	*PPAP2B*	1.24	3.60E-07	smoker	1.08	0.574	1.23	4.73E-07
rs4767234	A	0.59	12	113122231	*TBX5*	1.18	1.28E-06	smoker	1.15	0.242	1.17	6.32E-07
rs4534959	A	0.97	18	60028136	*SERPINB8*	0.57	4.19E-07	FEV_1 65%	0.91	0.844	0.58	6.90E-07
rs715921	A	0.31	13	23693489	*SPATA13*	1.22	5.18E-07	no asthma	1.07	0.551	1.20	7.60E-07
rs16889038	T	0.92	6	24414366	*DCDC2*	0.7	4.15E-07	FEV_1 65%	0.95	0.845	0.72	7.94E-07
rs9536318	A	0.83	13	52392695	*PCDH8*	0.81	5.47E-06	no asthma	0.75	0.051	0.80	8.24E-07
rs1997352	A	0.26	3	25513321	*RARB*	0.85	4.29E-06	all	0.82	0.076	0.84	8.64E-07
rs10759102	A	0.33	9	9900123	*PTPRD*	0.81	4.66E-06	FEV_1 65%	0.77	0.083	0.81	1.04E-06
rs13144621	T	0.32	4	109437378	*LEF1*	0.85	5.87E-07	all	0.98	0.850	0.86	1.20E-06
rs1982234	C	0.63	15	69478345	*THSD4*	1.16	4.80E-06	all	1.16	0.136	1.16	1.55E-06
rs7799265	C	0.95	7	28399001	*CREB5*	0.63	8.84E-07	FEV_1 65%	0.91	0.742	0.65	1.71E-06
rs181652	A	0.54	10	119369077	*EMX2*	1.18	8.12E-06	no asthma	1.19	0.097	1.18	1.94E-06
rs11766496	C	0.12	7	71026786	*CALN1*	1.34	3.90E-07	all	0.97	0.855	1.30	2.01E-06
rs2263638	A	0.37	10	94158777	*IDE*	0.78	4.68E-06	never smoker	0.80	0.275	0.78	2.53E-06
rs7850092	A	0.21	9	9899119	*PTPRD*	0.79	4.00E-06	FEV_1 65%	0.85	0.324	0.79	2.60E-06

Table 5. (Continued)

	Coded Allele					Discovery meta-analysis			Family Heart Study*		Combined meta-analysis	
SNP	**Allele**	**Freq**	**Chr**	**position**	**Closest gene**	**OR**	**P-value**	**analysis**	**OR**	**P-value**	**OR**	**P-value**
rs1329705	A	0.2	6	142795031	*GPR126*	0.79	3.75E-06	FEV_1 65%	0.85	0.342	0.79	2.63E-06
rs11209261	A	0.76	1	68557801	*GPR177*	0.81	6.76E-06	FEV_1 65%	0.80	0.191	0.81	2.78E-06
rs7607316	A	0.21	2	237186581	*CXCR7*	1.28	9.19E-06	never smoker	1.35	0.152	1.29	3.21E-06
rs9975851	T	0.57	21	26638525	*CYYR1*	1.18	4.92E-06	no asthma	1.11	0.332	1.17	3.66E-06
rs12505749	C	0.92	4	57028869	*SRP72*	0.76	3.06E-07	all	1.22	0.258	0.79	4.69E-06
rs1207393	C	0.36	22	24983362	*SEZ6L*	0.83	8.69E-06	FEV_1 65%	0.85	0.283	0.84	4.78E-06
rs12744110	T	0.25	1	56168897	*PPAP2B*	1.18	4.51E-06	smoker	1.06	0.681	1.17	5.95E-06
rs11097912	T	0.33	4	107219911	*MGC16169*	0.85	6.04E-06	smoker	0.92	0.497	0.85	5.95E-06
rs17086172	T	0.94	18	68378001	*CBLN2*	0.73	7.13E-06	no asthma	0.86	0.492	0.74	7.23E-06
rs2322734	A	0.96	3	4608492	*ITPR1*	0.67	7.19E-06	FEV_1 65%	0.83	0.518	0.69	7.52E-06
rs8036030	A	0.39	15	72503662	*SEMA7A*	0.84	5.71E-06	no asthma	0.95	0.653	0.85	8.31E-06
rs892961	A	0.41	17	72911695	*SEPT9*	0.85	8.00E-06	no asthma	0.93	0.479	0.85	8.91E-06
rs7629245	T	0.15	3	186624551	*MAP3K13*	1.23	0.00001	smoker	1.12	0.456	1.22	9.06E-06
rs2830165	T	0.59	21	26598463	*APP*	0.81	5.27E-06	never smoker	0.98	0.892	0.82	9.55E-06
rs7686928	T	0.14	4	188970823	*ZFP42*	1.21	8.77E-06	smoker	1.08	0.630	1.21	9.65E-06
rs9632471	C	0.72	5	128761894	*ADAMTS19*	0.76	5.54E-06	FEV_1 65%	0.95	0.776	0.77	1.07E-05
rs4837614	T	0.15	9	118350186	*ASTN2*	0.76	4.56E-06	FEV_1 65%	0.98	0.911	0.78	1.20E-05
rs12960805	A	0.41	18	7909707	*PTPRM*	1.27	6.22E-06	never smoker	1.00	0.995	1.25	1.27E-05
rs1799257	A	0.12	19	53664351	*PSCD2*	1.37	2.08E-06	FEV_1 65%	0.78	0.337	1.33	1.38E-05
rs1868466	A	0.79	16	76301059	*KIAA1576*	0.85	5.45E-06	all	1.00	0.997	0.86	1.39E-05

Table 5. (Continued)

	Coded Allele					Discovery meta-analysis			Family Heart Study*		Combined meta-analysis	
SNP	Allele	Freq	Chr	position	Closest gene	OR	P-value	analysis	OR	P-value	OR	P-value
rs10518948	C	0.93	15	69415023	*THSD4*	0.68	9.91E-06	never smoker	0.97	0.931	0.69	1.51E-05
rs6901575	A	0.1	6	28358963	*PGBD1*	1.24	6.76E-06	all	0.97	0.853	1.22	1.55E-05
rs3790728	T	0.97	1	215737150	*GPATCH2*	0.57	3.12E-06	FEV_1 65%	4.24	0.060	0.60	1.58E-05
rs9511117	A	0.09	13	23660045	*SPATA13*	0.73	6.78E-06	smoker	1.01	0.979	0.75	1.65E-05
rs4957070	A	0.63	5	600858	*SLC9A3*	0.85	9.09E-06	smoker	0.98	0.874	0.86	1.65E-05
rs3814818	T	0.9	14	94263130	*GSC*	0.74	2.51E-06	FEV_1 65%	1.24	0.357	0.77	1.68E-05
rs1895493	C	0.09	16	78122404	*MAF*	1.41	5.51E-06	never smoker	0.77	0.460	1.37	1.72E-05
rs12872078	A	0.91	13	64002324	*PCDH9*	1.43	4.81E-06	FEV_1 65%	0.96	0.876	1.37	1.75E-05
rs764593	T	0.11	3	3687236	*LRRN1*	0.76	3.89E-06	smoker	1.11	0.563	0.79	2.64E-05
rs1408298	T	0.7	6	17199273	*RBM24*	0.81	8.19E-06	no asthma	1.00	0.972	0.83	2.95E-05
rs2164220	T	0.08	7	157447986	*PTPRN2*	1.42	9.80E-06	never smoker	0.61	0.266	1.38	3.14E-05
rs10496694	A	0.09	2	133252637	*NAP5*	1.31	7.42E-06	smoker	0.86	0.512	1.27	3.30E-05
rs11023434	C	0.23	11	15083724	*INSC*	1.33	7.78E-06	never smoker	0.95	0.766	1.28	3.70E-05
rs1567398	T	0.43	8	8764214	*MFHAS1*	1.2	8.20E-06	FEV_1 65%	0.91	0.519	1.17	3.98E-05
rs1125729	T	0.81	8	93427586	*RUNX1T1*	0.8	1.63E-06	FEV_1 65%	1.59	0.014	0.83	4.89E-05
rs7163331	A	0.04	15	96260720	*ARRDC4*	1.55	6.81E-06	FEV_1 65%	0.56	0.132	1.46	6.34E-05
rs12265908	A	0.97	10	2339319	*ADARB2*	0.59	6.92E-06	FEV_1 65%	1.81	0.157	0.64	7.54E-05
rs7719062	T	0.08	5	1222044	*SLC6A19*	1.42	9.79E-06	smoker	0.81	0.393	1.34	7.71E-05

*Family Heart Study results are generated from phenotypes consistent with the discovery analysis indicated, with affected/unaffected sample sizes: 331/2550 all, 248/1003 smoker, 83/1547 never smoker, 266/2350 no asthma, 155/2550 FEV1 65%.

airflow obstruction, the chromosome 6 MHC locus at 6p21.33 was among the top results (smallest P value = 6.8×10^{-7}, rs3094013). The closest gene to the top SNP was HLA complex P6 (HCP5), although the extensive linkage disequilibrium in the region makes interpretation difficult. When discovery results were meta-analyzed with the replication studies, the previous associations were attenuated. The top SNP from the meta-analysis of discovery and replication results had an OR of 1.13 for the common allele (66%) and a P value of 6.03×10^{-6} near the HLA-A gene. Thirty-six SNPs in chromosome 6 with combined meta-analysis P values less than 1×10^{-4} are provided in Table E3.

On chromosome 15, after meta-analysis of airflow obstruction in ever smokers from discovery populations with replication studies, the order of the top hits was generally unchanged and P values improved, reaching 2.6×10^{-15}. The COPD case-control studies meta-analysis included only ever smokers, so the FamHS served as a sole replication study for the never smoker regional results. Figure 1 depicts the chromosome 15 regional association of the meta-analysis of combined discovery and replication cohorts for the separate groups of ever smokers (A) and never smokers (B), created using LocusZoom [25].

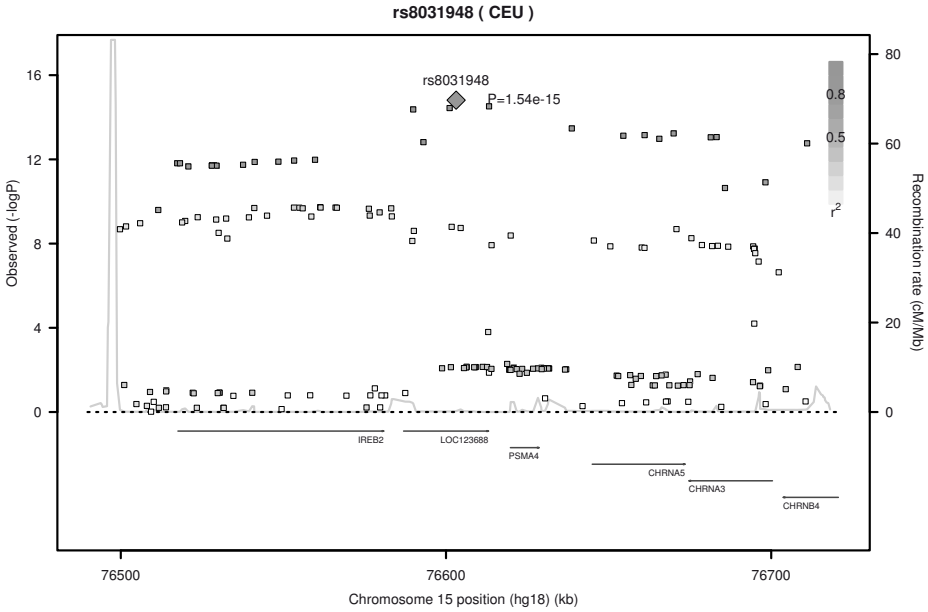

Figure 1A and 1B. Regional association plot for chromosome 15 presenting results from combined meta-analysis of discovery and replication studies. X-axis is Megabase (Mb) position. Y-axis is negative log of the p-values. Linkage disequilibrium to the named SNP (in purple) is depicted by degree of color according to the legend. Non-synonymous SNPs are depicted by an inverted triangle and other coding SNPs by a square. A) ever smokers B) never smokers.

Figure 2 is a forest plot presenting the study-specific results among never smokers that demonstrates similar effect sizes across the cohorts.

Replication of top 60 SNPs and combined meta-analysis

Table 5 presents the 60 SNPs selected for replication studies (not including the chromosome 6 and 15 SNPs included in the regional meta-analyses). A P value of 8×10^{-4}, representing Bonferroni correction for 60 tests at the $\alpha = 0.05$ level, was selected a priori as the threshold for statistically significant replication. No SNPs achieved the replication criterion. In a meta-analysis combining the discovery results with the FamHS, one SNP achieved genome-wide statistical significance (rs7733088 in *HTR4*) with a 38% frequent minor allele conferring an OR of 0.81 ($P = 4.09 \times 10^{-9}$). Of the top 60 SNPs, four had nominal association (P values < 0.05) in the COPD meta-analysis and a consistent risk allele; these SNPs were located in *ADAM19*, *RARB*, *PPAP2B*, and *ADAMTS19* (Table 6).

Association of spirometry-associated SNPs with airflow obstruction

Previous meta-analyses in the CHARGE and SpiroMeta consortia [3-5] identified 75 SNPs associated with either FEV_1 or FEV_1/FVC at genome-wide significance (P value $\leq 5 \times 10^{-8}$). We examined the association P values for airflow obstruction for these 75 SNPs in the meta-analysis results from all subjects and from ever smokers. Association for these 75 SNPs represents 58 independent tests using a multiple-testing correction that incorporates the linkage disequilibrium structure derived from HapMap European (CEU) samples [26]. Accordingly, we considered a P value of 8.6×10^{-4} as the criterion for statistically significant association with airflow obstruction (Bonferroni correction for 58 tests at the $\alpha = 0.05$ level) given the a priori association with spirometry. Among all

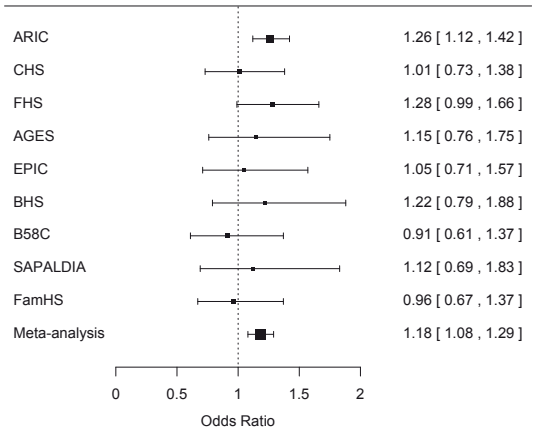

Figure 2. Forest plot depicting the association results for rs1051730 (*CHRNA3*) and airflow obstruction among never smokers in each cohort and the meta-analysis.

Table 6. Four SNPs with nominal association to COPD and consistent risk allele out of 60 SNPs selected for replication from airflow obstruction discovery GWAS.

	Coded Allele					Airflow obstruction meta-analysis			COPD meta-analysis	
SNP	Allele	Freq	Chr	position	Closest gene	analysis	OR	P-value	OR	P-value
rs11744671	T	0.92	5	156853809	*ADAM19*	no asthma	0.72	4.51E-07	0.8	0.027
rs1997352	A	0.26	3	25513321	*RARB*	all	0.84	8.64E-07	0.88	0.038
rs12744110	T	0.25	1	56168897	*PPAP2B*	smoker	1.17	5.95E-06	1.13	0.045
rs9632471	C	0.72	5	128761894	*ADAMTS19*	FEV_1 65%	0.77	1.07E-05	0.86	0.045

Definition of abbreviations: Chr = chromosome; COPD = chronic obstructive pulmonary disease; Freq = frequency; OR = odds ratio; SNP = singly-nucleotide polymorphism

participants, SNPs in *RARB*, *GPR126*, *HTR4*, *C10orf11*, near *HHIP*, and near *HLA-DRA* were statistically significantly associated with airflow obstruction. Among smokers, *HTR4*, *RARB*, *GPR126*, and *THSD4* were associated with airflow obstruction. Results for the 75 SNPs are presented in Tables E4 and E5. When only cohorts that did not contribute to the published spirometry findings [3-5] were considered (RS3, SAPALDIA, LBC1936, MESA, and COPACETIC) as an independent sample, power was reduced, and only the *ADAM19* SNP in smokers achieved the Bonferroni cutoff for significance (Table E6).

Gene expression results

Expression of *CHRNA3* and *CHRNA5* was evaluated in cDNA from human whole lung, peripheral blood mononuclear cells, and primary cultures of bronchial epithelial cells and airway myocytes, together with control tissues (kidney, brain, and placenta: see online supplement for methods). Both genes were expressed in all lung-derived tissues examined. Within the lung, expression of both *CHRNA3* and *CHRNA5* appeared strongest in airway myocytes and epithelial cells. The identity of reverse transcriptase–polymerase chain reaction products was confirmed by direct sequencing of bands of the relevant size from at least one tissue type for each gene.

DISCUSSION

These meta-analyses included 32,875 participants from population-based studies for discovery of loci associated with airflow obstruction. In addition, we attempted to replicate results in 2,881 participants from a population-based family study and a meta-analysis of 5,421 participants from case-control studies of clinically ascertained COPD. The present study confirms the previously identified [7] association between the chromosome 15q25 region and airflow obstruction among smokers. Although the number of

participants with airflow obstruction among never smokers was low (504 affected), and statistical power is therefore limited, analyses of airflow obstruction among never smokers also showed a nominal association to the Asp398Asn missense SNP in *CHRNA5* and to a synonymous SNP in *CHRNA3*.

Results from gene expression studies demonstrated that both *CHRNA5* and *CHRNA3* were expressed in whole lung, airway smooth muscle, and bronchial epithelial cells. In a publication reporting *CHRNA5* gene expression in normal lung tissue samples, the Asp398Asn genotype was strongly related to mRNA levels, with homozygosity of the risk allele (A) associated with 2.5-fold lower mRNA levels compared with homozygosity for the G allele [27]. A similar pattern was observed for rs1051730 in the sputum of COPD cases, in which the minor allele was associated with lower expression of *CHRNA5* [28]. The correlation between associated SNP genotypes and *CHRNA5* expression levels in the lung and sputum combined with our finding of increased risk for airflow obstruction in never smokers suggests that the variants in this region may have an influence on risk of airflow obstruction that is not simply mediated by an influence on nicotine dependence. Supporting a direct influence of variants in this region on lung phenotypes, a *CHRNA3/5* variant was recently found to be associated with bronchial hyperresponsiveness in children not exposed to cigarette smoke [29]. Silencing *CHRNA5* in bronchial epithelial cells was found to reduce expression of adhesion molecules, thereby increasing cell motility, which may influence the repair and remodeling processes that lead to COPD [30]. Our results suggest that the A allele of rs16969968 confers as much as a 20% increased odds of airflow obstruction, and based on the prior report, this increased risk may be mediated by lower mRNA levels in lung tissue [27].

In addition to the chromosome 15q region, SNPs in *HTR4* met genome-wide statistical significance in ever smokers. The *HTR4* (5-hydroxytryptamine {serotonin} receptor 4) gene was originally identified with association to FEV_1/FVC in CHARGE [3] and SpiroMeta [4], and subsequently showed a statistically significant association with COPD in a targeted gene analysis of six loci in the SpiroMeta cohorts [31]. Serotoninergic receptors have been demonstrated to regulate cytokine and chemokine release in human airway epithelial cells and have been implicated in the pathogenesis of asthma [32]. The reduced risk of airflow obstruction was strongest when limited to ever smokers, suggesting that variation in *HTR4* may contribute to the inflammatory response to cigarette smoke.

Several genes represented among the top SNP results were nominally replicated in the COPD case-control meta-analysis (*ADAM19*, *RARB*, *PPAP2B*, and *ADAMTS19*). Of them, both *ADAM19* and *RARB* have been previously implicated in GWAS of lung function as measured by spirometry [3-5]. *ADAM19* (a disintegrin and metalloprotease domain 19) was

originally shown to be associated with FEV_1/FVC in the CHARGE GWAS [3], and these SNPs were subsequently reported to be associated with COPD in a case-control study [33]. Here, we demonstrate that *ADAM19* is associated with airflow obstruction in population-based cohort studies. *ADAM19* is expressed in bronchial epithelial cells, bronchial smooth muscle, and interstitial inflammatory cells and may have a role in immune defense and the inflammatory process [34]. *ADAMTS19* (a disintegrin and metalloproteinase with thrombospondin motifs 19) has several of the same domains and has been shown to be expressed in fetal lung [35]. *PPAP2B* is a lipid phosphate phosphohydrolase, which are generally believed to influence surfactant secretion and have a role in lung injury and repair [36].

RARB (retinoic acid receptor β) was recently demonstrated to be associated with lung function measures at genome-wide significance in the combined CHARGE and Spiro-Meta meta-analysis [5]. Retinoic acid (RA) has been evaluated as a potential therapeutic agent for emphysema after results in rats demonstrated reversibility of experimentally induced emphysema with administration of RA [37]; however, subsequent studies in animal models had conflicting results [38], and a small feasibility study of RA for the treatment of emphysema did not show significant improvement in lung function [39]. The finding that *RARB* minor alleles were associated with lower risk of airflow obstruction may provide insight into which patients may benefit from RA therapy or suggest modifying the design of RA therapeutics to target the β receptor.

The *HHIP* region was associated with airflow obstruction in our look-up replication of spirometry-associated SNPs, which was expected given the prior findings of association with COPD in earlier GWAS [7,9] and further replication in targeted studies of *HHIP* and COPD [8]. This region of chromosome 4q31 including SNPs in *HHIP* and GYPA has also been shown to be associated with lung cancer [40]. Recently, a COPD risk haplotype upstream of *HHIP* was identified to be associated with reductions in *HHIP* promoter activity [41]. Our meta-analysis is able to confirm that rs6537296 is associated with airflow obstruction ($P = 3.2 \times 10^{-4}$), but the other SNP in the haplotype (rs1542725) was not studied. Also, previously identified SNPs in *GPR126*, *THSD4*, and near *HLA-DRA* were associated with airflow obstruction, and *GPR126* demonstrated a nominal association with COPD in a prior report focusing on clinically ascertained cases and control subjects [33]. It should be noted that the look-up replication that supports the relation of these genes with airflow obstruction is not statistically independent from the original meta-analyses of spirometry traits because of overlap between the samples. When only the cohorts not included in the earlier published meta-analyses [3-5] were analyzed separately, in this reduced sample size (567 affected, 2,922 unaffected) only the *ADAM19* gene achieved the cutoff criterion for significant association with airflow obstruction.

The chromosome 6 region identified in discovery meta-analysis did not replicate when additional studies were included in the meta-analysis. The regional meta-analysis results demonstrated modest association (P values $< 1 \times 10^{-4}$) across five megabases in the HLA region, including 17 SNPs in the histone gene cluster at 27.9 Mb. Our results are not able to clarify which gene or combination of genes may give rise to the underlying association signal given the extensive linkage disequilibrium in the MHC. Recently, a meta-analysis of the COPD case-control cohorts that served as replication cohorts in our study implicated a locus on chromosome 19q13 [24] as a COPD susceptibility locus; however, the rs7937 SNP identified is not replicated in the discovery meta-analyses described here (P values ranged from 0.12 among never smokers to 0.87 among ever smokers).

Our study has several limitations. Our cohorts had only prebronchodilator spirometry, and thus we could not examine the formal definition of COPD. Our main analysis used a definition of airflow obstruction that includes persons with very mild ventilatory impairment, and the participants who meet this definition may not all have COPD. Our definition of more severe airflow obstruction is likely to be more comparable to clinically ascertained COPD in the replication studies, but the numbers of affected participants were reduced. In addition, our ability to address asthma in the context of airflow obstruction was limited to a subset of cohorts with self-reported asthma diagnoses. Last, as our study was limited to white participants of European descent, the generalizability of these findings to other ethnic groups is unknown.

In summary, we performed meta-analyses and replication studies using data from more than 40,000 study participants of European ancestry to identify genetic loci influencing airflow obstruction as a categorical disease phenotype. We identified the *CHRNA3/5* genes and *HTR4* at genome-wide significance, and several genes that were implicated by previous GWAS of single spirometry measures as quantitative phenotypes (*ADAM19*, *RARB*) were among top results. Here we show, for the first time, that a *CHRNA5* missense SNP is associated with airflow obstruction in never smokers, suggesting a main effect on risk of airflow obstruction that is independent of the influence mediated through effects on smoking habits. This was supported by gene expression findings demonstrating the *CHRNA3/5* genes in relevant lung and airway tissues. Thus, *CHRNA3/5* variants may mediate airflow obstruction in both ever and never smokers.

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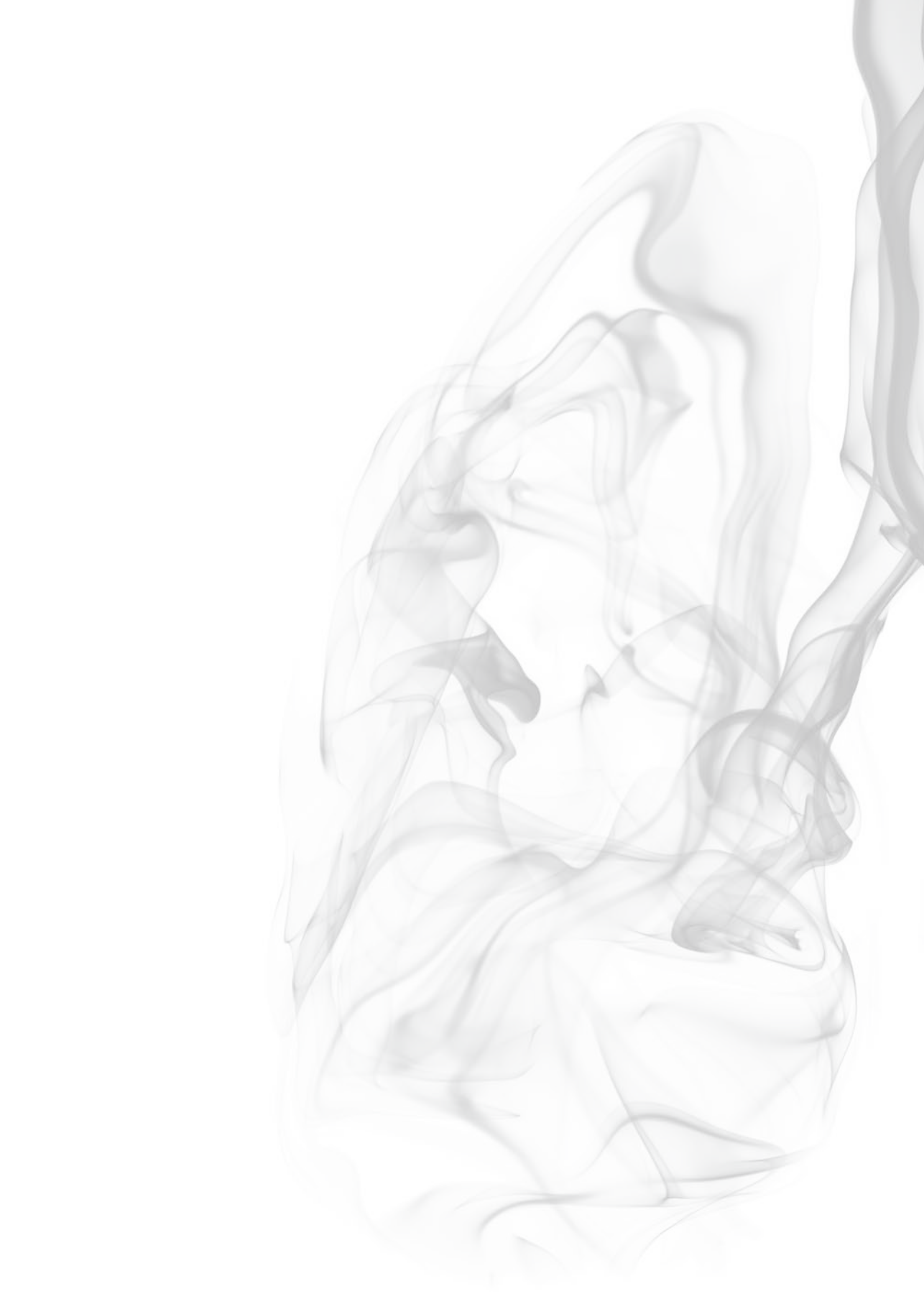
PART IV

Pharmacoepidemiology and the Lung



CHAPTER 4.1

Beta-blockers and pulmonary function in the general population: The Rotterdam Study

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Published in Br J Clin Pharmacol. 2013 Jun 17. (Epub ahead of print) PubMed PMID: 23772842.

ABSTRACT

Aim Beta-blockers have been used with caution in patients with obstructive lung diseases such as asthma or chronic obstructive pulmonary disease (COPD), due to the potentially increased airway reactivity and risk of bronchial obstruction. Cardioselective β-blockers have a more beneficial profile than non-cardioselective β-blockers and can be safely prescribed to patients with both cardiovascular disease and COPD. We hypothesized that also cardioselective β-blockers affect pulmonary function.

Methods This study was performed within the Rotterdam Study, a prospective population-based cohort study. Effects of cardioselective and non-cardioselective β-blockers on pulmonary function were analyzed using regression techniques with multivariable adjustment for potential confounders.

Results Current use of non-cardioselective β-blockers was significantly associated with a lower forced expiratory volume in 1 second (FEV_1) of -198 ml (95%CI -301; -96), with a lower forced vital capacity (FVC) of -223 ml (95%CI -367; -79) and with a decreased FEV_1/FVC of -1.38% (95%CI -2.74; -0.13%). Current use of cardioselective β-blockers was significantly associated with a lower FEV_1 of -118 ml (95%CI -157; -78) and with a lower FVC of -167 ml (95%CI -222; -111), but did not affect FEV_1/FVC. After exclusion of patients with COPD, asthma and heart failure the effects of cardioselective β-blockers remained significant for FEV_1: -142 ml (95%CI -189; -96) and for FVC: -176 ml (95%CI -236; -117).

Conclusion In our study both non-cardioselective and cardioselective β-blockers had a clinically relevant effect on both FEV_1 and FVC. In contrast to cardioselective β-blockers, use of non-cardioselective β-blockers was associated with a significantly lower FEV_1/FVC.

INTRODUCTION

Several highly prevalent cardiovascular diseases, including hypertension, ischemic heart disease, heart failure and arrhythmia, are important indications for β-blockers. Major adverse effects due to blockade of the β-adrenergic system are fatigue, decreased peripheral circulation and increased airway resistance. β-blockers have been used with caution in patients with obstructive lung diseases such as asthma or chronic obstructive pulmonary disease (COPD), due to the potentially increased airway reactivity and subsequent risk of bronchial obstruction in asthmatics [1]. β-adrenoreceptor blockade, especially β_2-blockade, can provoke bronchospasm in patients with airway hyperreactivity.

These possible adverse effects of β-blockers may complicate treatment of patients with concomitant COPD and cardiovascular disease (CVD), since both conditions often coexist [2-6]. Both diseases share common risk factors such as smoking and ageing. Moreover, both CVD (in case of heart failure) and COPD may elicit similar symptoms such as dyspnea, cough and wheezing, which can make it difficult to distinguish between both diseases. In the TORCH-study, 30% of mortality in patients with COPD was due to CVD [7]. The World Health Organisation estimated that CVD and COPD will be amongst the top four causes of death in 2030 [8]. This underlines that adequate treatment of both diseases is highly relevant for reducing mortality and morbidity in the general population.

β_1-selective (cardioselective) β-blockers are supposed to have a more beneficial profile than non-cardioselective β-blockers and can be safely prescribed to patients with both CVD and COPD or asthma [9-11]. A systematic review of 22 randomized trials to evaluate the effects of cardioselective β-blockers showed no significant change in respiratory symptoms or spirometry parameters of interest in participants with obstructive syndromes. The Forced Expiratory Volume in 1 second (FEV_1) did not change significantly nor did the response in FEV_1 to β_2-agonist treatment [9]. Another meta-analysis analyzed 19 randomized trials on single dose administration of cardioselective β-blockers and 10 studies on continuous treatment [10]. In this meta-analysis the authors concluded that the first dose of a cardioselective β-blocker produces a small decrease in FEV_1 that is not associated with adverse respiratory effects compared to placebo [10]. Sirak et al. stated that cardioselective β-blockers are routinely preferred to non-cardioselective β-blockers in treatment of congestive heart failure and can be safely prescribed [12]. Recently, a large observational study suggested that β-blockers are well tolerated in patients with COPD and may reduce mortality [11].

It is common knowledge that randomized clinical trials predominantly include highly selected, relatively young and healthy patients and that those results cannot always be generalized to clinical care in real life circumstances. Therefore, the objective of our study was to assess the effects of non-cardioselective and cardioselective β-blockers on pulmonary function in the general population.

MATERIALS AND METHODS

Setting

This study was performed in the Rotterdam Study, a prospective population-based cohort study which started in 1990 in Ommoord, a suburb of Rotterdam. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, The Netherlands, approved the study. At baseline, all participants were visited for a standardized questionnaire, and were subsequently examined at the research centre. The first cohort (RS I) consists of 7,983 participants, aged 55 years and over. Since the start of the initial cohort, two subsequent cohorts have been defined in the same area. The second cohort (RS II) started in 2000 and included 3,011 participants aged 55 years and over. The third cohort (RS III) was enrolled in 2006 and included 3,932 participants aged 45 years and over. As of 2009 the total of the combined cohorts encompasses 14,926 subjects aged 45 years or over. The main focus of the Rotterdam Study is on studying common diseases in the elderly. Every 3-5 years the participants undergo a round of interviews and physical examinations, of which pulmonary function tests are routinely performed during every study round as of 2002. To continuously monitor the cohort for major diseases and mortality, the database is linked to the electronic records of the general practitioner and the municipality. All prescriptions dispensed to the participants are periodically collected by linking to the seven pharmacies covering the Ommoord region. Main objectives and methods of the Rotterdam Study have been described elsewhere [13,14].

Study population

The study population consisted of all participants of the Rotterdam Study with a pulmonary function test during the fourth study round. These tests were analysed by two research physicians, and validated by a specialist in pulmonary medicine. During the validation process the quality control was performed by two researchers, of whom at least one was a physician. Normal spirometry, airflow obstruction and spirometry suggestive of restrictive syndromes were classified.

Exposure

To assess exposure to β-blockers, for participants with a pulmonary function test, prescription data was collected from the pharmacies. We collected prescription data on cardioselective and non-cardioselective β-blockers using the Anatomical Therapeutic Chemical (ATC) classification system [15] for C07AB (cardioselective β-blockers) and C07AA (non-cardioselective β-blockers). For every participant, the prescription period was calculated and if the date of the pulmonary function test fell within the prescription period of a β-blocker, the study participant was classified as exposed.

Outcome

Spirometry was performed by trained paramedical personnel using a SpiroPro® portable spirometer (Erich Jaeger, Hoechberg, Germany), according to American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines [16]. FEV_1, FVC and FEV_1/FVC-ratio were measured. Spirometry procedures that yielded results that did not meet ATS/ERS criteria for acceptability and reproducibility were classified as not interpretable [17]. For practical reasons, reversibility testing could not be carried out. Participants were asked to refrain from using any prescribed pulmonary medication before the center visit.

Covariables

Information on several potential confounders or effect modifiers such as age, sex, height, weight and body mass index (BMI) was gathered at the study round in which the pulmonary function test was performed. We collected data on medication prescribed for obstructive pulmonary diseases such as asthma and COPD. For our analyses, we included inhaled short-acting β-adrenoreceptor-agonists (SABAs), long-acting β-adrenoreceptor-agonists (LABAs), anticholinergic agents, inhaled glucocorticosteroids and combinations of the above mentioned substances. Furthermore, we collected information on use of oral glucocorticosteroids, xanthine derivatives and leukotriene-receptor antagonists.

Data on smoking was collected from the questionnaires and classified subjects were classified as current, former or never smokers. As a quantitative measure of tobacco exposure, we used the number of pack-years, with one pack-year defined as smoking 20 cigarettes per day for 1 year. We assessed COPD using spirometry and classified according to the GOLD criteria [18,19]. Heart failure at baseline in the Rotterdam Study was assessed using a validated score based on the definition of heart failure of the European Society of Cardiology [20]. As described previously, cases of incident heart failure were obtained by continuously monitoring participants of the Rotterdam Study during follow-up [21]. For these analyses, we assessed if participants were cases at the time of the pulmonary function test and considered cases defined before the test as prevalent heart failure patients. Using echocardiography, fractional shortening and E/A-ratio were calculated

as measures of systolic and diastolic cardiac function, respectively [22]. Echocardiography was done on the same day as the spirometry. The methods for echocardiography in the Rotterdam Study have been described elsewhere [22]. Systolic and diastolic blood pressures were measured twice from the right upper arm with a random-zero sphygmomanometer with the patient in a sitting position. The mean of the two readings was used to determine blood pressure levels.

Data analysis

Differences in baseline characteristics between cardioselective and non-cardioselective β-blocker users, and non-users were tested using a χ^2-test for dichotomous variables and a t-test for continuous variables. The effect of cardioselective and non-cardioselective β-blockers on pulmonary function measures was evaluated using linear regression analyses for FEV_1 and for FVC, selecting covariables by assessing whether there was a higher than 10% change in point estimate. Furthermore, we tested for possible interaction by using multiplicative interaction terms. We adjusted for sex, age, height, systolic blood pressure, smoking status, pack-years, heart failure, obstructive and restrictive syndromes and concomitant respiratory medication, with pulmonary function in non-users of β-blockers as a reference group. To avoid potential confounding by indication, we also performed analyses excluding patients with COPD and heart failure. We preferred this over adjustment because the occurrence of these conditions was considered as a contra-indication for β-blockers during a substantial number of years within the study period.

In 784 participants, consecutive measurements were available, with a mean follow-up of 6.1 years (±0.5) between consecutive center visits. In this subanalysis, exposure categories were classified as "never exposed" (no use of β-blockers at the first as well as the second measurement), "starters" (no use of β-blockers at the first measurement but use at the second measurement), "continuous users" (use of β-blockers at the first as well as the second measurement), and "discontinued use" (use of β-blockers at the first measurement but no use at the second measurement).

All analyses above were repeated for non-cardioselective β-blockers. All statistical analyses were performed with SPSS PASW software (IBM Corporation, Armonk, New York, USA). P-values <0.05 were considered statistically significant. All graphs were made using R 2.9.1. [23]

RESULTS

Of the total of 14,926 subjects from the Rotterdam Study cohorts, a total of 6,489 pulmonary function tests were performed; 4,324 spirometry tests were of sufficient quality to be validated as interpretable and could be used for our analyses.

Subject characteristics

The population consisted of 1,880 men (43.5%) and 2,444 women (56.5%) (Table 1). The mean age at lung function examination was 66 years (range 46-97 years). In our cohort 651 persons were exposed to cardioselective β-blockers at the time of the pulmonary function test. The mean FEV_1 in the cohort was 3.23 l (±0.9 l) for men and 2.34 l (±0.6 l) for women. The mean FVC was 4.27 l (±1.03 l) for men and 3.03 l (±0.74 l) for women. We defined airflow limitation according to the GOLD definition (FEV_1/FVC ratio < 0.70). In our sample there were 308 subjects with COPD GOLD stage I (7.1%), 330 with COPD GOLD stage II (7.6%) and 63 with COPD GOLD stage III (1.5%). There were no subjects with COPD GOLD Stage IV.

Furthermore, we identified 301 participants (7.1%) with lung function results suggestive of a restrictive syndrome. Of the participants with pulmonary function tests we had full data, including smoking history, in 4,033 subjects. These were included in our analyses. Missingness of data was independent of β-blocker-use and multiple imputations followed by combined analyses did not change effect estimates.

Cardioselective β-blockers

Exposure to cardioselective β-blockers was significantly associated with a lower FEV_1 in our population of -118 ml (95% CI -157; -78 ml). To investigate the effect of cardioselective β-blockers on FVC we tested similar models. Current use of cardioselective β-blockers was significantly associated with a lower FVC with a decrease of -167 ml (95% C.I. -222; -111 ml) (table 2). Testing for interaction between sex, age or COPD and ß-blocker exposure was non-significant but with suspected pulmonary restrictive disease interaction seemed to be present. When we subsequently analyzed the dataset stratified, the significant effect on FEV_1 only persisted in the non-diseased group and was stronger (-145, 95% C.I. -188; -103)

To assess the effect in subjects without known heart failure and an obstructive or a restrictive syndrome, we separately analysed participants without these diseases. As asthma or COPD patients might be prone to a prescription of cardioselective β-blockers because of the possible adverse effects of non-cardioselective β-blockers, we also

excluded participants with a prescription for inhalation medication and/or oral glucocorticosteroids.

Table 1. Baseline characteristics of the study population.

		Total	Unexposed	β_1-selective	Non-selective
Sex N (%)	Male (%)	1880 (43.5%)	1533 (42.8%)	303 (46.5%)	44 (50%)
	Female (%)	2444 (56.5%)	2052 (57.2%)	348 (53.5%)	44 (50%)
Age Years (SD)		65.9 (9.5)	65.2 (9.5)	68.9 (9.0)	71.8 (9.3)*
Height cm (SD)		168.9 (9.3)	169.0 (9.3)	168.1 (9.2)*	169.5 (8.7)
Smoking Status†† N (%)	Never	1418 (32.8%)	1208 (32.9%)	210 (32.2%)	22 (25%)
	Past	2074 (48.0%)	1734 (47.2%)	340 (52.2%)*	50 (56.8%)
	Current	828 (19.1%)	727 (19.8%)	101 (15.5%)*	16 (18.2%)
Pack Years py (SD)		15.5 (21.1)	14.9 (20.8)	18.5 (22.7)*	16.3 (19.1)
Prevalent Heart Failure N (%)		140 (3.2%)	93 (2.5%)	47 (7.3%)*	7 (8%)*
Blood Pressure mmHg (SD)	Systolic	142 (21)	141 (21)	150 (22)*	143 (20)
	Diastolic	81 (11)	81 (11)	82 (12)	78 (11)*
BMI kg/m^2 (SD)		27.6 (4.3)	27.3 (4.2)	29.0 (4.4)*	28.0 (3.6)
Hx myocardial infarction (%)		223 (5.2)	111 (2.6)	100 (15.4)*	12 (13.6%)*
Echocardiography¶	Fractional Shortening % (SD)	39.7 (6.7)	40.2 (6.3)	38.0 (7.8)*	35.0 (7.7)*
	E/A-Ratio	1.0 (0.3)	1.0 (0.3)	1.0 (0.3)	0.9 (0.3)
Total colesterol mmol/l (SD)		5.6 (1.0)	5.6 (1.0)	5.2 (1.0)*	5.3 (1.1)*
HDL cholesterol mmol/l (SD)		1.4 (0.4)	1.5 (0.4)	1.3 (0.3)*	1.3 (0.3)*
Obstructive Pulmonary Disease	No obstruction‡	3623 (83.8%)	3025(84.4%)	530 (81.5%)	68 (77.3%)
GOLD Classification, N (%)	I	308 (7.1%)	261 (7.3%)	41 (6.3%)	6 (6.8%)
	II	330 (7.6%)	248 (6.9%)	69 (10.6%)*	13 (14.8%)*
	III	63 (1.5%)	51 (1.4%)	11 (1.7%)	1 (1.1%)

BMI: Body Mass Index, E/A-ratio: ratio of early and late ventricular filling velocity, Hx: History, N: number of participants, SD: Standard Deviation * P-value < 0.05 for difference, exposed vs. unexposed † Smoking status missing: n=4 ‡ Spirometry suggestive for a restrictive syndrome: unexposed n=215, exposed to β1-selective n=72, exposed to non-selective n=14 ¶ Available in a random subset: n= 3629

Table 2. Effect estimates

Pulmonary function test	Effect, crude	95%CI	Effect, adjusted‡	95%CI	P-value, adjusted
FEV_1					
Non use	Reference		Reference		
Non-cardioselective*	-503 ml	-685; -321	-198 ml	-301; -96	<0.001
Cardioselective†	-307 ml	-378; -236	-118 ml	-157; -78	<0.001
FVC					
Non use	Reference		Reference		
Non-cardioselective	-524 ml	-752; -296	-223 ml§	-367; -79	0.001
Cardioselective	-379 ml	-468; -290	-167 ml	-222; -111	<0.001
FEV_1/FVC					
Non use	Reference		Reference		
Non-cardioselective	-3.52 %	-5.37; -1.66	-1.38%¶	-2.74; -0.13	0.048
Cardioselective	-0.63 %	-1.36; 1.01	0.12 %	-0.41; 0.65	0.654

* No. exposed non-cardioselective= 88, N unexposed = 3585 † No. exposed cardioselective= 651, N unexposed = 3585 ‡ Adjusted for: sex, age, height, pack-years, smoking status, inhalation medication, oral glucocorticosteroids, heart failure, obstructive or restrictive syndromes and systolic blood pressure and cohort.

§ As above plus BMI. ¶ Adjusted for: age, height, BMI, pack-years, smoking status, obstructive or restrictive lung diseases and inhalation medication and cohort.

In this sample (n = 3,157), the associations of cardioselective β-blockers exposure and a lower FEV_1 and FVC are comparable to the complete sample. Exposure to cardioselective β-blockers was associated with a lower FEV_1 of -142 ml (95% C.I. -189; -96 ml) and a change of FVC of -176 ml (95% C.I. -236; -117 ml). To minimize the effect of misclassification of heart failure, we performed a sensitivity analysis in which we also adjusted for echocardiography, which was available in a random subset of 3,629 participants. Fractional shortening and E/A-ratio were used as measures of systolic and diastolic function, respectively. Of these measures only fractional shortening was of influence in our models. The effect estimate of cardioselective β-blockers changed less than 5% and remained significant in this analysis.

Non-cardioselective β-blockers

In our cohort, exposure to non-cardioselective β-blockers was low; only 88 participants (2.2%) of our cohort of 4,033 participants had a prescription for non-cardioselective β-blockers. The majority of these users (75%) had a prescription for Sotalol. Exposure of non-cardioselective β-blockers was also associated with lower FEV_1 and FVC (table 2).

The effect of non-cardioselective β-blockers was -198 ml (95% C.I. -301; -96 ml) on FEV_1 and -223 ml (95% C.I. -367; -79 ml) on FVC. We also analysed the effect in the subset of participants without obstructive and restrictive syndromes, heart failure and inhalation medication. For FEV_1 the effect was -219 ml (95% C.I. -343; -94 ml) and for FVC the effect was -179 ml (95% C.I. -339; -19 ml).

FEV_1/FVC

No significant association was shown on the FEV_1/FVC-ratio in participants exposed to cardioselective β-blockers compared to unexposed participants, namely an effect of 0.12% (95% C.I. -0.41; 0.65). In the adjusted analysis, a significantly lower ratio of FEV_1 over FVC of -1.38 % (95% C.I. -2.74; -0.13 %) was seen in participants exposed to non-cardioselective β-blockers compared to unexposed subjects (figure 3). Compared to users of cardioselective β-blockers, the adjusted effect was -1.28 % (-2.49; -0.76).

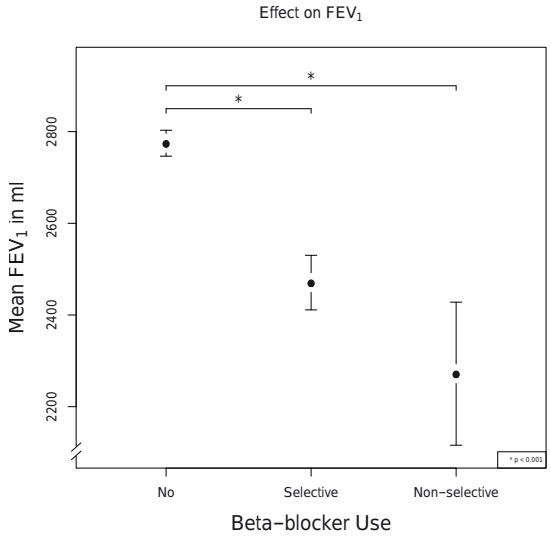

Figure 1. Effect of β-blocker exposure on mean FEV_1 in milliliters
Exposure is divided in three classes; No use, current use of cardioselective β-blockers and current use of non-cardioselective β-blockers. The whiskers represent the 95 % C.I.

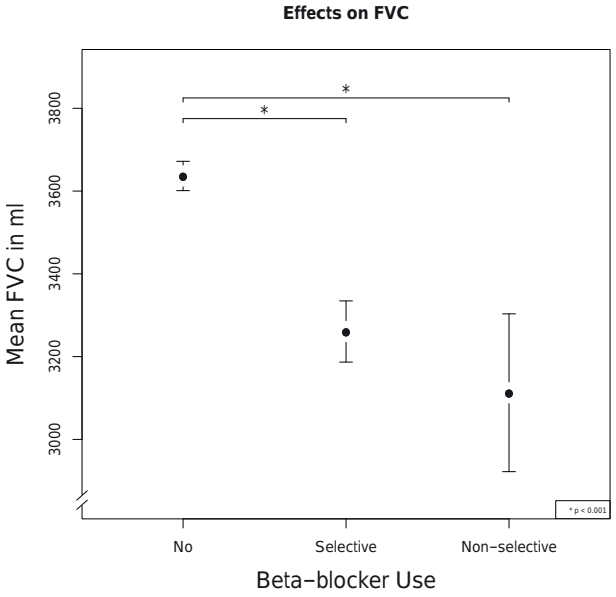

Figure 2. Effect of β-blocker exposure on mean FVC in milliliters.
Exposure is divided in three classes; No use, current use of cardioselective β-blockers and current use of non-cardioselective β-blockers. The whiskers represent the 95 % C.I.

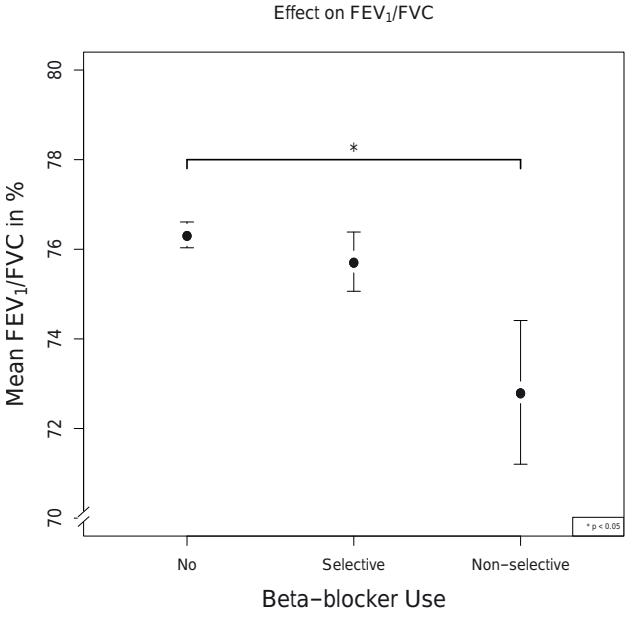

Figure 3. Effect of β-blocker exposure on mean FEV_1/FVC in percent.
Exposure is divided in three classes; No use, current use of cardioselective β-blockers and current use of non-cardioselective β-blockers. The whiskers represent the 95 % C.I.

Longitudinal effects

For 1 cohort (RS-I), consecutive spirometry measurements were available in 784 participants (table 3). Participants starting cardioselective β-blockers showed a significantly stronger percentual decrease in FVC than never users of β-blockers, i.e. respectively 13.8% versus 9.6% (figure 3).

For FEV_1 a similar effect was seen with a decrease of 13.5% for starters of β-blockers versus 9.4% for never users. When prescriptions ended between the measurements, participants who had been using cardioselective β-blockers showed less of a decrease in both FEV_1 and FVC compared to starters (figures 4 and 5). Linear regression with adjustment for age, sex, follow-up time, current smoking and pack years, showed a statistically significant stronger percentual decrease for both FEV_1 and FVC compared to never users (figure 6).

Table 3. Baseline characteristics of the subgroup with two spirometry tests.

		Total	Unexposed	β_1-selective
Characteristics				
Number of participants		784	648	136
Sex N (%)	Male (%)	337 (43.0%)	278 (43.0 %)	59 (43.4 %)
	Female (%)	447 (57.0 %)	370 (57.0 %)	77 (56.6 %)
Age baseline Years (SD)		72.3	72.3 (4.5)	72.7 (4.1)
Height cm (SD)		167.6 (9.0)	167.7 (8.9)	166.9 (9.4)
Smoking Status*† N (%)	Never	259 (33.0 %)	209 (32.3 %)	50 (36.8 %)
	Past	453 (57.8%)	376 (58.0 %)	77 (56.6 %)
	Current	72 (9.2%)	63 (9.7 %)	9 (6.6 %)
Pack Years py (SD)		15.5 (21.1)	15.6 (21.5)	14.8 (18.6)
Prevalent Heart Failure N (%)		23 (2.9%)	15 (2.3 %)	8 (5.9%)
Blood Pressure mmHg (SD)	Systolic	149 (20)	148 (20)	156 (20)
	Diastolic	80 (10)	80 (10)	81 (10)
BMI kg/m^2 (SD)		27.3 (3.9)	27 (3.8)	28.6 (3.8)
Hx myocardial infarction (%)		52 (6.6 %)	33 (5.1 %)	19 (14.0 %)
Chronic Obstructive Pulmonary Disease	No obstruction‡	579 (73.9 %)	483 (74.5 %)	96 (70.6%)
GOLD Classification, N (%)	I	65 (8.3 %)	54 (8.3 %)	11 (8.1 %)
	II	89 (11.4 %)	70 (10.8 %)	19 (14.0 %)
	III	8 (1.0 %)	8 (1.2 %)	0
Follow-up Time Years (SD)		6.6 (0.4)		

BMI: Body Mass Index, Hx: History, N: number of participants, SD: Standard Deviation

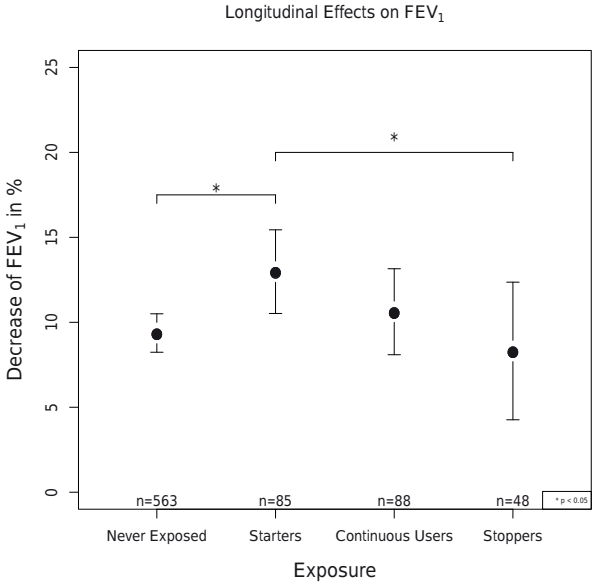

Figure 4. Longitudinal effects of cardioselective β-blocker use on FEV_1 in percentages.
Divided in four exposure categories; "never exposed", "starters", "continuous users", and "discontinued use".
The whiskers represent the 95% CI

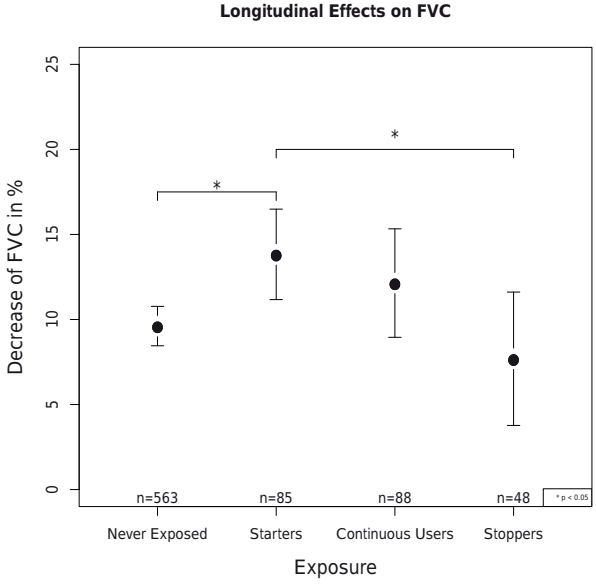

Figure 5. Longitudinal effects of cardioselective β-blocker use on FVC in percentages.
Divided in four exposure categories; "never exposed", "starters", "continuous users", and "discontinued use".
The whiskers represent the 95% CI

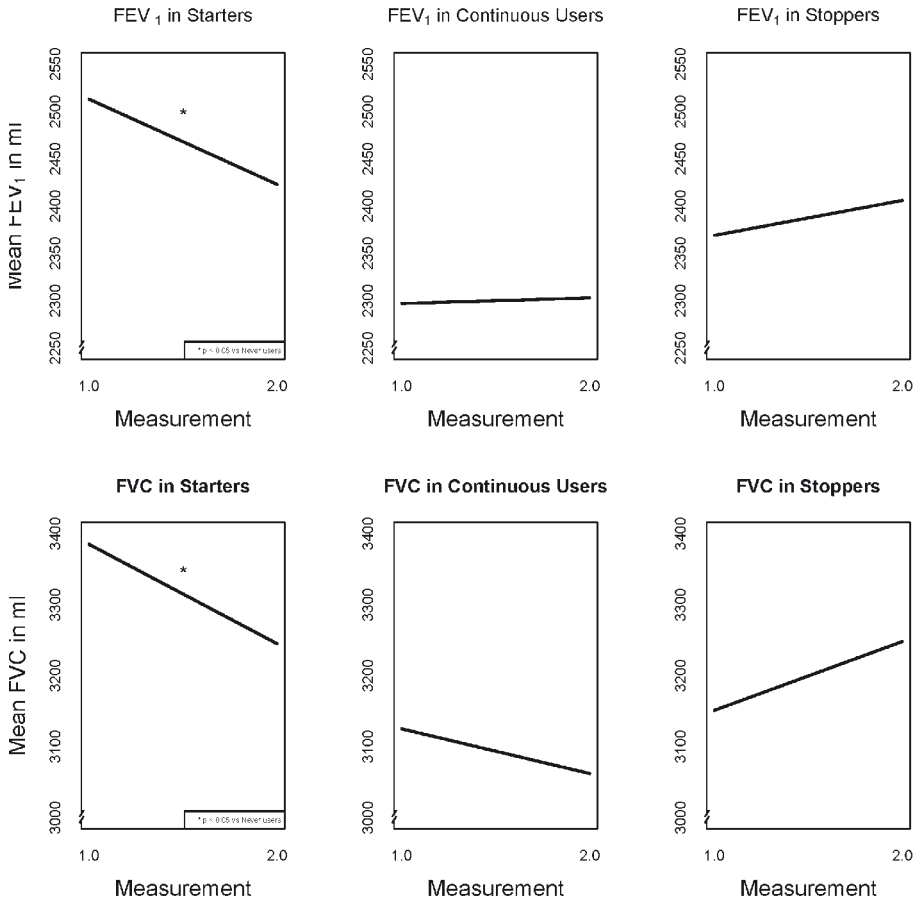

Figure 6. Longitudinal effects compared to stoppers
These figures represent the decrease or increase in FEV1 and FVC for separate types of β-blockers users compared to never users over the consecutive time points. The linear relation is corrected for age, sex, follow-up time, current smoking and pack-years.

DISCUSSION

In this population-based study, exposure to cardioselective β-blockers was associated with a lower FEV_1 of -118 ml, and FVC of -167 ml, respectively. For non-cardioselective β-blockers we found an association with a marginally lower FEV_1/FVC of -1.38% (-2.74;-0.13), a measure to assess airflow obstruction. For cardioselective β-blockers, although the sample was much larger, no such association was found. When we compared the effect on FEV_1/FVC within users of non-cardioselective β-blockers versus cardioselective β-blockers, the association remained significant. Longitudinal effects of exposure showed a stronger decrease of FVC over time in participants starting on β-blockers

versus never users. For FEV_1 a similar pattern was seen. When prescriptions ended between the two measurements, the decrease in FEV_1 and FVC was less in quitters than in never users, however this was not significant. Although our results suggest negative effects of cardioselective β-blockers on pulmonary function which can be defined as a clinically important difference [24,25], several studies suggest beneficial effects on mortality in patients with concomitant CVD and COPD. Therefore, negative effects on pulmonary function may very well be outweighed by the positive effects of cardioselective β-blockers on CVD with respect to morbidity and mortality. Apparently, also cardioselective β-blockers are not completely free of pulmonary effects. Importantly, non-cardioselective β-blockers seem to have greater association with an airflow obstruction than cardioselective β-blockers. Only non-cardioselective β-blockers had a significant association with a reduced FEV_1/FVC compared to non-exposed participants and compared to users of cardioselective β-blockers.

To our knowledge, this is the first population-based cohort study solely investigating the effects of β-blockers on pulmonary function. To deal with potential confounding by indication, we separately analysed the participants with heart failure and/or obstructive pulmonary disease. The results in this subset were consistent with the results in the complete set, suggesting that underlying obstructive pulmonary disease did not influence the magnitude of the effects. To minimize the possible misclassification of heart failure, we did a sensitivity analysis in which we adjusted for cardiac function, defined by fractional shortening and E/A-ratio. These measures hardly influenced the effect estimate. The decreased spirometry measures can possibly be explained by increased airway resistance as has been stated in the past [1]. Another possible reason could be that some participants with a prescription for β-blockers might have already developed heart failure, which was still unknown at the time of pulmonary function test. But as participants in The Rotterdam Study were also screened for heart failure this seems less likely [13,14].

Our findings suggest that also cardioselective β-blockers have an adverse effect on pulmonary function in the general elderly population, although this is less pronounced than in non-cardioselective β-blockers and there is no effect on airflow obstruction. One, somewhat similar, study by Schnabel showed a trend for a lower FEV_1 in people taking β-blockers and a significantly reduced FVC, but since the study focussed on other objectives, the association was not extensively investigated [26]. Our study confirms this association with FVC and shows an association with FEV_1.

Previous studies did not show a significant effect on FEV_1 or respiratory symptoms in meta-analyses of randomized clinical trials [9,10]. However, these meta-analyses included

primarily small randomized placebo controlled studies. In these studies, 80% of the participants were male and COPD severity was mild to moderate and according to the authors this could be a limitation of the study [9]. In a crossover trial, Jabbour et al. showed a difference in FEV_1 between patients with cardioselective and non-cardioselective β-blockers for chronic heart failure (CHF) with COPD. The authors reported a 150 ml lower FEV_1 in patients with CHF and COPD receiving non-cardioselective β-blocker than in patients receiving cardioselective β-blockers. No such difference could be detected for FVC [27]. This study in 51 males showed differences between patients receiving either of the β-blockers, but did not compare pulmonary function to non-users. Another study by Çamsari et al in 50 patients showed that metoprolol did not change pulmonary function according to symptoms, clinical findings and FEV_1 changes [28].Other studies focussed on morbidity and mortality in patients with CVD and COPD. Rutten et al. performed a large observational study using general practitioners records. The authors assessed all-cause mortality and exacerbations of COPD in the complete sample and in subsets. Rutten et al. showed a possible beneficial effect on survival and a reduced risk on COPD exacerbation in patients taking cardioselective β-blockers [11]. Misclassification of patients could be an issue, according to the authors, and patients could have had concealed cardiac disease instead of COPD, which would show a positive effect of β-blockers on survival [2,11]. On the other hand, since heart failure and COPD exacerbations were suggested to be closely linked with regard to mortality [29,30], treating patients with a multidrug regimen might provide disease-specific benefits and reduce mortality [31].

Chen et al. found that β-blocker use after an acute myocardial infarction, in patients with COPD without β-agonist, use significantly reduced one year mortality. The authors suggest that these patients may have a mild form of COPD and that the benefits of β-blocker therapy outweigh the risk of bronchospasm [32].

In a recent paper by Short [33], the investigators defined a cohort of COPD patients and compared mortality across different treatment regimens for COPD and CVD. The investigators found a 22% overall reduction of all-cause mortality for patients with COPD if the treatment regimen included β-blockers. Spirometry measurements were available in a subset of patients. The authors found no deleterious effect of β-blockers on FEV_1 and FVC. This observational study, like any observational study, is at risk for confounding by indication and post hoc treatment changes by physicians can influence the results [34]. Furthermore, the analysis of a subset with spirometry tests could be influenced by some form of selection bias in a retrospective database study.

The strengths of our study are the prospective, population-based cohort design and the large number of study subjects with pulmonary function tests. The population-based character and high participation rate makes selection bias less likely. Due to the prospec-

tive nature of the data collection, also information bias is unlikely and information about several covariables was collected. Another strong point is that we in the Rotterdam study we have both prescription and interview data to check adherence to therapy. An earlier analysis in the Rotterdam Study showed that there was a very high concordance between pharmacy dispensing data of β-blockers, and actual use during interview [35].

Although longitudinal data shows interesting patterns, numbers of repeated measurements were limited. Due to the observational character of our data, residual confounding cannot be ruled out completely. We adjusted for confounding by (contra-) indication by excluding participants with COPD, and as a proxy for asthma, we excluded patients with prescriptions for inhalation medication to study the effects in patients without obstructive pulmonary disease. Since spirometry was introduced in 2002, some form of survival bias could have occurred, but our expectation is that this would not explain the fact that we observed a difference between users and non-users of β-blockers. Lastly we could not include disease-specific mortality data into these analyses to see if this reduction in FEV_1 and FVC was associated with a higher mortality rate.

In conclusion, in this population-based study, we demonstrated that not only non-cardioselective, but also cardioselective β-blockers had a clinically significant effect on both FEV_1 and FVC in some patients. Unlike cardioselective β-blockers, non-cardioselective β-blockers were, beside FEV_1 and FVC, significantly associated with a marginally decreased FEV_1/FVC.

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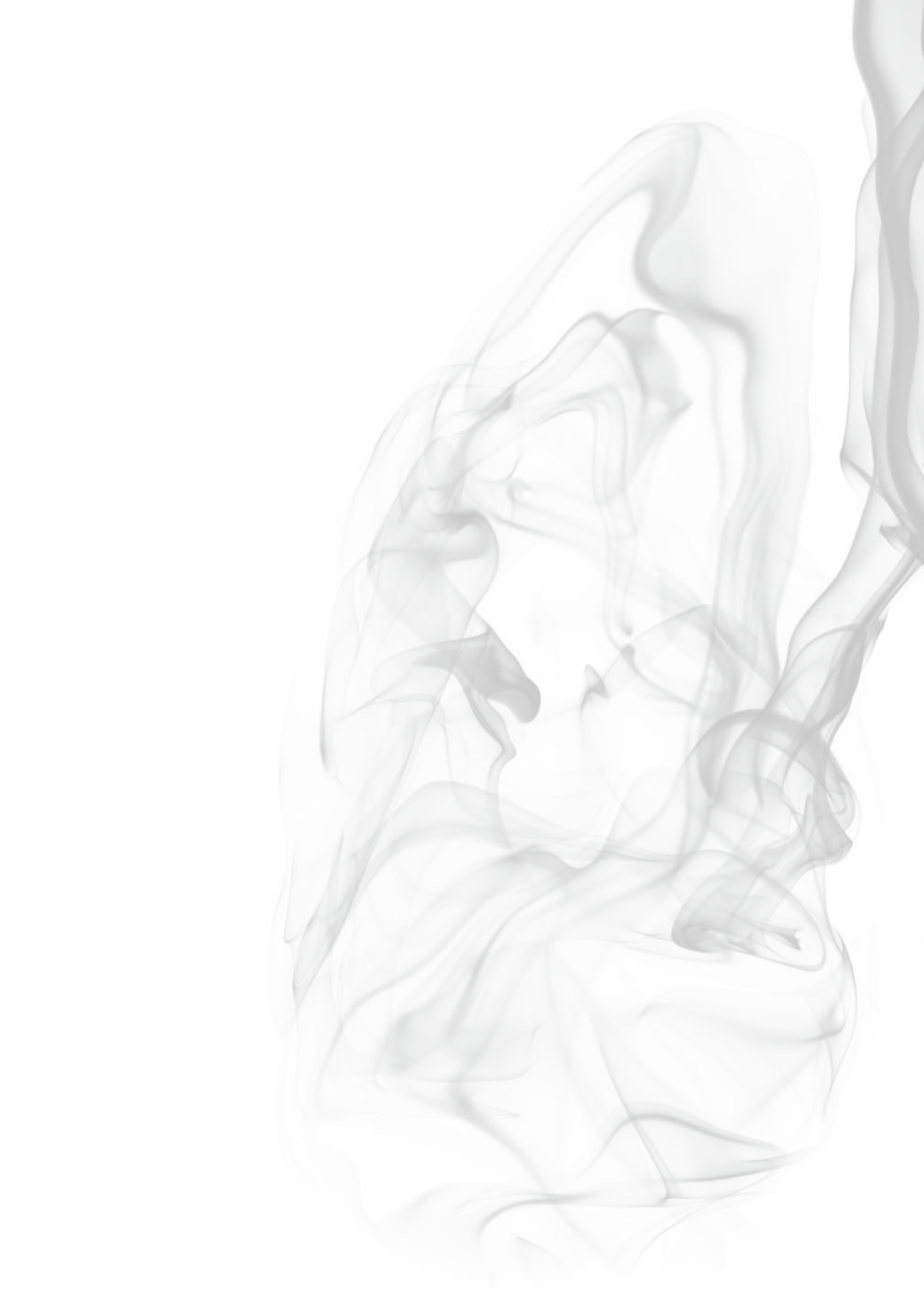

CHAPTER 4.2

Statins, systemic inflammation and risk of death in COPD: The Rotterdam Study

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Published in Pulmonary Pharmacology & Therapeutics. 2013 Apr;26(2):212-7. Epub 2012 Nov 7. PubMed PMID: 23142156.

ABSTRACT

Background Studies suggest that statins decrease mortality in COPD patients but it is unknown which patients might benefit most.

Objectives We investigated whether statins were associated with reduced mortality in COPD patients and whether effects differed according to baseline high-sensitivity C-reactive protein (hsCRP) concentration, a marker of systemic inflammation.

Methods This nested case-control study was part of the Rotterdam Study, a prospective population-based cohort study among 7983 subjects ≥ 55 years. Using automated pharmacy records, we evaluated statin use of 363 cases (COPD patients who died during follow-up of 17 years) with 2345 age and sex matched controls (COPD patients who survived the follow-up period of the index case).

Results Compared to never use, long-term statin use (> 2 years) was associated with a 39% decreased risk of death in COPD patients. Stratified according to the level of systemic inflammation, long-term statin use was associated with a 78% reduced mortality if hsCRP level > 3 mg/L, versus a non-significant 21% reduced mortality if hsCRP level ≤ 3 mg/L.

Conclusions Statin use is associated with a beneficial effect on all-cause mortality in COPD, depending on the baseline level of systemic inflammation.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of morbidity and mortality worldwide. In industrialized countries about 400,000 COPD deaths occur each year and the mortality is expected to increase further.[1] The disease is characterized locally by a chronic inflammation of small airways and destruction of alveoli and systemically, in a subset of COPD patients, by increased markers of inflammation, including high-sensitivity C-reactive protein (hsCRP) and interleukin 6 (IL6) [2-4]. The subset of COPD patients with persistent systemic inflammation has recently been shown to be associated with poor clinical outcomes despite similar lung impairment.[5] The chronic low-grade inflammation might be the key link to the occurrence of various comorbidities in COPD including cardiovascular diseases and lung cancer. Moreover, these comorbidities are the main causes of death in mild to moderate COPD; therefore, with the increased recognition of the prognostic role of comorbidities in COPD, all-cause mortality has become one of the major endpoints in the evaluation of novel therapies[6].

Recent observational studies suggest that statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) may reduce morbidity and mortality in COPD patients[7-9]. Statins are a class of drugs mainly used to treat hypercholesterolemia and to prevent cardiovascular events. Statins reduce cholesterol synthesis by inhibition of HMG-CoA reductase in the liver and increase low density lipoprotein-cholesterol uptake from the circulation. In addition to their lipid-lowering effect, statins also possess pleiotropic anti-inflammatory and immunomodulating properties, and are able to reduce levels of inflammatory markers such as CRP [10]. CRP is a validated biomarker of systemic inflammation in COPD and increased CRP levels in patients with COPD are associated with increased mortality [11,12]. Lee et al. recently showed in a randomized controlled trial with COPD patients, that pravastatin treatment significantly decreased CRP and IL6 levels compared to placebo and that the improvement of exercise tolerance was greater in those with a greater decrease of hsCRP levels and higher baseline CRP levels [13]. However, to our knowledge, studies exploring whether the beneficial effect of statins on all-cause mortality in COPD is greater in those with increased systemic inflammation, have not yet been published.

Therefore, the objective was to investigate whether statins have a beneficial effect on mortality in COPD patients with increased baseline hsCRP-levels in the Rotterdam Study, a large prospective population-based cohort study with long-term follow-up.

METHODS

Study population and design

We performed a nested case-control analysis in all COPD cases within the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly [14]. The Rotterdam study cohort includes 7983 participants aged ≥ 55 years, living in Ommoord, a well-defined suburb of Rotterdam, the Netherlands. Almost all participants (99.8%) are of Caucasian descent. Baseline data were collected from 1989 until 1993 and each participant visits the research centre every 2 to 3 years. In addition, participants are continuously monitored for the onset of major events which occur during follow-up through automated linkage with files from general practitioners. Nearly all participants (99.7%) are registered at one or more of seven automated pharmacies serving the Ommoord area. From these pharmacies, records of all filled prescriptions were available as of January 1st, 1991. The medical ethics committee of the Erasmus Medical Centre, Rotterdam, and the review board of The Netherlands Ministry of Health, Welfare and Sports, approved the study. Participants gave written informed consent.

Definition of cases and controls

Cases and controls were nested in all participants of the source population of whom hsCRP was measured at baseline and of whom COPD was diagnosed by an obstructive spirometry (proportion of the forced vital capacity exhaled in the first second (FEV_1/FVC) < 0.7) during the research centre or pulmonologist visits or by a general practitioner. Physician diagnosed asthma patients were excluded. The incident COPD date was defined as the date of COPD diagnosis in the medical records, or the date of a first COPD medication prescription or the date of obstructive lung function examination, whichever came first. To ensure at least three months medication history for every subject, participants of whom study follow-up started before April 1st, 1991 were excluded. Cases were COPD subjects who died between April 1st, 1991 and January 1st, 2008. The mortality date was taken as the index date. Controls were COPD subjects matched on sex and age (+/- 1 year) who were still alive on the same day of follow-up as their matched case. The duration of COPD was determined as the time between the incident COPD date and the index date.

Statin exposure

Complete information on all filled prescriptions on a day-to-day basis was obtained in automated format from the pharmacies. Subjects were classified as statin users if they had received at least one prescription for statins between start and index date. The duration of a prescription was calculated as the total number of delivered units divided by

the prescribed daily number of units. The studied statins were simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin and rosuvastatin. All drugs under study are only available on prescription in the Netherlands.

All-cause mortality & primary cause of death

Information on vital status of the Rotterdam Study participants was obtained from general practitioners and from municipal records. Mortality follow-up started at baseline and was complete until January 1st, 2008. Causes of death during follow-up in the Rotterdam Study were coded according to the International Classification Of Diseases (ICD)-10 [15]. The following categories were applied for description in the text: cardiac mortality (ICD-10: I21-I73, R96), pulmonary mortality (ICD-10: J15-J44), death from bronchial carcinoma (ICD-10: C34), death from other malignancies (ICD-10: C15-C96 except C34) and other causes of death (ICD-10: all other used codes).

Covariables

The nested case-control approach makes it possible to account for age and gender by matching and to adjust for other drug use at the index date. Therefore, we adjusted mortality risk estimates for use of cardiovascular drugs (antihypertensives, diuretics, β-blockers, calcium channel blockers and ATC C02, C03, C07, C08 & C09 respectively), antidiabetics (ATC A10) and corticosteroids for systemic use (ATC H02) on the index date. Furthermore, the duration of COPD at index date and the following covariables at baseline were considered as potential confounders: pack-years of cigarette smoking, total serum cholesterol, hsCRP, systolic blood pressure, body-mass index (BMI), diabetes mellitus and cardiovascular covariables (myocardial infarction, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, atrial fibrillation and heart failure); and their assessment has been described previously [2,3]. One prerequisite to be a confounder is that the variable is associated with the exposure, here statin use. Therefore, the relationship between the potential confounder and statin use (yes/no) was evaluated for categorical variables with a Chi Square test and for continuous variables with a Mann-Whitney U test.

Statistical analysis

Crude and adjusted odds ratios for mortality were estimated using conditional logistic regression. All models were adjusted for covariates that were significantly related to the exposure and changed the point estimate by more than 10%. Statin drug use was categorized as no use (0 days), short-term (1-30 days), mid-term (31 days-2 year) and long-term use (≥ 2 year). Because every participant should have the opportunity to be a long-term user, COPD subjects with an incident date from January 1st, 2006 onwards, were excluded from analyses. Never use of statins was the reference category in all

analyses. To gain power in the subdivided cause-specific mortality analyses, the groups mid-term and long-term use were combined. hsCRP serum levels were categorized as high versus average and low, based on the American Heart Association classification [16]. Total serum cholesterol levels were categorized as high versus borderline and desirable, based on the Adults Treatment Panel III classification [17]. All statistical analyses were performed using SPSS version 18 (SPSS Inc, Chicago, IL). P values below the conventional level of significance ($p < 0.05$) were considered as statistically significant.

RESULTS

Baseline characteristics of the study population

Within the source population of 7983 subjects, hsCRP was successfully measured in 6658 participants at baseline. (Figure 1) Of these, 758 COPD patients were identified with a study start date after April 1st, 1991 to ensure at least three months of medication history. The vast majority (82.5%) of COPD patients was confirmed by an obstructive spirometry. Seventy-one patients with an incident COPD date after January 1st, 2006 were excluded from analyses because at least two years of follow-up between incident COPD and death were required to study the association of long-term statin use on mortality. During the potential follow-up of 17 years (1991-2008), 363 COPD patients deceased and were determined as cases. For each case, an average of six age- and sex-matched

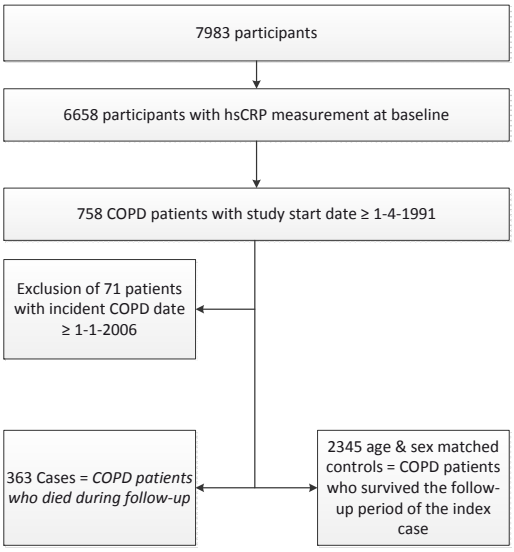

Figure 1. Study flowchart.

controls with COPD who survived the follow-up period of their matched case, were selected for a total of 2345 controls. Because non-deceased participants with COPD could serve as a control in several case-control sets, the total number of controls is larger than the total number of incident cases of COPD. Table 1 shows the baseline characteristics

Table 1. Baseline characteristics

		Cases	Controls
Number of participants		363	2345
Age at index date (years)		81 (75-85)	78 (74-81)
Male		249 (69%)	1722 (73%)
Smoking behaviour			
Never smoker		39 (11%)	252 (11%)
Current smoker		145 (40%)	820 (35%)
Former smoker		164 (45%)	1170 (50%)
Missing		15 (4%)	103 (4%)
Median pack-years		30 (14-50)	28 (13-47)
Body mass index (kg/m^2)		25 (23-28)	26 (24-28)
Diabetes		43 (12%)	161 (7%)
Systolic blood pressure (mmHg)		139 (123-155)	137 (124-152)
Cardiovascular covariables[§]		163 (45%)	724(31%)
hsCRP (mg/L)		2.1 (1.1-4.0)	2.7 (1.3-4.8)
hsCRP categories[a]	**≤ 3 mg/L**	202 (56%)	1524 (65%)
	>3 mg/L	161 (44%)	821 (35%)
Total serum cholesterol (mmol/l)		6.4 (5.6-7.2)	6.4 (5.7-7.3)
Total cholesterol	**< 240 mg/dL**	166 (46%)	1055 (45%)
categories[b]	**≥ 240 mg/dL**	197 (54%)	1290 (55%)
Drug use at index date			
Antidiabetics		41 (11%)	194 (8%)
Cardiovascular drugs[b]		209 (58%)	1249 (53%)
Oral corticosteroids		138 (38%)	303 (13%)
Duration of statin use			
None		299 (82%)	1856 (79%)
1-30 days		5 (1%)	22 (1%)
31 days-2 years		18 (5%)	197 (8%)
> 2 years		41 (11%)	270 (12%)
Duration of COPD at index date (years)		7 (3-11)	5 (2-8)

Categorical variables are expressed as count (percentage). Values of continuous variables are expressed as median (25-75 percentiles). Cases: COPD patients who deceased during follow-up; controls: COPD patients who survived the follow-up period of the index case. §Cardiovascular covariables included myocardial infarction, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, atrial fibrillation and heart failure. a hsCRP categories based on the American Heart Association [16], total cholesterol categories based on the Adults Treatment Panel III classification [17]. b; Cardiovascular drugs include antihypertensives, diuretics, β-blockers and calcium channel blockers.

of cases and controls. Cases were more frequently current smokers and had a higher prevalence of cardiovascular disease at baseline in comparison to the controls. Although age- and sex-matched, cases seem older and less often males as a consequence of the fact that there were more controls for males and lower age-categories.

Mortality in COPD patients

The cumulative survival of COPD patients (n=758) was worse in those with a baseline hsCRP of more than 3 mg/L than in those with an hsCRP of 3 mg/L or less (Figure 2; log-rank: $p < 0.0001$). In contrary, COPD patients with a total cholesterol of 240 mg/dL or more had a better survival than those with less than 240 mg/dL at baseline (data not shown; log-rank: $p = 0.008$). The most frequent causes of death in COPD patients are listed in Table 2. Most cases died due to cardiovascular causes (38.3%), followed by pulmonary complications of COPD (COPD exacerbations, emphysema or pneumonia;19.6%) and bronchial carcinoma (10.5%).

Statin use and the risk of mortality in COPD

Table 3 shows the association between statin use and the risk of all-cause mortality in COPD in a first model which is already adjusted for age and sex by matching and in a second model adjusted for all confounders related to the exposure which changed the

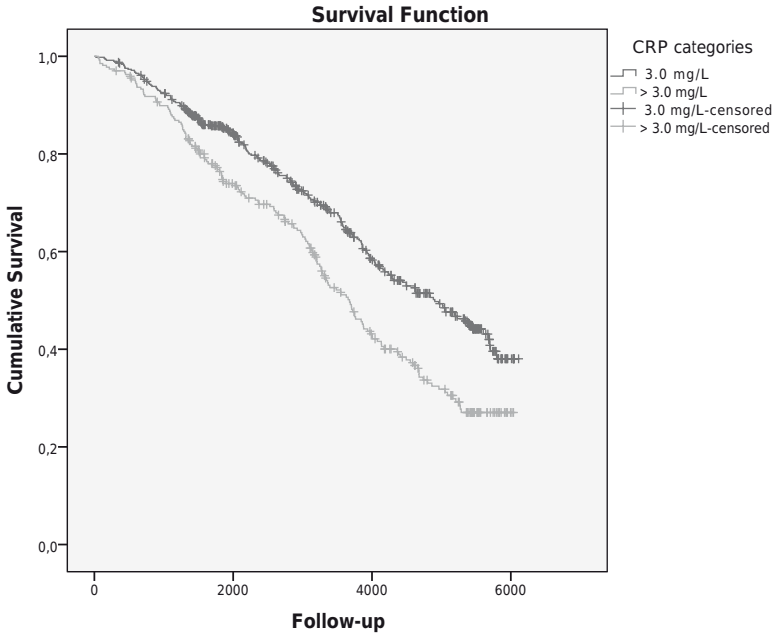

Figure 2: Kaplan-Meier survival curve of COPD patients (n=758) according to the hsCRP level (at baseline).

Table 2. Causes of death in the COPD study population

		n	% of Total
Pulmonary		71	19.6
	COPD	59	16.3
	Pneumonia	12	3.3
Cardiovascular		139	38.3
Mainly	Heart failure	31	8.5
	Stroke	30	8.3
	Sudden (cardiac) death	30	8.3
	Cardiac arrest	18	5.0
	Acute myocardial infarction	12	3.3
	Chronic ischaemic heart disease	6	1.7
Cancer		88	24.2
Mainly	Malignant neoplasm of bronchus and lung	38	10.5
	Malignant neoplasm of colon	8	2.2
	Malignant neoplasm of pancreas	6	1.7
	Malignant neoplasm of prostate	6	1.7
Other		65	17.9
Mainly	Other ill-defined and unspecified causes of mortality	19	5.2
	Dementia	8	2.2
	Fracture of femur	7	1.9
	Total:	363	

Table 3. Association between statin use and the risk of (all-cause) death in COPD

	Crude model[a]				**Adjusted model**[b]			
		95% CI				95%CI		
	OR	Lower	Upper	p-value	OR	Lower	Upper	p-value
No statin use	*Reference*				*Reference*			
1 - 30 days	1.34	0.49	3.67	0.572	1.61	0.48	5.48	0.443
31 days - 2 years	0.68	0.40	1.16	0.156	0.64	0.35	1.15	0.136
> 2 years	0.82	0.55	1.22	0.331	**0.61**	**0.38**	**0.99**	**0.045**

[a]Crude model: adjusted for age and sex by matching

[b]Adjusted model: adjusted for age and sex by matching; adjusted in the analyses for the use of cardiovascular drugs, antidiabetics, oral corticosteroids and duration of COPD at index date, and pack-years of cigarette smoking, total serum cholesterol, body-mass index and cardiovascular covariables at baseline

Abbreviations : CI, confidence interval; OR, Odds ratio

point estimate by more than 10%. Baseline hsCRP-level was ruled out as confounder because it was not associated with statin prescriptions (nor as categorical variable neither continuously); however, hsCRP was evaluated as a potential effect modifier. Compared to never use, long-term statin use (> 2 years) was associated with a 39% decreased risk

of (all-cause) death in COPD patients (95%CI, 0.38-0.99) independent of age, sex, other drug use, duration of COPD, pack-years, total serum cholesterol, BMI and cardiovascular covariables.

Regarding cause-specific mortality in COPD, statin use (>30 days) compared to never use was associated with a significant 64% decrease in risk of pulmonary mortality (95%CI, 0.13-0.97). (Table 4) In the adjusted model, there was a trend of decrease in risk of pulmonary and cardiovascular mortality. Statin use did not affect cancer mortality in both, crude and adjusted analyses.

Statin use and the risk of (all-cause) death in COPD according to the level of systemic inflammation

When stratified according to the level of systemic inflammation, long-term statin use was associated with a 78% reduced risk of death in COPD patients with a hsCRP level > 3 mg/L (95% CI, 0.06-0.74), versus a non-significant 21% reduced risk of death in COPD patients with a hsCRP level ≤ 3 mg/L (95% CI, 0.41-1.55). (Table 5) When we excluded COPD patients exclusively diagnosed by GP, the point estimator did not change substantially (OR 0.49 in the crude model and OR 0.31 in the adjusted model, investigating the effect of long-term statin use compared to no use in COPD subjects with hsCRP > 3mg/L). A sensitivity analysis restricting the subjects to smoking COPD patients with a hsCRP level > 3 mg/L, confirmed that long-term statin use compared to no use was associated with a 85% reduced risk of death (95% CI, 0.04-0.61, adjusted model). In Table 6, analyses were stratified according to the total serum cholesterol level at baseline. The reduced risk of

Table 4. Association between statin use and the risk of cause-specific mortality in COPD

	Crude model[a]				**Adjusted model**[b]			
		95% CI				95% CI		
Pulmonary mortality	OR	Lower	Upper	p-value	OR	Lower	Upper	p-value
No statin use	*Reference*				*Reference*			
>30 days of statin	**0.36**	**0.13**	**0.97**	**0.044**	0.37	0.13	1.08	0.068
Cardiovascular mortality								
No statin use	*Reference*				*Reference*			
>30 days of statin	0.87	0.54	1.41	0.579	0.58	0.33	1.01	0.053
Cancer mortality								
No statin use	*Reference*				*Reference*			
>30 days of statin	0.89	0.49	1.61	0.700	0.92	0.48	1.75	0.790

[a]Crude model: adjusted for age and sex by matching

[b]Adjusted model: adjusted for age and sex by matching; adjusted in the analyses for the use of cardiovascular drugs at index date, and total serum cholesterol and cardiovascular covariables at baseline

Abbreviations : CI, confidence interval; OR, Odds ratio

Table 5. Association between statin use and the risk of death in COPD, stratified according to the serum level of hsCRP (at baseline)

		Crude model[a]				**Adjusted model**[b]			
			95% CI				95% CI		
		OR	Lower	Upper	p-value	OR	Lower	Upper	p-value
hsCRP ≤ 3 mg/L	No statin use	*Reference*				*Reference*			
	1 - 30 days	0.66	0.14	3.16	0.606	0.77	0.14	4.09	0.756
	31 days - 2 years	0.70	0.35	1.43	0.335	0.60	0.27	1.34	0.212
	> 2 years	0.87	0.51	1.50	0.620	0.79	0.41	1.55	0.496
hsCRP > 3 mg/L	No statin use	*Reference*				*Reference*			
	1 - 30 days	1.83	0.29	11.49	0.520	*NA*			
	31 days - 2 years	0.77	0.30	1.96	0.584	0.95	0.33	2.73	0.917
	> 2 years	**0.44**	**0.20**	**0.97**	**0.042**	**0.22**	**0.06**	**0.74**	**0.015**

[a]Crude model: adjusted for age and sex by matching

[b]Adjusted model: adjusted for age and sex by matching; adjusted by model for the use of cardiovascular drugs, antidiabetics, oral corticosteroids and duration of COPD at index date, and pack-years of cigarette smoking, total serum cholesterol, body-mass index and cardiovascular covariables at baseline.

Abbreviations : hsCRP, high-sensitivity CRP; CI, confidence interval; OR, Odds ratio; NA, Not applicable (due to low numbers)

Table 6. Association between statin use and the risk of death in COPD, stratified according to the total serum cholesterol level (at baseline)

		Crude model[a]				**Adjusted model**[b]			
			95% CI		p-		95% CI		p-
		OR	Lower	Upper	value	OR	Lower	Upper	value
Total cholesterol < 240 mg/dL	No statin use	*Reference*				*Reference*			
	1 - 30 days	1.99	0.36	10.85	0.428	5.68	0.38	84.61	0.208
	31 days - 2 years	0.32	0.07	1.40	0.130	0.17	0.02	1.42	0.102
	> 2 years	0.41	0.13	1.30	0.129	**0.16**	**0.03**	**0.91**	**0.039**
Total cholesterol ≥ 240 mg/dL	No statin use	*Reference*				*Reference*			
	1 - 30 days	1.24	0.22	7.01	0.806	1.22	0.07	21.19	0.891
	31 days - 2 years	0.69	0.37	1.32	0.266	0.65	0.32	1.33	0.238
	> 2 years	0.79	0.47	1.31	0.355	**0.50**	**0.26**	**0.95**	**0.034**

[a]Crude model: adjusted for age and sex by matching

[b]Adjusted model: adjusted for age and sex by matching; adjusted by model for the use of cardiovascular drugs, antidiabetics oral corticosteroids and duration of COPD at index date, and pack-years of cigarette smoking, body-mass index and cardiovascular covariables at baseline.

Abbreviations : CI, confidence interval; OR, Odds ratio

death in COPD patients by long-term statin use was significant in both categories (<240 mg/dl and ≥ 240 mg/dl).

DISCUSSION

This is the first prospective study in a general population showing that the beneficial effect of long-term statin use on the risk of mortality in COPD patients is modified by the baseline level of systemic inflammation. The results suggest that the subset of COPD patients characterized by increased markers of systemic inflammation might benefit most from long-term statin therapy. One in three COPD patients in our study died from cardiovascular causes - figures which have also been described by other authors [6,18]. Although the protective effect of statins in COPD patients could represent solely an indirect effect on the cardiovascular comorbidities associated with COPD, our results also suggest an effect on respiratory mortality by statin use compared to never use.

Increasing insights into the pleiotropic effects of statins unravel several possible mechanisms for the beneficial effects seen in COPD patients. Beyond their known ability to inhibit endogenous cholesterol synthesis, statins exert immunomodulating effects in both systemic and pulmonary cytokine driven inflammation by inhibiting guanosine triphosphatase proteins [19]. Statins down regulate the expression of adhesion molecules involved in the recruitment of inflammatory cells, and of chemokines which are increased in COPD, such as CCL2 and CXCL8 [20]. Furthermore, simvastatin reduces the expression of matrix metalloproteinases (MMPs) involved in COPD matrix remodelling, such as MMP2, MMP9 and MMP12 [21,22]. In support of a direct effect of statins in COPD, Lee et al. demonstrated in a rat model of smoking-induced emphysema that simvastatin ameliorated the structural and functional derangements of the lungs partly by suppressing inflammation and matrix MMP9 induction [23]. Statins may even reduce oxidative stress, related to their ability to scavenge oxygen derived free radicals [24].

The 39% reduced risk of death in COPD patients by using (long-term) statin therapy is consistent with findings of previous (retrospective) observational studies [8,9]. Similar to the retrospective study of Frost et al., we found a protective effect of statin use on the risk of pulmonary mortality in COPD [25]. Furthermore, our results showed that having an hsCRP baseline level of more than 3 mg/L was associated with increased mortality and therefore we expected, if statins were effective, that the pre-specified subset of COPD patients with high hsCRP levels would benefit most of treatment. Surprisingly but consistent with other prospective population-based studies in the elderly, we found that COPD patients with a total cholesterol of 240 mg/dL or more had a better survival [26,27]. Selective survival or changes in the arterial wall by aging might yield individuals resistant to the effects of high cholesterol concentrations in the blood or high cholesterol levels might be associated with less frailty.

Importantly, we demonstrate a significant interaction between statin use and the degree of systemic inflammation because the protective effect of statins was only significant in COPD patients with the highest hsCRP serum level. There was no significant effect in COPD patients with a low-to-moderate degree of systemic inflammation, which cannot be explained due to lack of power as numbers were even higher in this stratum. The same phenomenon has also been reported in cardiovascular studies and one RCT in COPD patients. Kinjo et al. described a decreased hazard by statin therapy for 1-year mortality in patients with a CRP level above 2.9 mg/L who had an acute myocardial infarction [28]. In addition, the JUPITER trial of apparently healthy persons without hyperlipidemia but with elevated hsCRP levels, demonstrated that rosuvastatin significantly reduced the incidence of all-cause mortality and was associated with a 60% decreased risk for cardiovascular endpoints [29]. However, a recent RCT in persons at high risk of vascular events, could not confirm that these vascular benefits of statin therapy were affected by the baseline CRP concentration [22]. The RCT in COPD patients by Lee et al. demonstrated that the improvement in exercise time by statin treatment is modified by plasma hsCRP-levels [13]. Because both the inflammation and therapeutic interventions in COPD have been studied merely in (ex)smoking subjects, we furthermore restricted the analyses to smoking COPD patients, which confirmed the beneficial effects of long-term statin use in the subgroup with greater inflammation.

Because of its observational character, a possible limitation of our study is that treatment with statins was not randomly assigned. Our results could therefore be flawed by confounding by indication. However, because statins are selectively prescribed in subjects who have substantial comorbidities such as diabetes mellitus and cardiovascular disease, this kind of confounding would only underestimate the protective effect of statins on the risk of death. Consequently, this would mean that the true protective effect is even stronger than we measured. Secondly, we cannot exclude a healthy user bias if statins are prescribed more readily in health-conscious, medical-attention-seeking patients or in those patients who are expected to live long enough to benefit from statin treatment. Importantly, however, this would not explain effect modification by baseline hsCRP as this measurement was not used for usual patient care. Finally, of a minority of COPD patients diagnosed by GP (17,5%), we do not have the certainty that diagnosis was confirmed by an obstructive spirometry. However, exclusion of this subgroup did not change the point estimator substantially.

The strengths of this study are the high quality information available about exposures prior to outcome with a prospective data collection, the general population based setting, the large number of subjects that participated in the Rotterdam Study and the long duration of follow-up. The high response rate and virtually complete follow-up for every

participant makes information and selection bias for these data unlikely. An important advantage is the availability of continuous pharmacy dispensing data with complete pharmacy records of all filled prescriptions on practically all members of the cohort, providing very specific and detailed information on drug use and thus minimizing the risk of exposure misclassification. Furthermore, the nested case-control approach made it possible to account for other drug use at index date, a major confounder in the association between statins and mortality. It is important that these results should be further investigated in randomized controlled trials before recommending widespread statin use in COPD patients. According to Clinicaltrials.gov, several randomized clinical trials are ongoing.

In conclusion, statin use is associated with a decreased risk of all-cause mortality in patients with COPD, depending on the degree of systemic inflammation. These results suggest that in an older population of COPD patients, CRP levels might guide the clinician better than total cholesterol levels in his decision to start lipid lowering therapy. This study may provide a rationale for undertaking more definitive randomized clinical trials to confirm the impact of statin use on the outcome of COPD and to elucidate the mechanisms by which they may work.

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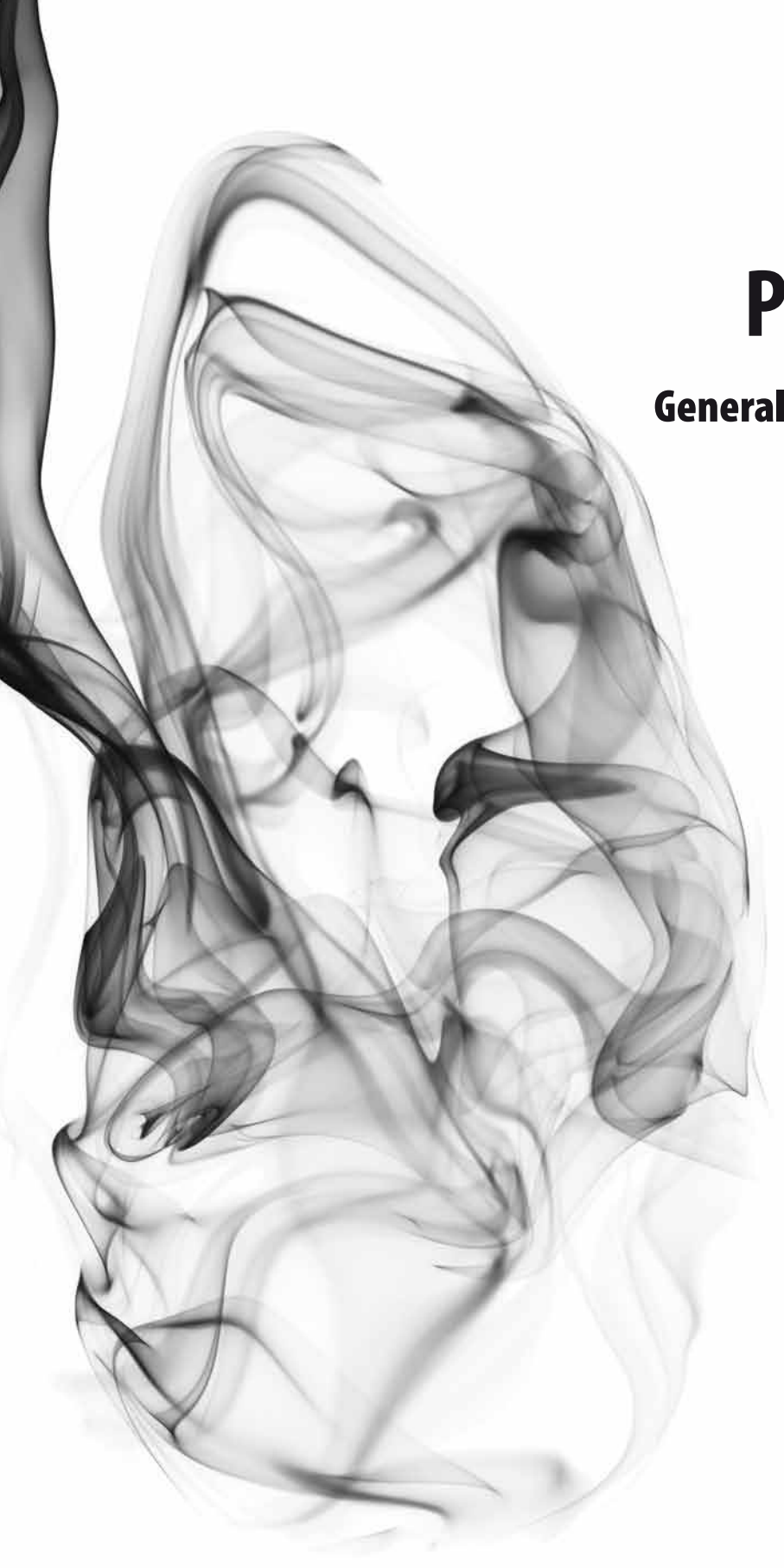

PART V

General Discussion

INTRODUCTION

"Chronic Obstructive Pulmonary Disease (COPD), a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients." [1]

This is the definition as stated by the Global initiative for Chronic Obstructive Lung Disease (GOLD). The incidence of COPD is increasing and the disease is estimated to be the third leading cause of death worldwide[2]. Although inhalation of noxious particles and gases is the main cause of COPD, there seems to be a discrepancy between the number of smokers and the occurrence of COPD. Common and rare genetic factors may explain part of the susceptibility to the damage inflicted by these particles and gases. Furthermore, genetics may account for impaired lung growth during fetal development, childhood and adolescence, resulting in a maximum lung capacity which is already reduced compared to the population average[3]. During the course of adult life, lung function diminishes which can result in disability, oxygen dependency or death [4]. COPD is a very heterogeneous disease, but one of the cornerstones of diagnosis of COPD remains spirometry, a highly reproducible and fairly easy to implement assessment of lung volumes and function. Due to the heterogeneity of the etiology of COPD and the assumption that complex diseases consist of a compound of quantitative traits [5], spirometry measurements have been proven to be valuable to investigate common genetic polymorphisms [6-8] and to identify genes like Human Hedgehog Interacting Protein (*HHIP*) which seem to have a strong relationship to COPD [9].

The main objectives of this thesis were to investigate the association between spirometry and COPD in the general population. This includes, but is not limited to, genetic components of and genetic susceptibility to COPD. This discussion places the results of our research in perspective, elaborates on general methodological considerations and provides some future perspectives of genetics and pharmacogenetics with respect to obstructive lung diseases.

MAIN FINDINGS

Spirometry in an aging population

Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) and the ratio of FEV_1 over FVC (FEV_1/FVC) are the most commonly used measures of pulmonary func-

tion and essential in the diagnosis of COPD, assessing disease severity, and monitoring treatment responses [1,10,11]. Pulmonary function also predicts mortality in the general population even among people who have never smoked, who have only modestly reduced pulmonary function, and who have no respiratory symptoms [12]. The peak level of pulmonary function is attained in early adulthood and declines subsequently over time[3,4]. Many of the currently used reference values were calculated several decades ago and did not include elderly participants due to the lack of data in large groups of elderly [13-17]. Being above or below the cut-off for the FEV_1/FVC, in diagnosis of COPD, is one of the most important diagnostically used evaluations. The most frequently used cut-off is the fixed ratio of 70% [1]. One important drawback of using reference values, which have been evaluated only in younger people, is that healthy elderly, who have a normal lung function for their age but have a FEV_1/FVC below 70%, will be incorrectly considered to have an airflow limitation. This will lead to an overestimation of COPD in the healthy elderly. One of the possible consequences could be unnecessary treatment with (inhalation) medication[15].

In chapter 2.1 the objective was to investigate the spirometry measures from a large group of healthy elderly from the Rotterdam Study. For the selection of the study population of healthy elderly, we excluded current and ex-smokers and participants with symptoms of respiratory diseases, participants taking pulmonary (inhalation) medication and β-blockers. The exclusion of β-blockers was considered necessary as we showed an association between β-blockers use and spirometry (see chapter 4.1). We decided to select such a strict subset in order to remove any participants who might suffer from (previously) undiagnosed COPD and were not comparable to the entire population. Although this decreased the size of the study group, we were still able to investigate the pulmonary function tests in 1,125 people of whom more than 25 % was aged 72 years and over. The spirometric measures from these people were used to calculate reference equations using quantile-regression. Interestingly, although we allowed for several mathematical transformations of the outcome and predictors, FEV_1 and FVC followed a linear decrease in the elderly, whereas in children and adolescents the trajectory is all but linear. The ratio of FEV_1/FVC decreased in females more rapidly between 50 and 70 years of age after which it seems to decrease at a slower rate. The majority of female participants had an age between 61 and 77, so perhaps the estimates for the younger age categories are not as precise as we had aimed for. Compared to two of the most commonly used references [15,18], we showed a narrow range for prediction of pulmonary function across several different ages and mean heights. Furthermore, after evaluating prediction formulas, we concluded that healthy elderly can have lower spirometry values (FEV_1, FVC and FEV_1/FVC) without classifying them as diseased.

These data hopefully shed some extra light on the issue of inadequate reference values in the elderly and may contribute to the evidence that the fixed ratio, as it is still proposed by GOLD [1], could use more flexibility. Our study has of course some limitations, of which sample size is one. Although we have a relatively large study compared to other single study analyses, specific studies or large scale collaborations aimed to analyze lung function in healthy elderly, and especially in people over 80 years old, are still necessary. Another limitation of calculating reference values in relatively small age strata, is that they cannot be collided with reference values from adjacent age categories, introducing uncertainty about the equations at the ends of the stratum.

During the same period as our study, a large effort has been made to calculate the reference values for people aged 8 – 95 years[19], and while this study was much larger than our study, the number of subjects in the people aged over 80 years decreased drastically compared to all other categories. We were also not able to investigate pulmonary function in people from other than European descent. Quanjer and colleagues did analyze the results from subjects from other than European descent, but there are almost no data in the age categories over 80 years old in people from Asian or African descent. These are some major issues which need to be addressed in future research especially since spirometry is not only useful in assessing pulmonary disease, but can play a role in the prediction of mortality, even in absence of respiratory disease [20-25].

Because spirometry is also closely related to cardiac function, we evaluated the correlation between the association between echocardiographic measures and pulmonary function in the general population (chapter 2.2). Although the presence of elevated pulmonary arterial systolic pressure (PASP) in patients with a lung disease is a well-known occurrence, the relation between pulmonary ventilatory function and PASP is less well investigated. Furthermore, data on both pulmonary arterial pressure and spirometry measurements, from the general population are scarce. We found indeed strong evidence for an association between PASP and spirometry, but the effect sizes were limited. Therefore, it remains to be seen if this is merely normal physiological variation or if this can further elucidate the etiology of secondary pulmonary hypertension or be of use in developing treatment of pulmonary hypertension. This highlights the fact that lung function extends its effects to more organs than only the lungs and that more research is needed to grasp the clinical implications and diagnostic opportunities of these associations.

Genome-wide association studies of spirometry

After the initial genome-wide association studies (GWAs) which have been performed in single study analyses, consortia of several population-based cohorts were formed to

increase sample size and thereby power to detect small effects in the common variants. The first GWAs in 2.5 million common variants across the genome was done in 2009 [6] and showed evidence for the association between Human Hedgehog Interacting Protein (*HHIP*) and airflow obstruction as defined by a combination of a decreased FEV_1/FVC and FEV_1. After this initial GWAs, a working group was founded within the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium or CHARGE. This consortium is founded by the five following population-based cohort studies: Age, Gene/Environment, Susceptibility—Reykjavik (AGES), Atherosclerosis Risk in Communities Study (ARIC), Cardiovascular Health Study (CHS), Framingham Heart Study (FHS), and Rotterdam Study (RS). The large scale meta-analysis in 20,890 individuals resulted in the association of seven previously unidentified loci for FEV_1 and FEV_1/FVC and the confirmation of *HHIP* as gene associated with lung function [8]. Parallel to these analyses, the Europe based consortium SpiroMeta carried out a similar analysis in 20,288 individuals, resulting in the identification of five new loci. The results from these analyses have been cross-checked in both cohorts. The identified loci together only explained a small proportion of the heritability, leading to the hypothesis that many more common variants remained undetected. After these separate analyses, the consortia decided to combine the samples in order to be able to meta-analyze all cohorts and thereby increase power.

This collaboration included a combined sample size of meta-analysis and follow-up of 94,612 subjects and has been very productive in identifying new loci. The results from this collaboration can be found in chapter 3.1. We brought the total number of identified variants associated with FEV_1 and/or FEV_1/FVC up to 26. Table 1 below provides a brief overview of the loci for spirometry, discovered by the most recent GWAs. In general, GWAs in spirometry seem to identify loci which influence lung development, e.g. *HHIP*, *PTCH1*, and *RARB* which are involved in branching morphogenesis of the airway during embryological development [26-28]. Other interesting genes that were discovered in these analyses were *MMP-15* and *ADAM19* which are involved in airway remodeling and repair, and therefore could probably be associated with respiratory diseases. Genes from the same family of proteases, either matrix metalloproteases (MMPs) or disintegrins and metalloproteases (ADAMs), were associated with asthma and lung function; e.g. *MMP-12* and *ADAM33* [29-31].

As shown in the table, the first GWAs in spirometry did not include Forced Vital Capacity (FVC). Therefore, we proposed and performed an analysis plan to focus on the genetic determinants of FVC. The description of this effort can be found in chapter 3.2. FVC is an approximation of total lung capacity and can be measured by spirometry by assessing a person's maximal in- and expiration. A reduced ratio of FEV_1 to FVC (FEV_1/FVC) indicates airflow obstruction when FEV_1 is reduced disproportionately relative to FVC. In contrast,

Table 1. Genes implicated to be associated with spirometry

Trait / Article	FEV_1	FVC	FEV_1/FVC	Proportion variance explained (%)
Wilk e.a.			*HHIP*	**n.a.**
Hancock e.a. 2010[8]	*INTS12-GSTCD-NPNT*		*HHIP*, *GPR126*, *ADAM19*, *AGER-PPT2*, *FAM13A*, *PTCH1*, *PID1*, *HTR4*	FEV_1: 0.07-0.14% FEV_1/FVC: 0.09 %
Repapi e.a. 2010 [7]	*GSTCD*, *TNS1*, *HTR4*		*AGER*, *THSD4*	
Soler Artigas e.a. 2011[35] (**chapter 3.1**)	*MECOM*, *ZKSCAN3/ ZNF323*, *CDC123*, *c10orf11*, *CCDC38*		*MFAP2*, *TGFB2*, *HDAC4*, *RARB*, *SPATA9*, *NCR3*, *ARMC2*, *CDC123*, *LRP1*, *MMP15*, *CFDP1*, *KCNE2*	FEV_1: 1.5%, FEV_1/ FVC: 3.2%
Loth e.a. (**chapter 3.2)**		6 newly identified loci, currently unpublished		FVC: 1%

FEV_1 = Forced Expiratory Volume in 1 second, FVC = Forced Vital Capacity.

a decreased FVC in combination with an elevated FEV_1/FVC, suggests a restrictive ventilatory defect. In clinical practice, FVC is often used as a surrogate measure of disease progression in patients with established restrictive lung disorders, such as idiopathic pulmonary fibrosis[32,33]. Reduced FVC is a strong predictor of mortality in the general population, independently of the forced expiratory volume in 1 second (FEV_1) and standard risk factors such as age and cigarette smoking[22-25,34]. In conjunction with FEV_1, FVC is used to diagnose various respiratory diseases. Comparable to the previous CHARGE-SpiroMeta collaborations, we meta-analyzed data from 26 cohorts in a first stage, after which we followed up the sentinel SNPs showing most evidence (n = 7) for association with FVC in 7 independent cohorts encompassing a total sample size of n = 77,093. Lastly we tested expression in lung tissue, expression quantitative trait locus-analysis in whole blood and replicated the top genetic associations in African-American subjects.

In the last 4 years, genome-wide research has taken major leaps forward in identifying genetic variations. Altogether, however, these loci explain only 1.5% for FEV_1, approximately 1% for FVC and 3.2% for FEV_1/FVC. When putative undiscovered variants with a similar distribution are taken into account, the proportion variance explained may reach 3.4% for FEV_1 and 7.5% for FEV_1/FVC[35,36]. This technique[36] to include currently undiscovered loci into account assumes a heritability of lung function of 40% [37,38]. The proportion variance explained is low but comparable to other quantitative traits [36,39]. However, this is a major drawback of the results from many GWAs. The great power available to detect associations often leaves many questions regarding the true causal relationship and determinants of heritability unanswered. On the other hand, although these loci may

not exert massive effects, the identification contributes to the understanding of the pathogenesis of lung diseases and lung development. The transcripts of genes usually do not act on a single target, but are part of bigger pathway networks. Associating loci to spirometry can help disentangling these networks and identifying pathways. Eventually, this can help in the development of new therapeutic agents.

To evaluate somewhat more complex associations between genetics and spirometry, we followed two approaches. In chapter 3.3, we explored the association between smoking and genetic variants, a so called gene-by-environment interaction (G x E). Since smoking is one of the strongest risk factors for pulmonary diseases, this is usually taken into account as a co-variable in the regression models. However, some patients seem to be more susceptible to nicotine dependency and the harmful effects of smoking than others[40,41], implying interaction between genetic variants and the environment. Despite the direct exposure to cigarette smoke, interaction between smoke exposure and certain genetic traits on spirometry values had previously only been investigated through candidate gene analyses but without consistent results. In order to fully investigate the (multiplicative) interaction between smoking and genes on spirometry, we investigated FEV_1 and FEV_1/FVC and the relation of smoking status. Secondly, we investigated the dose response by analyzing the interaction of genes with pack-years smoked (pack-year = packs smoked per day × years as a smoker) over time and spirometry measures. This collaboration included spirometry from 50,047 subjects and we investigated the interaction using a meta-analysis of SNP_{main} and $SNP_{interaction}$ effects ('two degrees of freedom' test of the null hypothesis $\beta_{SNP} = 0$ and $\beta_{INT} = 0$) [42]. This method has been compared to regular G x E analysis and main effect analyses and had a greater power to detect effects than the two methods separately [43]. We showed associations for FEV_1 & FEV1/FVC by ever-smoking (*DNER*, *HLA-DQB1*/HLA-DQB2) and for FEV_1/FVC by pack-year (*KCNJ2*/*SOX9*). The Major Histocompatibility Complex, where *HLA-DQB1*and HLA-DQB2 are localized, is a highly polymorphic area with complex linkage disequilibrium, making it currently too difficult to further investigate the associated genes and their modes of action. For *DNER* (associated with FEV_1/FVC) and KJNC2/*SOX9* (associated with FEV_1) expression was detected in lung tissue. The *DNER* protein product is a ligand of the Notch signaling pathway which is a critical controller of cellular differentiation in multiple organs including the lung [44,45]. This region is enriched for long-range regulatory elements for *SOX9*, although the possibility of this region containing *KCNJ2* regulatory elements cannot be discounted [46]. *KCNJ2* is a member of the inwardly-rectifying potassium channel family, which regulates membrane potential and cell excitability and is expressed in many tissues including myocardium, neurons, and vasculature. This potassium channel also affects human bronchial smooth muscle tone and may play a role in airflow limitation [47]. *SOX9* is a transcription factor that is essential for cartilage formation, *SOX9*–/– and

SOX9+/– mice have multiple skeletal anomalies and severe tracheal cartilage malformations and die prematurely from respiratory insufficiency [48,49]. Although we were looking for interaction effects, the p-values for interaction were not significant, implying that the loci are associated with SNP_{main}-effects. One main problem with G x E-interaction analysis is sample size;most studies (including ours) are grossly underpowered to detect multiplicative interaction[50]. With the approach we used, the model is fully saturated for smoking as a covariable, thereby improving power to detect SNP_{main} effects. However, power to detect true and straightforward interaction effects was still insufficient. Apparently, interaction GWAs cannot fully replace the need for additional candidate gene studies.

A second interaction analysis that we carried out was the interaction between genetics and time-varying lung function (chapter 3.4). The curve of FEV_1 over time, first shown by Fletcher and Peto[4], shows a decrease of lung function over the years. However, this graph is a representation of the mean decrease over time of the population and does not account for the individual decrease of pulmonary function over time. While the decrease is dramatically accelerated by smoking, it is hypothesized that also genetics play an important role [51,52]. Family- and twin studies of the longitudinal change in lung function report heritability estimates between 10 and 39%[53,54]. Within the CHARGE and SpiroMeta consortia 14 cohorts with at least two spirometry measurements carried out a linear mixed effects GWA-analysis. Of these 14 cohorts, 5 cohorts had more than two measurements over the course of time. A separate analysis was carried out, in order to achieve greater precision of estimating the trajectory. The results of this study were looked up in a population based cohort (AGES-Reykjavik) and a COPD cohort (Lung Health Study). In the total consortium, across approximately 27,000 persons with at least 2 spirometry assessments, this only yielded suggestive evidence for a single genetic association. The subset analyses across 5 cohorts with at least 3 measurements yielded one genome wide hit. Since these types of analyses are extremely time-consuming it is vital to identify the technical difficulties associated with these analyses. Beside lack of power, which is usually a problem in GWAs, heterogeneity across cohorts might cause the absence of strong signals. An important observation in the current study is the phenotypic variation among the cohort studies in the meta-analyses, in aspects such as baseline age and cigarette smoking, and in factors that are of special importance to this longitudinal meta-analysis, such as the number of FEV_1 measurements per participant and follow-up duration, and changes in the lung function apparatus over time within individual cohorts. Phenotypic heterogeneity represents a general challenge in genetic epidemiology, particularly in the investigation of longitudinal phenotypes.

While we have explained the importance of quantitative phenotypes above, eventually we would like to identify causes for pulmonary diseases and to be able to predict COPD. In the final chapter (chapter 3.5) of the genetic analyses, we describe our approach to COPD as a disease state rather than the independent quantitative traits underlying the diagnosis. According to the previous and very widely used GOLD guidelines, COPD is diagnosed by spirometry (FEV_1/FVC < 70%). The staging is according to the predicted percentage which a person can exhale in the first second (FEV_1 percent predicted). As we addressed in chapter 2.1, these fixed cut-offs may not be applicable to elderly participants. In our analysis we assessed COPD according to the prediction equations proposed by Hankinson [15]. With a case-control analysis across 3,368 affected and 29,507 unaffected participants from fifteen population based cohort studies one region was associated with airflow obstruction which included *CHRNA5*/*CHRNA3* which encodes for nicotinic acetylcholine receptor subunit alpha-3 and 5. These genes have been previously associated with both COPD [55] and nicotine dependency [41,54], but the close correlation between smoking and COPD makes it difficult to disentangle any causal relation. In our cross-sectional analysis, we show evidence for an independent association between*CHRNA5*/*CHRNA3* and COPD. Results from gene expression studies demonstrated that both *CHRNA5* and *CHRNA3* were expressed in whole lung, airway smooth muscle, and bronchial epithelial cells [56]. The correlation between associated SNP genotypes and *CHRNA5* expression levels in the lung and sputum, combined with our finding of increased risk of airflow obstruction in never smokers suggests that the variants in this region may be associated with a risk of airflow obstruction that is not simply mediated by an influence on nicotine dependency. Supporting a direct influence of variants in this region on lung phenotypes, a *CHRNA3/5* variant was recently found to be associated with bronchial hyperresponsiveness in children not exposed to cigarette smoke [57]. On the other hand, colleagues have analyzed the direct link with *CHRNA3/5* and lung function decline and found no evidence for an independent effect but through smoking habits [58]. Nevertheless this is a very interesting region which, through smoking habits or directly, influences lung function and the risk to develop COPD. It would be very interesting to further investigate this region.

Spirometry, COPD and therapeutic agents

In the fourth part of this thesis, we explored the association between spirometry or COPD, and two drug groups which are commonly used in people with pulmonary diseases.

In chapter 4.1, we demonstrated an association between β-blockers and spirometry. In this population-based study, exposure to cardioselective β-blockers was associated with a lower FEV_1 of -118 ml, and FVC of -167 ml, respectively. For non-cardioselective β-blockers we found an association with a marginally lower FEV_1/FVC of -1.38%, a mea-

sure to assess airflow obstruction. For cardioselective β-blockers, although the sample was much larger, no such association was found. The difference of effects (adjusted for several co-variables) between cardioselective and non-cardioselective β-blockers and the lack of a strong association with FEV_1/FVC implies that the non-cardiac effects are in fact limited, but not completely absent. Our objective was to investigate whether this association was modified by common variants in the *ADRB2*-gene, encoding for the Beta-2-adrenergic receptor which is the target of cardioselective β-blockers, to identify markers for a higher susceptibility to this unwanted effect. Unfortunately, our dataset was not powerful enough to detect such effect or there was no effect modification between the genetic variants.

While we show a deleterious effect on pulmonary function, there is ample evidence that β-blockers are beneficial in the treatment of cardiovascular disease (CVD) in COPD patients. Since heart failure and COPD exacerbations are associated with an increased risk of mortality [59,60], treating patients with a multidrug regimen might provide disease-specific benefits and reduce mortality [61]. Our findings are probably outweighed by the beneficial effects of the treatment on CVD but might have clinical consequences in those with end-stage COPD.

Lastly, we investigated another very frequently used class of prescription drug in CVD, i.e. statins and their effect on survival in patients with COPD. The airflow obstruction is most notable in COPD patients, but there is evidence that COPD is also a systemic inflammatory disease and that statins might decrease the risk of mortality in patients with COPD. In addition to their lipid-lowering effect, statins also possess pleiotropic anti-inflammatory and immunomodulating properties, and are able to reduce levels of inflammatory markers such as CRP [62]. This is the first prospective study in a general population showing that the beneficial effect of long-term statin use on the risk of mortality in COPD patients is modified by the baseline level of systemic inflammation. Although the protective effect of statins in COPD patients might represent solely an indirect effect on the cardiovascular co-morbidities associated with COPD, our results also suggest a favorable effect on respiratory mortality by statin use compared to never use. One in three COPD patients in our study died from cardiovascular causes – figures which have also been described by other authors [61,63].

We describe both positive and negative effects from treatments mainly used in CVD with respect to the lungs. This further emphasizes the close correlation between the heart and lungs. While one drug class (β-blockers) may have apparent negative effects on spirometry, in COPD patients it is still beneficial. Although statins have not been marketed for the indication COPD or advertised as being beneficial to COPD patients, our results

show effects on mortality independent of cardiovascular mortality. That means that we might in the future prescribe statins to COPD patients irrespective of their cardiovascular risk profile or cholesterol levels. However, before we reach that conclusion, large scale placebo controlled trials would be needed, with the specific purpose of evaluating the inflammatory risk profile, to further clarify the effects of statins in COPD.

METHODOLOGICAL CONSIDERATIONS

Per chapter, most relevant methodological issues have been addressed in the discussions. In these paragraphs we will further highlight some general methods and problems we encountered.

Study design and setting.

All studies in this thesis were embedded in the Rotterdam Studies I, II & III. The Rotterdam Study is a prospective population-based cohort study which started in 1990 in Ommoord, a suburb of Rotterdam. The objectives and design of the Rotterdam Study have been described earlier [62,64]. In brief, at baseline, all participants were visited for a standardized questionnaire, and were subsequently examined at the research centre. The first cohort (RS-I) consists of 7,983 participants, aged 55 years and over. Since the start of the initial cohort, two subsequent cohorts have been defined in the same area. The second cohort (RS-II) started in 2000 and included 3,011 participants aged 55 years and over. The third cohort (RS-III) was enrolled in 2006 and included 3,932 participants aged 45 years and over.

Every 3-5 years, participants undergo a round of interviews and physical examinations, of which pulmonary function tests were routinely performed during every study round as of 2002. To continuously monitor the cohort for major diseases and mortality, the database is linked to the electronic records of the general practitioner and the municipality. All prescriptions dispensed to the participants are periodically collected by linking to the seven pharmacies covering the Ommoord region.

Spirometry was performed by trained paramedical personnel using a SpiroPro®portable spirometer (Erich Jaeger, Hoechberg, Germany) and as of 2009 with the CareFusion MasterScreen ™ PFT, according to American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines [10]. FEV_1, FVC and FEV_1/FVC-ratio were measured. Spirometry procedures that yielded results that did not meet ATS/ERS criteria for acceptability and reproducibility were classified as 'not interpretable'[65].

During the investigations at the research center, all measurements are limited to strict timeslots in order to keep the process efficient and make it possible for the elderly to within without demanding too much effort. This time restriction does, in some cases, prohibits us from repeating the spirometry test until sufficient quality tests are obtained. Most of the times we can acquire up to 5 tests. The failure rate, being less that 10%, is very low. Participants were asked to refrain from smoking and/or using any prescribed pulmonary medication before the center visit. For practical reasons, reversibility testing could not be carried out which might be considered as a methodological limitation. Although asthma prevalence figures in the elderly are not very well investigated, estimates of asthma occurrence range from 4 to 8 % [63,66]. In order to be able to take asthma into account in our analyses, asthma cases were ascertained by 1) Doctor's diagnosis of Asthma and 2) no conflicting respiratory diagnosis. Additionally, prescriptions for respiratory medicines were used for case finding.

The great advantages of the Rotterdam Study, as our study base, are the prospectively (unbiased) data collection, long-term follow-up and detailed information on a large variety of phenotypes. This allows for association testing with a relatively low chance of confounding by indication or protopathic bias in an observational study. Furthermore, we were able to adjust for many co-morbidities and co-variables. The follow-up allows for survival analyses like in chapter 4.2.

Genome-wide association studies

In the third part of this thesis, we showed several straightforward cross-sectional GWAs and two interaction analyses. The quantitative cross-sectional analyses proved to be most successful in identifying associations, a success mainly due to their large sample sizes. The drawback of these consortia is that they consist of many cohorts, increasing the heterogeneity across the studied phenotypes and genotypes. Hereby, we will only be able to identify very consistent effects of SNPs in all cohorts. Due to geographical differences between cohorts, there might be specific variants which we were not able to detect. Thereby, we may have missed large parts of unidentified genetic variants with tiny effects. The clinical relevance of these undiscovered loci may be limited but the evidence provides pieces of the puzzle, a puzzle of which we do not know size and character yet.

Gene–environment interactions have been proposed to hold a key to a proportion of unexplained heritability. The sample sizes needed for such studies are a major hurdle, illustrated by our G x E analysis in chapter 3.3. The most direct interaction expected to be demonstrable in pulmonary medicine would be the one of smoking by genetic susceptibility. However, even with a very powerful statistical technique we were not able

to identify clear-cut interaction effects. This poses a problem when studying gene-drug interactions in which we would like to identify patients who have a good response as well as those who are more susceptible to the harmful effects of medication. Since we were not able to identify an interaction by one of the strongest and most widely used exposures (smoking), it is a great challenge to identify gene-drug interactions. This is partially due to power issues, but also the difference in exposure windows create extra complexity. For smoking, the effects occur after chronic exposure for years, in drug-gene interaction one would expect acute effects within the exposure window. Finally, most of the genes are part of complex biological networks which attributes to Gene-Gene and Gene-Gene-Environment interactions. Although very interesting, these are currently difficult to analyze because of a lack of sufficient sample size and computational resources.

FUTURE DIRECTIONS

To identify more of the genetic heritability and pharmacogenetic effects, new tools are needed.

Beside the new arrays such as the exome chip, we are standing at the brink of extensive sequencing of DNA in the individuals of the Rotterdam Study and similar population-based cohort studies. The analyses to be performed will most likely focus firstly on the fine mapping of GWA results and identification of rare variants with small or moderate effects. Besides that, we should widen our scope and look into the candidate gene approach for testing hypotheses on gene-environment (drugs) interactions. By sequencing and analyzing the exomes, the functional parts of the genes, we may increase power to detect interactions with prescribed medication or assess why some patients experience more adverse effects than others. Further down the pathway, more research is needed with respect to the modification proteins, coded by the genes, undergo (proteomics) and the metabolites of the cellular processes (metabolomics). These might provide more insight into interindividual variation.

CONCLUDING REMARK

The identification of genetic determinants underlying the heritability of spirometry is far from complete. However, it has been more powerful than the genetic case-control analyses of COPD. The discoveries in this thesis do provide information on the pathogenesis of chronic obstructive pulmonary diseases. New approaches may be more successful in identifying gene-drug interactions than the current tools we have and pave the path

to personalized medicine in respiratory diseases. In the meantime, we should not overlook candidate gene analyses and given part IV, non-genetic pharmacoepidemiology remains an important tool.

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SUMMARY

Spirometry is a standardized and reliable measure to assess pulmonary ventilatory function and evaluate lung volume. Spirometry is mainly used to diagnose obstructive respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD), and to assess treatment response. COPD is a common respiratory disease which is primarily caused by inhalation of noxious particles and gases from e.g. cigarette smoke, air pollution or indoor fossil fuel combustion. COPD is characterized by a not fully reversible airflow limitation in combination with several symptoms, such as chronic cough with or without excess sputum production. The disease is usually progressive. In addition to the severity of airflow limitation, the number of co-morbid conditions, and the frequency and severity of exacerbations determine the overall survival in COPD patients. While cigarette exposure is the strongest risk factor for COPD, susceptibility seems heritable and some genetic determinants are known to contribute to the development of COPD. A homozygous mutation in *SERPINA1* leading to Alfa-1 Antitrypsin-deficiency is one of the most well-known genetic determinants for emphysema. Affected smokers develop COPD much earlier in life, before 40 years of age, than affected non-smokers and unaffected smokers. Other genetic determinants such as hedgehog interacting protein (*HHIP*) and Transforming Growth Factor Beta-1 (*TGFB1*) have been associated with COPD, but cannot fully explain the heritability.

The main objectives of this thesis were, 1) to explore the epidemiology of (ab)normal spirometric measurements in elderly, 2) to identify genetic determinants for lung function, smoking susceptibility, lung function decline and COPD, 3) to assess the effects of drugs on lung function or disease progression and to elucidate the genetic- and environmental modifiers of drug response. Four of the studies in this thesis were conducted within the Rotterdam Study, a large population-based cohort study. In five of the studies, the Rotterdam Study collaborated with up to 33 similar ohorts from Europe, the United States and Australia.

In Part II, two epidemiological topics regarding spirometry were studied. In chapter 2.1 we calculated reference values for three spirometry measures; Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) and the ratio of FEV_1 over FVC. These findings showed that healthy elderly may have a physiologically decreased lung function, even if they do not suffer from obstructive lung disease. Possible misclassification of COPD in the elderly may overestimate the incidence and prevalence of COPD in population-based studies. In chapter 2.2 we studied the relationship between pulmonary and cardiovascular function, showing evidence for a strong correlation between spirometric measurements and pulmonary artery systolic pressure. We described a physiological

relationship which need to be further explored to identify if, and if so, which clinical implications this may have.

In Part III we described the results of three "standard" genome-wide association analyses across two large consortia (CHARGE and SpiroMeta) for: 1) FEV_1 and FEV_1/FVC, 2) FVC and 3) COPD. Furthermore, we showed the results from a gene-environment interaction of cigarette smoke exposure on FEV_1 and FEV_1/FVC. Lastly in this series, we evaluated the interaction between genes and time on FEV_1. For FEV_1 and FEV_1/FVC, we undertook a large scale meta-analysis with several look-up strategies in order to identify new loci associated with both traits (chapter 3.1). We were able to identify 16 new loci for FEV_1 and FEV_1/FVC bringing the total amount of loci known for these traits up to 26. The loci implicated in pulmonary function may lead to a better understanding of the embryologic development of the lungs, their damage repair system and related immunological processes. In chapter 3.2, we identified nine loci associated with FVC and although FVC and FEV_1 are highly correlated, we identified loci to be independently associated with FVC. Furthermore, we confirmed two loci to be associated with both FEV_1 and FVC.

Chapters 3.3 and 3.4, report on two interaction analyses. In chapter 3.3, we explored the interaction between genetics and the environment. One of the most direct exposures influencing lung function is (cigarette) smoking. As mentioned previously, susceptibility to the damaging effects differs. We undertook analyses with smoking status (ever/never) and cumulative exposure described in pack years. Remarkably, our statistical approach proved to be more powerful to identify SNP effects than effects of interaction with smoking. Two reasons may underlie this observation. Firstly, the models are fully saturated for the exposure, thereby reducing confounding to a minimum. Secondly, for pure interaction effects, we were probably underpowered although the sample size exceeded 50,000 participants. For interaction with time (chapter 3.4), we were able to find suggestive evidence for loci associated with an accelerated lung function decline. However, we were not able to replicate our findings in a separate independent cohort. Possible heterogeneity across cohorts (e.g. in participants, lung function apparatus and timing/interval between lung function measurements) and power issues were probably limiting factors in these analyses.

Lastly, we identified a potential independent effect of the nicotinic acetylcholine receptors (*CHRNA3/5*) on the risk of airflow obstruction. These results were independent of smoking and can be found in chapter 3.5.

In part IV we assessed the effects of pharmacological treatment for cardiovascular diseases on pulmonary function and COPD. Use of β-blockers was associated with

a lower FEV_1 and FVC (chapter 4.1). This effect was stronger in non-cardioselective β-blockers than in selective β-blockers, which is consistent with the different receptors these classes target and the less harmful risk profile selective β-blockers tend to have. Althoughβ-blockers may decrease pulmonary function, recent studies have shown that β-blockers are beneficial in patients with COPD and cardiovascular disease, mainly due to a reduction of the high cardiovascular mortality in COPD patients. Lastly, we showed that statins reduce mortality in COPD patients. This reduction in all-cause mortality is modified by the baseline level systemic inflammation, as evidenced by serum levels of high-sensitivity C-reactive protein and may be due to anti-inflammatory and immuno-modulatory properties of this drug class.

Part V gives a general interpretation of our results, places the findings into perspective and highlights some of the methodological issues regarding our studies. The successes of the genome-wide association studies, but also the limitation in sample size are highlighted. Furthermore, some general considerations and future perspectives are given to further explore (pharmaco)genetic susceptibility to diseases and treatment.

SAMENVATTING

Spirometrie is een gestandaardiseerde en betrouwbare methode om de functie van de longen te meten en het volume van de longen te schatten. Spirometrie wordt met name gebruikt voor de diagnose van obstructieve longziekten zoals astma en chronic obstructive pulmonary disease (COPD) en het evalueren van de therapie effecten. COPD is een veel voorkomende longziekte, welke met name wordt veroorzaakt door de inhalatie van schadelijke deeltjes en gassen in bijvoorbeeld sigarettenrook, luchtvervuiling of het binnenshuis verbranden van fossiele brandstoffen. COPD wordt gekarakteriseerd door een niet volledig omkeerbare luchtwegobstructie in combinatie met verscheidene symptomen zoals chronische hoest en overmatige slijmproductie. De ziekte is meestal progressief. Naast de ernst van de luchtwegobstructie, wordt de mortaliteit beïnvloed door het aantal comorbiditeiten en de frequentie en ernst van de COPD exacerbaties. Hoewel sigarettenrook de sterkste risicofactor voor COPD is, lijkt er tevens een erfelijke component te bestaan en van sommige genetische determinanten is bekend dat ze het risico op het ontwikkelen van COPD verhogen. Een homozygote mutatie in het *SERPINA1* gen, resulterend in een Alfa-1 Antitrypsine-deficiëntie is 1 van de reeds lang bekende genetische determinanten voor het ontwikkelen van emfyseem. Rokers die aangedaan zijn door deze mutatie ontwikkelen COPD veel vroeger, vaak reeds voor het 40ste levensjaar, dan niet-rokers met deze mutatie of rokers zonder de mutatie. Andere genetische determinanten, zoals Hedgehog Interacting Protein (*HHIP*) en Transforming Growth Factor Beta-1 (*TGFB1*) zijn geassocieerd met COPD, maar kunnen de erfelijkheid niet volledig voorspellen.

De doelstellingen van dit proefschrift waren, 1) het bestuderen van de epidemiologie van normale en afwijkende metingen bij spirometrie in ouderen, 2) het identificeren van genetische determinanten voor longfunctie, gevoeligheid voor roken, longfunctievermindering en COPD, 3) het analyseren van effecten van geneesmiddelen op de longfunctie of het ziekteverloop en het identificeren van genetische en omgevingsfactoren die de mogelijke geneesmiddeleneffecten beïnvloeden. Vier van de onderzoeken in dit proefschrift zijn uitgevoerd binnen de Rotterdam Studie, een grote cohort studie in de algemene populatie. In vijf onderzoeken zijn we de samenwerking aangegaan met 33 vergelijkbare studies uit Europa, de Verenigde Staten en Australië.

In Deel II hebben we twee epidemiologische onderwerpen bestudeerd. In hoofdstuk 2.1 hebben we referentiewaarden berekend voor drie spirometriematen: Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) en de ratio van deze twee maten (FEV_1/FVC). Deze bevindingen tonen aan dat gezonde ouderen een fysiologisch verminderde longfunctie kunnen hebben ondanks het feit dat ze niet lijden aan een obstructieve longziekte. Mogelijk worden hierdoor de prevalentie en incidentie van COPD

in studies bij ouderen overschat. In hoofdstuk 2.2 onderzochten we de relatie tussen spirometrie en de cardiale functie. We tonen een sterke correlatie aan tussen maten van longfunctie en de druk in de pulmonaalarterie. We beschrijven een fysiologische relatie, maar verder onderzoek zal moeten uitwijzen wat de klinische consequenties van deze relatie zullen zijn.

In Deel III, beschrijven we drie associatie studies over het gehele genoom in twee grote consortia (CHARGE en SpiroMeta) voor: FEV_1, FEV_1/FVC, 2) FVC en 3) COPD. Verder laten we resultaten zien van een gen-omgeving interactiestudie tussen blootstelling aan sigarettenrook en FEV_1 en FEV_1/FVC. Tenslotte hebben we interactie tussen genen en tijd geëvalueerd ten aanzien van het verloop in longfunctie over de jaren. Voor FEV_1 en FEV_1/FVC hebben we een grote meta-analyse verricht en verschillende strategieën gebruikt om nieuwe genetische varianten te voor deze spirometriematen te identificeren en te bevestigen (hoofdstuk 3.1). We hebben met dit onderzoek 16 varianten kunnen identificeren voor FEV_1 en FEV_1/FVC, waarmee we het totaal aantal bekende genetische loci naar een totaal van 26 hebben gebracht. De loci die geassocieerd zijn met longfunctie leiden mogelijk tot een beter begrip over de longontwikkeling gedurende de embryonale fase, de herstelmechanismen van de long en de immunologische processen. In hoofstuk 3.2, identificeerden we negen loci, die geassocieerd zijn met FVC. Hoewel FVC en FEV_1 zeer gecorreleerd zijn, waren we in staat om loci te identificeren die onafhankelijk met FVC geassocieerd zijn. Tevens hebben we opnieuw twee loci aangetoond die we eerder met FEV_1 en FVC geassocieerd hadden.

Hoofdstukken 3.3 en 3.4, beschrijven 2 interactie analyses. In hoofdstuk 3.3, hebben we de interactie onderzocht tussen genen en omgevingsfactoren. Een van de meest directe en sterke factoren, die de longfunctie beïnvloed, is sigarettenrook. Zoals eerder genoemd, is de gevoeligheid voor de schadelijke effecten verschillend per persoon. We hebben analyses uitgevoerd met rookstatus ('ooit'/'nooit') en met de totale cumulatieve blootstelling, uitgedrukt in zogeheten 'pak-jaren'. Opmerkelijk genoeg, bleek onze statistische benadering gevoeliger voor het ontdekken van effecten van genetische varianten dan voor het ontdekken van interactieeffecten. Twee mogelijke redenen kunnen hieraan ten grondslag liggen. Ten eerste, waren de statistische modellen volledig verzadigd met correctiefactoren voor roken, teneinde de verstorende factoren van roken tot een minimum te beperken. Ten tweede, om pure interactie effecten te identificeren, hadden we een té kleine onderzoekspopulatie ondanks het feit dat het aantal onderzochte deelnemers groter was dan 50.000. Voor interactie met tijd (hoofdstuk 3.4), waren we in staat om ondersteunend bewijs te leveren voor loci die geassocieerd zijn met een versnelde achteruitgang van de longfunctie. Helaas waren we echter niet in staat om deze bevindingen in een onafhankelijke populatie te bevestigen. Mogelijke

heterogeniteit tussen de cohorten (bijvoorbeeld in deelnemers, longfunctiemetingen en tijd/interval tussen de longfunctiemetingen) en te weinig statistische 'power' waren mogelijk beperkende factoren.

Voorts vonden we aanwijzingen voor een mogelijk onafhankelijk effect op obstructieve uitademing geassocieerd met de genen die coderen voor nicotine acetylcholine receptoren (*CHRNA3/5*). Deze resultaten waren onafhankelijk van roken en staan beschreven in hoofdstuk 3.5.

In deel IV hebben we de effecten van farmacotherapie voor cardiovasculaire aandoeningen op longfunctie en COPD onderzocht. Het gebruik van β-blokkers was geassocieerd met een lagere FEV_1 en FVC (hoofdstuk 4.1). Dit effect was sterker in gebruikers van niet-cardioselectieve β-blokkers dan in gebruikers van selectieve β-blokkers, hetgeen consistent is met het verschil in farmacologische aangrijpingspunten van deze twee klassen en het gunstiger risicoprofiel dat selectieve β-blokkers hebben. Hoewel β-blokkers de longfunctie lijkten te verminderen, hebben recente onderzoeken aangetoond dat ze wel degelijk van therapeutische betekenis zijn bij patiënten met COPD en cardiovasculaire ziekten, dankzij verlaging van de cardiovasculaire mortaliteit.

Tenslotte hebben we laten zien dat statines de sterfte bij patiënten met COPD verlagen (hoofdstuk 4.2). Deze reductie in totale mortaliteit (alle sterfteoorzaken) lijkt te worden beïnvloed door de aanwezigheid van systemische inflammatie, aangetoond door verhoogde bloedwaarden van high-sensitivity C-reactive protein. Dit is mogelijk het gevolg van de anti-inflammatoire en immuunmodulerende eigenschappen van deze geneesmiddelenklasse.

Deel V geeft een algemene interpretatie van de resultaten, plaatst de bevindingen in perspectief en licht enige methodologische problemen toe van onze studies. De successen van de analyses over het gehele genoom, maar ook de beperkingen in de grootte van de studiepopulatie worden hierbij besproken. Vervolgens geven we enige algemene overwegingen en bespreken mogelijkheden voor toekomstig onderzoek met betrekking tot de genetische risicofactoren voor het ontstaan van COPD en voor de behandeling bij ouderen van deze toenemend frequente aandoening met ernstige consequenties.

DANKWOORD

Het traditionele dankwoord van een proefschrift is waarschijnlijk het deel wat het meest gelezen wordt, ondanks het feit dat het niet peer-reviewed is.

Allereerst mijn promotores, prof. dr. B.H.Ch. Stricker, prof. dr. G.G. Brusselle en prof. dr. H.G.M. Leufkens. Beste Bruno, bedankt voor de kans die je me gegeven hebt. Je wetenschappelijke sturing was essentieel. Daarnaast gaf je me de vrijheid en verantwoordelijkheid om mijn eigen weg te vinden. Je anekdotes en maatschappelijke overpeinzingen, doorspekt met droge humor, waren welkome intermezzo's in de werkdagen. Dit gold voor zowel in het Erasmus als bij de Inspectie voor de Gezondheidszorg. Meermaals bij inspecties heb ik moeite gehad mijn gezicht in de plooi te houden en ik denk ook niet dat dat geheel gelukt is. Tenslotte, denk ik dat je je promovendi veel meer leert dan 'alleen' wetenschap bedrijven, waarvoor dank.

Beste Guy, bij de genetische studies zorgde je ervoor dat de klinische perspectieven en de integratie van de bevindingen in de kliniek van de longziekten niet uit het oog verloren werden. Je ongeremde nieuwsgierigheid en onuitputtelijke kennis van de literatuur hebben zeer boeiende brainstormsessies opgeleverd met Bruno. Daarnaast nam je me vaak op sleeptouw op internationale congressen zoals American Thoracic Society en European Respiratory Society. Als je zalen met vele honderden professionals naar jou ziet luisteren, besef je pas wat voor mazzel je als jonge onderzoeker hebt om bij jou te mogen promoveren.

Beste Bert, hoewel onze contacten minder intensief waren dan bij de andere promotores wil ik je graag bedanken voor je input en wetenschappelijke visie, met name gedurende het laatste jaar van mijn promotietraject.

Prof.dr. C.M. van Duijn, prof.dr. H.M. Boezen en prof.dr. H.C. Hoogsteden wil ik graag bedanken voor het plaatsnemen in de kleine commissie en het beoordelen van het proefschrift. Beste Cornelia, dank voor je input in de genetica van de Rotterdam Studie. Beste Marike, hoewel Groningen niet heel dichtbij is, kwamen we elkaar voornamelijk veel verder van huis tegen op ATS of ERS, bedankt voor de succesvolle samenwerking. Beste prof. Hoogsteden, op uw afdeling heb ik de variëteit van de longgeneeskunde ontdekt en de kans gekregen in Zuid-Afrika een deel van mijn co-schap te mogen doen, waarvoor dank.

A large part of this thesis is a result of the collaborative effort of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. Dear Stephanie,

your guidance has made the pulmonary group into a very successful and productive working group. I would like to thank you for all your help throughout the last four years but especially with the last paper. Although it is almost impossible to address everybody in person, I would like to acknowledge a couple of colleagues with whom I have had the pleasure of working closely: Dana, Jemma, George, Graham, Josée, Pat, Sina and Wenbo.

Several papers would not have been possible without our colleagues from the Spiro-Meta cohort. I would like to thank Martin Tobin, María Soler Artigas, Ian Hall and Louise Wain for all their effort, input and hard work.

Prof. Hofman, beste Bert, een regeltje in het dankwoord staat in het niet tot het werk wat u verricht hebt voor de Rotterdam Studie en een dankwoord van een promovendus van de Epidemiologie is niet compleet zonder uw naam. Eveneens dank voor de mogelijkheid een kijkje achter de schermen te kunnen nemen bij de management-team besprekingen. De Rotterdam Studie zou in het geheel niet mogelijk zijn geweest zonder de deelnemers aan de studie. Het verzamelen van de onderzoeksgegevens, het 'fuppen' van de statussen van meer dan 14.000 ERGO-deelnemers en het managen van dit geheel is een monsterklus. Ik wil graag alle deelnemers, onderzoeksmedewerkers, fuppers, computerondersteuning, databeheerders en het secretariaat van de Epidemiologie bedanken voor hun bijdrage.

Gedurende een groot deel van mijn promotieonderzoek heb ik parttime gewerkt bij de Inspectie voor de Gezondheidszorg, graag zou ik al mijn collega's van Programma 8 willen bedanken. In het bijzonder mijn directe collega's van farmacovigilantie en good clinical practice (GCP). Janny, je was de gids in het woud van procedures en formulieren, zonder jou zou ik waarschijnlijk nooit een succesvolle declaratie ingediend hebben. Dank voor je gezelligheid. Tijdens mijn inspectiejaren heb ik twee vaste partners in crime gehad met eenzelfde dubbele aanstelling, Eline en Rikje. De etentjes, borrels en melige buien waren een zeer welkome afwisseling van het doorworstelen van SOP's. Diederike, mijn (bijna) vaste flexplek collega, heel veel succes met je nieuwe baan. Maris, Toke, Robert-Jan, Jeanet, Jits, Judith en Willem, bedankt voor de leuke samenwerking.

Gedurende de afgelopen vier jaar heb ik het geluk gehad met veel verschillende collega's samen te werken. Graag zou ik iedereen de credits geven die ze verdienen, maar dan zou ik een tweede boekje moeten drukken. Ik ben in ieder geval alle (ex-)collega's van de Epidemiologie veel dank verschuldigd. Verder wil ik graag een aantal mensen in het bijzonder bedanken. Mark, dank voor al je hulp gedurende de beginjaren van mijn onderzoek en de gezelligheid in al de jaren. Janine, Arfan en Abbas, veel dank voor alle (genetische) hulp door de jaren heen. Mijn reisgenoten voor de CHARGE vergaderingen

Bouwe en Symen. Bouwe, ik vond het een eer je paranimf te zijn en verwacht niet anders dan dat je een goede dokter wordt. Succes met je co-schappen. Symen, geniet van je onderzoekstijd en niet met mate, voor je het weet is het voorbij (Let's Go Rockets!). Toke, bedankt voor de samenwerking en gezelligheid in Den Haag en Rotterdam. De teksten die je er soms uitflapt zijn onnavolgbaar vermakelijk. Virginie, koningin van de flauwe woordgrap, onze gesprekken waren altijd leuk, sporadisch nuttig. Geniet van je tijd in Singapore. En tenslotte, mijn enige long-epi collega Lies, je nauwgezetheid en je organisatorische kwaliteiten heb ik zeer gewaardeerd en de vrijdagbesprekingen met Bruno en Guy werden door jou altijd in goede banen geleid. Inmiddels is "the blue journal" je huisblaadje geworden en kan een prachtig proefschrift niet uitblijven.

Ook wil ik mijn nieuwe collega's uit het Amphia bedanken. Bedankt voor de steun, het begrip en warme ontvangst. En natuurlijk het fantastische tripje naar Boedapest.

Graag wil ik mijn (schoon)familie en vrienden bedanken voor alle steun en interesse. Bedankt voor jullie aanwezigheid vandaag.

Maarten, mijn paranimf, een mooi voorbeeld hoe je als collegae bevriend kunt raken en zowel zakelijk als privé door één deur kunt (hoewel jij vaak moet bukken dan). Bij tijden kritisch, maar altijd vol humor. Bedankt dat je mij wil bijstaan.

Lieve Julie, hoewel ik je niet echt mijn kleine zusje kan noemen, blijf je dat toch wel. Dank voor alles, niet alleen de laatste vier jaar. Ik vind het fijn dat ik vandaag op mijn kleine zusje kan bouwen.

Papa en mama, dankzij jullie onvoorwaardelijke steun heb ik nooit getwijfeld over de keuzes in mijn leven. Ik kan niet in drie regels aangeven hoe belangrijk jullie voor me zijn. Jullie hebben me gemaakt tot wat ik ben en daar ben ik jullie oneindig dankbaar voor.

Lieve Désirée, jou ben ik het meest dankbaar. Gewoon, omdat je er bent. Omdat je er altijd voor me bent. Omdat ik ontzettend gelukkig met je ben, samen in ons luchtkasteel.

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Chapter 2.1

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Epub 2013 Mar 29.

Chapter 2.2

Pulmonary Function is associated with Pulmonary Artery Systolic Pressure in the general population: The Rotterdam Study

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Manuscript in preparation

Chapter 3.1

Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function

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Nature Genetics, 2011 Sep 25;43(11):1082-90. doi: 10.1038/ng.941.

Chapter 3.2

Genetics of forced vital capacity: genome-wide association study meta-analysis and follow-up identifies six new loci

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Manuscript submitted

Chapter 3.3

Genome-Wide Joint Meta-Analysis of SNP and SNP-by- Smoking Interaction Identifies Novel Loci for Pulmonary Function

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Chapter 3.4

Genome-Wide Association Studies of Longitudinal Change in Adult Lung Function

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Manuscript submitted

Chapter 3.5

Genome-Wide Association Studies Identify *CHRNA5*/3 and *HTR4* in the Development of Airflow Obstruction

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Published in American Journal of Respiratory and Critical Care Medicine, 2012 Oct 1;186(7):622-32. doi: 10.1164/rccm.201202-0366OC. Epub 2012 Jul 26.

Chapter 4.1

Beta-blockers and pulmonary function in the general population: The Rotterdam Study

Daan W Loth, Guy G Brusselle, Lies Lahousse, Albert Hofman, Hubert G M Leufkens, Bruno H Stricker

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Chapter 4.2

Statins, systemic inflammation and risk of death in COPD: The Rotterdam Study

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Manuscripts in preparation

Common genes underlying asthma and chronic obstructive pulmonary disease? A genome-wide association study on the Dutch hypothesis

J. Smolonska, G.H. Koppelman, C. Wijmenga, J.M. Vonk, P. Zanen, M. Bruinenberg, I. Curjuric, M. Imboden, G.A. Thun, L. Franke, N.M. Probst-Hensch, P. Nürnberg, R. Riemersma, O. van Schayck, D.W. Loth, G.G. Brusselle, B Stricker, A Hofman, A Uitterlinden, L Lahousse, S London, L Ross Loehr, A Manichaikul, GR Barr, P Pare, Y Bosse, K Hao, M van den Berge, H.M. Groen, JW Lammers, W. Mali, H.M. Boezen, D.S. Postma

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Susceptibility to chronic mucus hypersecretion, a genome wide association study

AE Dijkstra, J Smolonska, M van den Berge, C Wijmenga, P Zanen, MA Luinge, M Platteel, JWJ Lammers, M Dahlback, K Tosh, P Hiemstra, P Sterk, A Spira, J Vestbo, M Benn, BG Noordestgaard, M Dahl, WM Verschuren, HS Picavet, HA Smit, M Owsijewitsch, HU Kauczor, U von Seydlitz-Kurzbach, HJ de Koning, F Mejza, P Nastalek, E Nizankowska-Mogilnicka, CC van Diemen, JM Vonk, MH Cho, EK Silverman, JD Crapo, TH Beaty, DA Lomas, P Bakke, A Gulsvik, Y Bosse, L Franke, D.W. Loth, L Lahousse, F Rivadeneira, AG Uitterlinden, A Hofman, BH Stricker, GG Brusselle, CM van Duijn, U Brouwer, GH Koppelman, MC Nawijn, HJM Groen, W Timens, HM Boezen, DS Postma

Submitted

Replication of five common variants for nonalcoholic fatty liver disease in the elderly and interaction with insulin resistance

Edith M. Koehler, Jeoffrey N.L. Schouten, Daan Loth, Bettina E. Hansen, Albert Hofman, Andre Uitterlinden, Bruno H. Stricker, Harry L.A. Janssen

Manuscript in preparation

Gene variants in the interferon gamma receptor 2 gene are independently associated with liver stiffness in the general population: Results from the Rotterdam Study

Elisabeth P.C. Plompen, Jeoffrey N.L. Schouten, Daan W. Loth, Bettina E. Hansen, Albert Hofman, André G. Uitterlinden, Bruno H.Ch. Stricker, Frank W.G. Leebeek, Harry L.A. Janssen

Manuscript in preparation

Clopidogrel use is associated with an increased prevalence of cerebral microbleeds in a stroke-free population: the Rotterdam Study

Sirwan K.L. Darweesh, Maarten J.G. Leening, Saloua Akoudad, Daan W. Loth, Albert Hofman, M. Arfan Ikram, Meike W. Vernooij, Bruno H. Stricker

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