Genetic variations in scavenger and β_2-adrenergic receptors and risk of pulmonary disease

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THE THREE ORIGINAL ARTICLES ARE:

1. Thomsen M, Nordestgaard B, Tybjærg-Hansen A, Dahl M. Scavenger Receptor AI/II Truncation,Lung Function and COPD in 48,700 Individuals. J Intern Med 2011; 269:340-8.
2. Thomsen M, Nordestgaard BG, Kobzik L, Dahl M. Genetic Variation in Scavenger Receptor MARCO, COPD, and lung infection in 10,604 individuals. Respiration 2013; 85:144-53.
3. Thomsen M, Nordestgaard BG, Sethi AA, Tybjærg-Hansen A, Dahl M. β_2-Adrenergic receptor polymorphisms, asthma and COPD: two large population-based studies. Eur Respir J 2012; 39:558- 5.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that usually is progressive and associated with an abnormal inflammatory response in the airways and the lung to noxious particles or gases [1]. COPD is one of the leading causes of death worldwide and projections predict that morbidity and mortality of COPD will continue to increase in the years to come [2,3], representing a major public health problem. The main causative risk factor is smoking. However, a substantial proportion of smokers do not develop COPD [4] suggesting that other interacting factors must be important. Familial clustering indicates that genetic susceptibility may be important for the development of the disease [5]. However, apart from the relatively rare α_1-antitrypsin mutations which account for 1-2% of cases, the additional genetic factors leading to increased risk of COPD remain poorly understood.

In recent years advances in genotyping technologies have facilitated the advent of genome-wide association studies that surveys the entire genome to identify new loci without prior hypotheses. Implementation of these studies has markedly increased the number of genetic variants associated with pulmonary disease. However, the loci identified collectively account for only a small fraction of the observed hereditability in lung function and COPD and initial promising associations have failed consistent replication [6]. One explanation could be that the common polymorphisms discovered through genome-wide association studies alter gene function in relatively subtle ways, but in combination with other common variants have an additive or multiplicative effect on risk of disease [7]. Another explanation could be the existence of many rare variants with profound functional consequences on gene expression that remain to be discovered. For this purpose large-scale candidate gene studies seem to remain a valuable approach. These genes are selected due to their involvement in pathways that may affect the pathophysiology of COPD and rely on an underlying biological plausible hypothesis. Genes encoding scavenger receptors involved in pulmonary pathogen clearance and the β_2-adrenergic receptor, an important regulator of airway smooth muscle tone, are plausible candidate genes for COPD.

Scavenger receptors SRA-I/II and MARCO

In the lungs scavenger receptors on the surface of alveolar macrophages are essential for recognition and removal of harmful airborne microorganisms and inhaled oxidants [8]. Scavenger receptor A-I/II (SRA-I/II) and macrophage receptor with collagenous structure (MARCO) are two dominant scavenger receptors on alveolar macrophages and thus important for normal non-specific host defence in the lungs [9-12]. These receptors have the same overall domain structure, but differ in that MARCO has a longer extracellular domain and lacks an α-helical coiled coil domain [13,14]. SRA-I and SRA-II are two similar receptors generated through alternative splicing of the *macrophage scavenger receptor-1* gene (*MSR1*) [15]. Reduced function of SRA-I/II and MARCO due to genetic variations could lead to impaired pathogen clearance that may be important in the pathophysiology of COPD. In support, alveolar macrophages from COPD patients are deficient in phagocytosis of common respiratory bacterial pathogens and apoptotic cells compared to alveolar macrophages from health nonsmokers [16]. A rare variant in *MSR1* that could impair pathogen clearance is Arg293X (rs41341748). This variant truncates the distal collagen-like domain of the receptor that is essential for ligand recognition [17,18] and has previously been associated with increased risk of prostate cancer [19]. However, Arg293X and genetic variants in the less well characterized *MARCO* gene have not previously been examined in relation to lung function and risk of COPD in humans.

The β_2-adrenergic receptor

The β_2-adrenergic receptor (ADRB2) is a G protein-coupled transmembrane receptor located on airway smooth muscle cells [20]. Receptor activation causes smooth muscular relaxation in response to endogenous catecholamines, and this is important for

the regulation of airway smooth muscle tone. Pharmacological targeting of this receptor is a widely used therapeutic approach for controlling bronchoconstriction associated with asthma and COPD [20]. There are three known polymorphisms in the coding region of the *ADRB2* gene that alter the function of the receptor [21,22]. The rare variant, Thr164Ile, reduces the receptor ligand binding affinity [21], whereas the two common polymorphisms, Gly16Arg and Gln27Glu, determine the extent of receptor down regulation following agonist exposure [22]. Several studies have investigated these polymorphisms and related haplotypes to assess their potential contribution to risk of asthma and COPD. The majority of studies have examined their relationship with risk of asthma, and have found positive associations with airway reactivity [23], nocturnal asthma [24], and asthma severity [25]. However, a large population-based study and meta-analyses have shown conflicting results [26-29]. For COPD a single study found an elevated risk of disease for Arg16 homozygotes and for carriers of the Arg16/Gln27 haplotype [30], but these results have not been replicated by others [31,32]. Also, studies using computed tomography to characterize structural changes in the lungs according to Arg16Gly and Gln27Glu genotype have not been conclusive [33,34]. The rare Thr164Ile has profound consequences on receptor function, but has not previously been examined in relation to COPD and asthma, presumably because large sample sizes are needed.

OBJECTIVES

The three papers included in this thesis had the following objectives:

- In paper 1, we tested the hypothesis that the A293X variant in *MSR1* is associated with reduced lung function and risk of COPD in the general population. To test this hypothesis, we genotyped 48,741 individuals from the adult Danish general population for Arg293X and recorded spirometry and hospital admissions for COPD. We then compared lung function and risk of COPD in Arg293X homozygotes and heterozygotes versus non-carriers. In addition, we also tested for potential interaction with other known environmental and genetic determinants of low lung function such as MZ genotype (rs28929474) in the α_1-*antitrypsin* gene [35], E1I1 (rs8192287/rs8192288) in the *superoxide dismutase-3* gene [36], and F508del (rs332) in the *cystic fibrosis transmembrane conductance regulator* gene [37].
- In paper 2, we tested the hypothesis that genetic variations in *MARCO* associate with reduced lung function, COPD, and lung infection. For this purpose we first screened 760 individuals with extreme lung phenotypes in the Copenhagen City Heart Study to identify variants that potentially could influence risk of pulmonary disease in the general population. We next genotyped the entire cohort consisting of more than 10,000 individuals for the nonsynonymous variants identified and tested whether these genotypes were associated with reduced lung function, increased risk of COPD and increased risk of lung infection. As a secondary aim we also tested for association with asthma.
- In paper 3, we tested the hypothesis that genetic variation in *ADRB2* is associated with reduced lung function, and increased risk of asthma or COPD. For this purpose we genotyped 8,971 individuals from the Copenhagen City Heart Study for the three most important functional polymorphisms in the *ADRB2* gene, Thr164Ile, Gly16Arg, and Gln27Glu. Because previous studies found that genetic effects of *ADRB2* polymorphisms may be influenced by tobacco smoke [23,38,39], we also performed the statistical analyses stratified for tobacco smoking. Findings for the rare Thr164Ile polymorphism were validated in the Copenhagen General Population Study.

METHODS

Study populations

The three papers in this thesis are based on two similar study cohorts sampled from the adult Danish population, The Copenhagen City Heart Study and the Copenhagen General Population Study. The Copenhagen City Heart Study was used in all of the three original articles included in this thesis. In paper 1, the Copenhagen City Heart Study and the Copenhagen General Population Study were analyzed as one collective cohort to obtain maximal statistical power in examining the association between the rare truncating variant in the gene encoding SR-AI/II and pulmonary disease. Pooling of data from the two studies may magnify existing association and/or introduce novel bias and errors. However, this approach seems reasonable because participants in the two studies share the same ethnicity, there is no overlap of individuals between the two studies, and data collection in the two studies was almost identical. In paper 2, a subsample of the Copenhagen City Heart Study was used to identify novel mutations in the *MARCO* gene using the extreme phenotype approach. The entire cohort was subsequently genotyped for the variants identified and used to examine the association with pulmonary disease. Finally, in paper 3 the Copenhagen General Population Study was used to replicate associations found in the Copenhagen City Heart Study between genetic variants in *ADRB2*, reduced lung function, and risk of COPD.

The Copenhagen City Heart Study

The Copenhagen City Heart Study is a prospective cohort study initiated in 1976. The cohort consisted of randomly selected age-stratified inhabitants living in Copenhagen. Individuals were selected on the basis of the national Danish Civil Registration System to reflect the population aged 20 to 100 years. The first examination took place in 1976-1978 and included 14,223 participants (response rate 74%). Follow-up examinations were conducted in 1981-1983, 1991-1994, and 2001-2003. At the second examination in 1981-1983, 500 individuals aged 20-24 years were supplemented to the original cohort and of those invited 12,698 participated (response rate 70%). At the third examination in 1991-1994, 3000 individuals aged 20-49 years were supplemented and of those invited 10,135 participated (response rate 61%). Finally, at the fourth examination in 2001-2003, 1040 individuals aged 20-34 years were supplemented and of those invited 6238 participated (response rate 50%). More than 99% of invited individuals were Whites of Danish descent. At all four examinations participants filled out a questionnaire reviewed by an examiner at the day of attendance, had a physical examination performed, and had blood samples drawn. At the third and fourth examinations, additional blood samples were drawn for DNA extraction.

The Copenhagen General Population Study

The Copenhagen General Population Study is a prospective cohort study initiated in 2003 and still recruiting. Inhabitants from counties in the greater Copenhagen area were randomly selected on the basis of the national Danish Civil Registration System to participate in the examination. Among individuals aged 20-39 years, 25% of the population were invited, while all individuals above 39 years of age were invited. All participants were Whites of Danish descent, defined according to the Danish Civil Registration System and requiring that the participant and both parents are Danish citizens born in Denmark. The participation rate was 45%. Data on each participant collected in this study are very similar to those in the Copenhagen City Heart Study. All participants had blood samples drawn for DNA extraction.

Selection bias

Selection bias is caused by systematic errors in selecting groups of participants to study within the cohort. This type of bias may affect the estimated effect sizes, the internal validity, and thus the legitimacy of the conclusions made. One potential source of selection bias may be non-response by invited participants. Individuals who do not respond to study invitations tend to have different socioeconomic status, lifestyle, and medical characteristics compared to those who respond, leading to possible overrepresentation of relatively healthy participants and underestimation of disease prevalence. Indeed, compared to responders cumulative incidences of all-cause mortality and COPD hospitalizations in the Copenhagen City Heart Study were increased among non-responders (Figure 1). A low response rate combined with differences in responders and non-responders may limit generalizability and the external validity of the study. However, it will only introduce a selection bias affecting the estimated effect sizes and the internal validity if non-response is associated to the exposure and to outcome. In paper 1, genotype distribution of the rare Arg293X polymorphism in *MSR1* was not in Hardy-Weinberg equilibrium and this could theoretically be due to selection bias against this genotype. However, this departure was mainly because the predicted number of homozygotes differed from the observed numbers (two and five, respectively) and the information provided by the Hardy-Weinberg equation may be limited for rare genetic variants due to the low number of homozygotes. If however selection bias did exist, we may have underestimated the effect of Arg293X homozygosity on lung disease. Genotype distributions in paper 2 and 3 were in Hardy-Weinberg equilibrium arguing against selection bias of these genotypes.

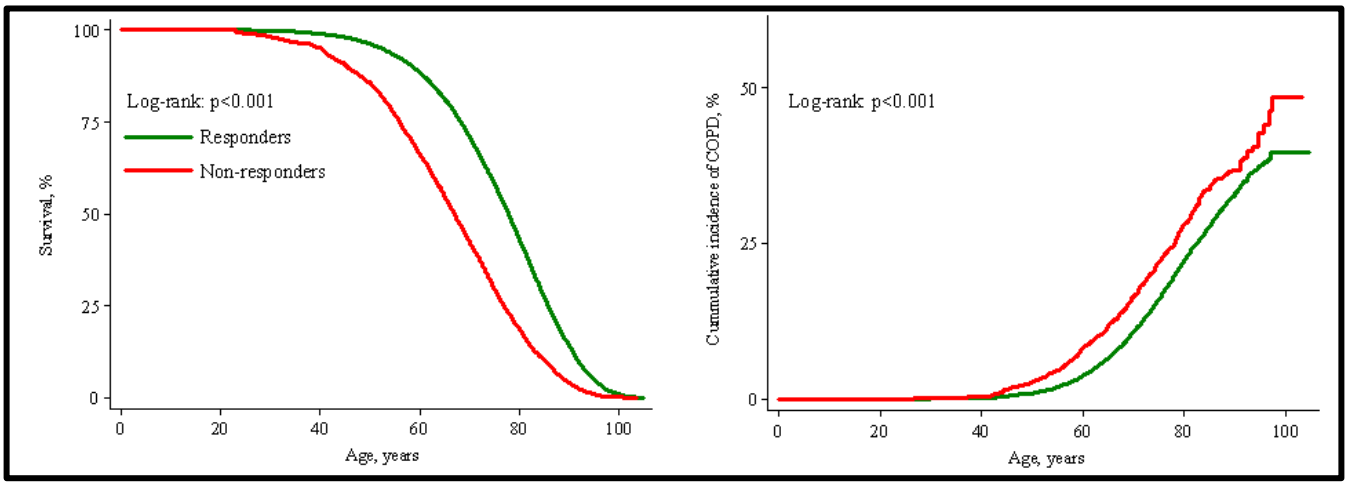
Measurements

Resequencing of *MARCO*

To increase the likelihood of detecting genetic variations in *MARCO* associated with lung disease in the general population, we performed resequencing of the *MARCO* gene using the extreme phenotype approach. The risk populations with extreme phenotypes in this cohort were defined as those with the earliest onset of COPD and asthma, individuals with interstitial lung disease, individuals with the highest FEV_1% predicted and the lowest FEV_1% among smokers and nonsmokers. These participants were sampled from the Copenhagen City Heart Study and included 760 individuals. When selecting participants with low FEV_1 % predicted, smoking status was taken into account (122 smoking and 118 non-smoking participants) whereas high FEV_1 % predicted were based on all participants (n = 140). As it is not clear exactly what phenotype people with functional genetic variants in *MARCO* may have, we cannot be sure that our selection criteria for our extreme risk population are optimal for detecting all genetic variants in *MARCO*. However, as our pre-specified hypothesis was to examine risk of pulmonary disease, sequencing confined to this extreme risk population seems reasonable. Another strategy could be sequencing of the entire cohort, but this would be costly and time-consuming.

Screening of the *MARCO* gene for genetic variations was performed with a 384-well LightScanner, a high resolution DNA melting curve analysis technique for variant detection with a high sensitivity and specificity. PCR is performed in the presence of a fluorescent double-strand DNA binding dye. During analysis, the temperature increases and the PCR amplicons denature. Denaturerization of the double stranded amplicon releases the dye and results in a marked drop in fluorescence and appearance of melting curves. Fourteen PCR fragments were amplified and screened for mutations, covering all 17 protein coding exons of *MARCO* and the intron-exon boundaries. PCR fragments with DNA melting curves differing from noncarrier control DNA were subsequently sequenced. We performed reruns on all undetermined samples. Using this method we identified all previously reported common genetic variations in the coding region of the gene in addition to several new mutations. However, the Lightscanner is a screening tool, and it is therefore possible that we have not detected all important variants in the *MARCO* gene. Although samples within a cluster would be expected to have the same genotype it is possible that different alleles may have been presented in one cluster causing us to overlook certain variants. It may be especially difficult to distinguish melting curves from noncarriers from

FIGURE 1:



Cumulative incidence of all-cause mortality and COPD hospitalization as a function of age among responders and non-responders in the Copenhagen City Heart Study.

minor allele homozygotes as they both form homoduplexes. One way of ensuring a better separation could be by adding noncarrier PCR-product to each sample after an initial read of melting curves allowing homozygous DNA to form heteroduplexes with DNA from noncarriers. However, we did not include this step, because our primary goal was to find novel mutations and as these mutations would be expected to be rare, it seems unlikely that we would find any homozygotes without identifying any heterozygotes for a specific genetic variant. Another limitation is that we did not screen the promoter region and may have missed important mutations in this site influencing gene expression and regulation.

Genotyping

Participants were genotyped for the genetic variants in *MSR1* and *MARCO* using TaqMan based assays. This technique was also use to genotype participants in the Copenhagen General Population Study for the Thr164Ile variant in *ADRB2*. The principle of TaqMan assays is based on two allele specific probes with a common quencher dye, but with different fluorescent reporter dyes. When the probe is intact, the quencher prevents fluorescence from the probe. During PCR, each probe anneals to complementary sequences between forward and reverse primer sites and the Taq-polymerase cleaves hybridizing probes. Cleavage separates the reporter dye from the quencher, which results in increased fluorescence. The intensity of this fluorescence signal is quantified on the ABI PRISM 7900HT Sequence Detection System allowing for determination of the genotype. In the Copenhagen City Heart Study genotyping of the three variants in *ADRB2* was performed using a Nanogen NMW 1000 Nanochip Molecular Biology Workstation. This technique uses a chip with DNA applied at different test sites. The DNA is then hybridized with allele-specific probes labelled with dye and fluorescence signals are subsequently scanned and quantified. This technique is no longer used in our laboratory due to the development of more time and cost-efficient methods like the TaqMan-based assays.

Misclassification of genotypes may arise especially if there are unknown genetic variants flanking the binding site of probes resulting in impaired binding. Other causes of genotyping errors could be poor DNA quality, biochemical artefacts, equipment failure, or human errors in sample or data handling. Nondifferential genotyping errors, that is, those that do not differ systematically according to outcome, would dilute risk estimates and bias associations towards the null-hypothesis and are therefore important to detect. To ensure correct genotyping, we sequenced a random selected subgroup of samples from each genotype or included controls diagnosed using another genotyping method. Also, comparison of the observed genotype to that predicted by the Hardy-Weinberg equilibrium served as an important check for misclassification. This equation uses the frequency of alleles to predict genotypes, but may be influenced by disturbances such as non-random mating, selection according to genotype, and gene flow. All genotype distributions except from Arg293X in *MSR1* were in Hardy-Weinberg equilibrium. However, control sequencing verified that Arg293X was genotyped correctly.

Spirometry

Forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) were determined without inhalation of a bronchodilator using a dry wedge spirometer (Vitalograph; Maids Moreton, Buckinghamshire, UK) in the Copenhagen City Heart Study and in the first 14,624 participants of the Copenhagen General Population Study. In the remaining participants of the Copenhagen General Population Study an EasyOne Spirometer (ndd Medizintechnik, Zurich, Switzerland) was used. Three sets of FEV_1 and FVC values were obtained and as a criterion for correct performance of the procedure at least two measurements differing by less than 5% had to be produced together with the correct visual appearance of the spirometry tracings. The highest obtained values for every single participant of both FEV_1 and of FVC were used.

As FEV1 and FVC are affected by gender, age, and height the actual measurements were adjusted for these factors prior to evaluation and compared with reference values derived from a healthy population. At the time of data analyses the existing Danish reference material consisted of a relatively small number of individuals and did not include individuals older than 70 years of age. We therefore chose to derive reference equations internally using linear regression with age and height as covariates for men and women separately for each spirometer. In paper 1 and 2, this was done on a subsample of never smokers whereas in paper 3, this was done on all individuals. Results were similar regardless of the sample used.

Questionnaire-based covariates

In both studies, participants filled out a questionnaire reviewed by an examiner at the day of attendance aiming to avoid any misinterpretations and to minimize questions with missing information. Information on smoking habits, packyears, occupational exposure to dust or fumes, and history of pulmonary infections were collected from this questionnaire and used to stratify or adjust statistical analyses. To ensure that all participants are included in the multivariable analyses, we used the missing-indicator method for categorical covariates and single imputation with age and sex as predictors for continuous covariates to account for missing information. The missing-indicator method assumes that data are missing completely at random, but may bias associations in studies with small sample sizes or a large number of missing values and in these cases single or multiple imputation procedures are preferable.

Endpoints

Spirometrically defined COPD

In paper 1 and 2, COPD was defined as FEV_1/FVC below 0.7 and FEV_1 below 80% of predicted corresponding to COPD in grades 2-4 defined in accordance with the Global Initiative for Chronic Obstructive Lung Disease (GOLD). Consequently, this definition excludes individuals with mild COPD, but increases the likelihood of identifying individuals having symptomatic airway limitation. We did not exclude individuals with asthma and this may have led to misclassification between these two similar and common diseases. However, if analyses were performed excluding individuals with self-reported asthma results were similar to those presented. In paper 3, self-reported asthmatics were excluded from analyses as this was an independent endpoint. Also, in this paper COPD was defined as FEV_1/FVC below 0.7 including individuals in all GOLD grades. Alternatives definitions of COPD that could have been used was FEV_1/FVC below the lower limit of normal (5^{th} percentile of a frequency distribution adjusted for age, sex and height) that limits the risk of overdiagnosis especially in elderly participants or to combine spirometric airflow limitation with the presence of a self-reported respiratory symptom to increase the likelihood that these individuals have clinical COPD.

Registry-based endpoints

Information about hospitalizations of COPD, pneumonia, and sepsis was obtained by linking the participants to the national Danish Patient Registry and the national Danish Causes of Death Registry, using each participant's unique Central Person Register number. Diagnoses were collected from the national Danish Patient Registry from 1977 to end of follow-up and defined according to World Health Organization International Classification of Diseases, 8th and 10th edition. COPD was ICD8: 491-492; ICD10: J41-J44, pneumonia was ICD8: 480-486; ICD10: J12-J18, and sepsis was ICD8:38; ICD10:A40-A41, A49.9.

Advantages of registry-based diagnoses are readily available data and high completeness. However, lack of correct registration may underestimate the number of actual events. As lung function is not routinely measured at hospital admittance some COPD patients with dyspnoea may receive a more unspecific diagnosis of respiratory failure at admittance or may falsely be misclassified as having another disease e.g. heart failure. Indeed, although the positive predictive value of COPD in Danish registries seems high there is a substantial underrecording of COPD among patients hospitalized with other primary diagnoses such as pneumonia. Although such misclassification would be independent of genotype it would cause us to underestimate the actual number of events and reduce the statistical power available for detecting a difference between genotypes. Diagnoses of pneumonia and sepsis used in paper 2 are more likely valid as these infectious endpoints often are supported by a more distinct clinical appearance of the patient, altered biochemical analyses, detection of bacterial pathogens, and/or diagnostic imaging.

Questionnaire-based endpoints

Information on respiratory infections and asthma used in paper 2 and 3 was self-reported. History of respiratory infections may be influenced by recall bias as this potential risk factor is ascertained retrospectively and since a person with impaired lung function may report past events differently compared to individual who have a normal lung function. Furthermore, the use of a participant's self-report of asthma will lead to some degree of misclassification, as some participants with COPD may regard their disease as asthma. However, one previous study from the Copenhagen City Heart Study showed that the prevalence of self-reported asthma did not increase with age and that the presence of asthma had a consistent negative effect on lung function decline regardless of smoking status. This suggests that the response to this simple question will indeed identify individuals with asthma and misclassification of asthma and COPD is unlikely to have biased our results to a major extent.

Statistical analyses

Statistical analyses were performed using Stata statistical software version 10.0-11.1 (StataCorp, College Station, TX, USA).

Comparing groups

For comparison of lung function across genotype groups we used two-sided unpaired Student's t-test. The assumptions for this test are that observations are independent, normally distributed with homogeneity of variances. The assumption of approximate normal distribution and homogeneity of variances can be assessed graphically. Also, formal statistical tests such as Shapiro-Wilk test for departure from normality and Levene's test for equality of variance can be used to test these assumptions. If violations are observed, data can be mathematically transformed or in the case of unequal variances the Welch test may be an alternative. Another alternative is to use non-parametric tests based on ranks. These tests are more robust and have fewer assumptions, but less statistical power than the parametric methods. In studies with large sample sizes like the ones included in this thesis, there are generally only minor differences in using parametric or non-parametric tests. In the presented analyses, FEV_1% predicted, FVC% predicted, and FEV_1/FVC were approximately normally distributed with equal standard deviations between groups. Thus, the assumptions for using Student t-test were met. For test of trend across genotype groups we used Cuzick's test for trend, which is an extension of the non-parametric Wilcoxon rank-sum test. For this test, genotype groups were coded 0, 1, and 2 assuming an ordinal relationship from noncarriers across heterozygotes to homozygotes; however, this assumption may not have been met perfectly for all genotypes.

Interaction

Interaction or effect modification is important to consider whenever there are two or more explanatory variables. Interaction is present if the effect of a change in the exposure variable on the outcome depends on the value of a second explanatory variable. One way of exploring the possibility of interaction is to stratify analyses for the other explanatory variable or compute interaction plots to see if an association between the exposure and outcome is equally strong in each strata. The effect/association may be greater or less than expected in the different strata indicating synergism or antagonism, respectively. Interaction can also formally be tested by including an interaction term in analysis of variance models (ANOVA) or in various regression models mentioned later. A statistically significant p-value obtained from introducing a multiplicative interaction term in the model indicates the presence of an interaction. Assumptions are the same as for the Student's t-test and models can be extended to include several covariates. Covariates tested for interaction with the exposure variable should be selected *a priori* and potential effect modification should be biologically plausible to avoid increase in type I errors due to multiple testing.

Repeated measures analysis

Because repeated measurements of FEV_1% predicted and FEV_1/FVC were available for subjects in the Copenhagen City Heart Study (30% of participants had four measurements, 39% three measurements, 21% two measurements, and 10% one measurement), lung function was also analyzed in a mixed-model repeated-measures ANOVA to illustrate the relationship between the Thr164Ile genotype and lung function over time in paper 3. This approach can be used when measurements are repeated under different experimental conditions e.g. several lung function measurements on the same participant at different ages. In a mixed-model ANOVA, one factor (a fixed effects factor) is a between-subjects variable (e.g. Thr164Ile genotype) and the other (a random effects factor) is a within-subjects variable (e.g. time). The assumptions of repeated measures ANOVA are an approximately normal distribution of the repeated measures (lung function) and homogeneity of the variances within-subjects. Furthermore, as the variability of measurements for within-subjects over time is correlated, it is necessary to specify a form for these general residuals. In our analyses, an unstructured covariance type for residuals was used. This means that no constraints are placed on the structure of the residuals and that each residual is estimated uniquely from the data. Consequently, this results in the best possible fit of the model, but reduces degrees of freedom.

Logistic regression

Logistic regression models were used to examine the association between genotype and binary endpoints. This approach models the logit of the outcome variable as a linear combination of included independent variables and compared to general linear models does not have assumptions of normality or homoscedasticity. However, the model assumes that continuous variables included display linearity on the logit scale; that is, that the change in the log odds per unit change in the continuous variable is constant. Test of nonlinearity can be done using Box-Tidwell transformation models. These models are implemented in the statistical software and provide a p-value for test of linearity and suggest transformations of the variable for the best model fit. Another option is to categorize the continuous variable. Categorical variables will always meet the assumption of linearity due to their ordinal nature.

In the papers included in this thesis, we applied logistic regression analyses to estimate odds ratios for our primary endpoint which was spirometrically defined COPD. This was ascertained at baseline leading to a cross-sectional study design. For consistency we chose to apply logistic regression to all other endpoints included in the papers. However, for the registry-based endpoints that are collected prospectively follow-up time for individuals included will vary, and although presumably independent of genotype, this may influence estimates obtained by logistic regression. Alternative analyses for prospectively collected endpoints could have been Cox proportional hazards regression models or competing risk proportional subhazard models by the method of Fine and Gray, taking competing risk of death into account. In our analyses, if a continuous variable did not meet the assumptions described above, it was categorized. If results were similar using the categorized variable, the crude continuous variable was included to simplify the model and to adjust best possibly for the covariates.

Confounding

Effects of potential confounders are important in interpreting any associations found. These factors are associated with both exposure and disease, but not part of the biological pathway linking the two together. Many potential confounders are usually collected for each participant at baseline allowing for stratification or adjustment in statistical analyses and thereby increasing the likelihood of isolating the true effect of exposure on risk of disease. However, there is always the risk that an apparent association is mediated through an unknown confounder and the undoubtedly best way to circumvent known and unknown confounding factors is randomisation. In genetic studies, where alleles are randomly assigned at gametogenesis, confounders will often be evenly distributed among genotypes. However, in the statistical analyses performed we have still chosen to adjust analyses for age, sex, and smoking because they are the most important confounders for risk of pulmonary disease and because most of the variants are rare, confounders may not necessarily be evenly distributed. Ethnicity may be another important confounder in genetic studies. However, in the Copenhagen General Population Study all participants were Whites of Danish descent, defined according to the Danish Civil Registration System and requiring that the participant and both parents are Danish citizens born in Denmark. Similarly, the Copenhagen City Heart Study consisted of more than 99% Whites of Danish descent making it unlikely that ethnicity have influenced our results.

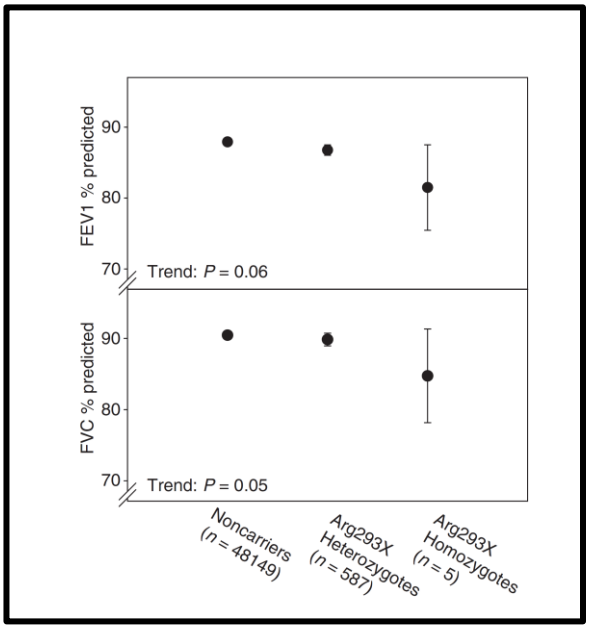
RESULTS

SRA-I/II truncation, lung function, and risk of COPD

The truncating variant Arg293X in *MSR1* was rare and had at most borderline statistical significant effect on lung function in all individuals combined. Arg293X homozygotes (n=5) and heterozygotes (n=587), compared with noncarriers (n=48,149) had 6% and 1% lower FVC% predicted (trend, p=0.05) (Figure 2). Corresponding reductions in FEV_1% predicted were 7% and 1%, respectively (trend, p=0.06). However, male Arg293X heterozygotes had 4% reduced FEV_1% predicted (trend, p=0.0004) and 4% reduced FVC% predicted (trend, p=0.0003) compared with noncarriers. In line with this, Arg293X heterozygosity was associated with spirometrically defined COPD among men with an odds ratio of 1.7 (95% confidence intervals, 1.1-2.4). In addition to the observed interaction with gender, the Arg293X also interacted with other genetic determinants of low lung function. Individuals with a combination of both Arg293X and MZ heterozygosity had 14% reduced FEV_1% predicted (trend, p=0.03) and 11% reduced FVC% predicted (trend, p=0.04) compared with MZ heterozygotes alone (Figure 3). Corresponding reductions for the combination of Arg293X and E1I1 heterozygosity compared to E1I1 heterozygosity alone were 9% and 8%, respectively (trend, p=0.04 and p=0.03). Finally, individuals with a combination of both Arg293X and F508del had 16% reduced FEV_1% predicted (trend, p=0.12) and 13% reduced FVC% predicted (trend, p=0.14) compared with F508del heterozygotes alone.

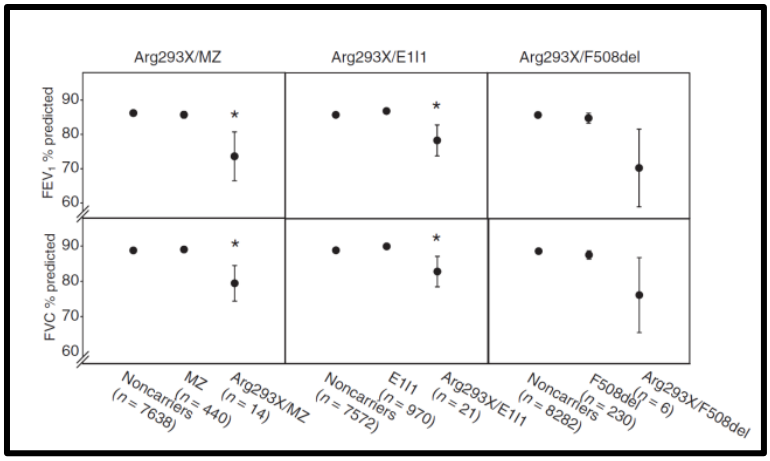
Our findings are supported by animal studies, in which mice with genetic depletion of *MSR1* was found to have increased susceptibility to bacterial pneumonia [11] and increased inflammatory responses to inhaled environmental particles [12]. Furthermore, a study using a murine model mimicking the typical features of COPD found impaired phagocytosis, progression of emphysema and absent expression of SRA-I/II on alveolar macrophages after infection with Haemophilus influenza compared to controls [16]. Previous human studies on polymorphisms in *MSR1* and pulmonary disease are scarce. One genomic region near *MSR1* was found to associate to low values for FEV_1 in a genome-wide association study [40] and another study found an association between the coding variant Pro275Ala and COPD in smokers [41]. Future studies are needed to confirm these results and to explore further the influence of genetic variation in *MSR1* on lung function among subgroups of individuals, in particular among individuals with variants in other COPD susceptibility genes.

FIGURE 2:



Lung function in Arg293X homozygotes and heterozygotes versus noncarriers in the Copenhagen City Heart Study and the Copenhagen General Population Study combined. Values represent mean ± 2 SE. P-values are by Cuzick's test for trend.

FIGURE 3:



*Lung function in Arg293X/MZ, Arg293X/E1I1 and Arg293X/F508del individuals versus MZ, E1I1 and F508del heterozygotes. Values represent mean ± 2 SE. *P<0.05 on Student's t-test comparing Arg293X/MZ and Arg293X/E1I1 individuals versus MZ and E1I1 heterozygotes, respectively. Data for MZ, E1I1, and F508del genotypes were only available for the subset of subjects from the Copenhagen City Heart Study.*

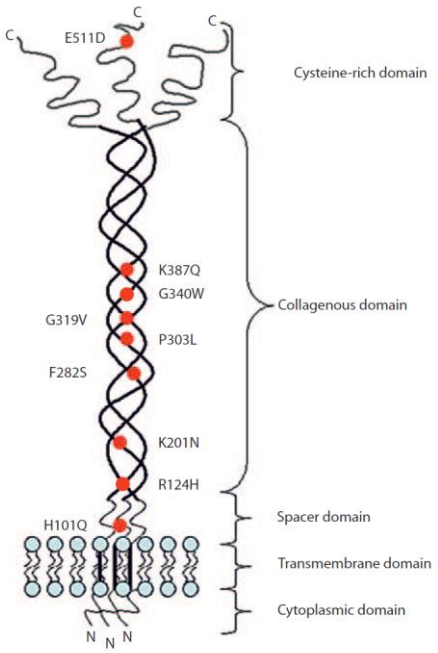
Resequencing of MARCO

Resequencing of the *MARCO* gene in 760 individuals with extreme lung phenotypes identified a total of 42 genetic variations. Of these, 16 were in protein coding regions and 9 were nonsynonymous variations. Figure 4 shows a schematic view of the location of the 9 nonsynonymous variants in relation to the previously described structure of *MARCO* [14]. The F282S, G319V, and G340W variants changes polarity of the amino acid substituted and could be of functional relevance to *MARCO*. Also, E511D located in the cystein rich domain required for ligand binding [14] could be of functional importance to the MARCO receptor. We genotyped 10,604 individuals from the Copenhagen City Heart Study for the 9 nonsynonymous variations identified. Minor allele frequencies were low ranging from 0.005% to 5%. The R124H, K201N, P303L, G340W, and K387Q mutations had minor allele frequencies below 0.01% and were not further analyzed due to insufficient statistical power.

Genetic variation in *MARCO*, lung function, risk of COPD and infections

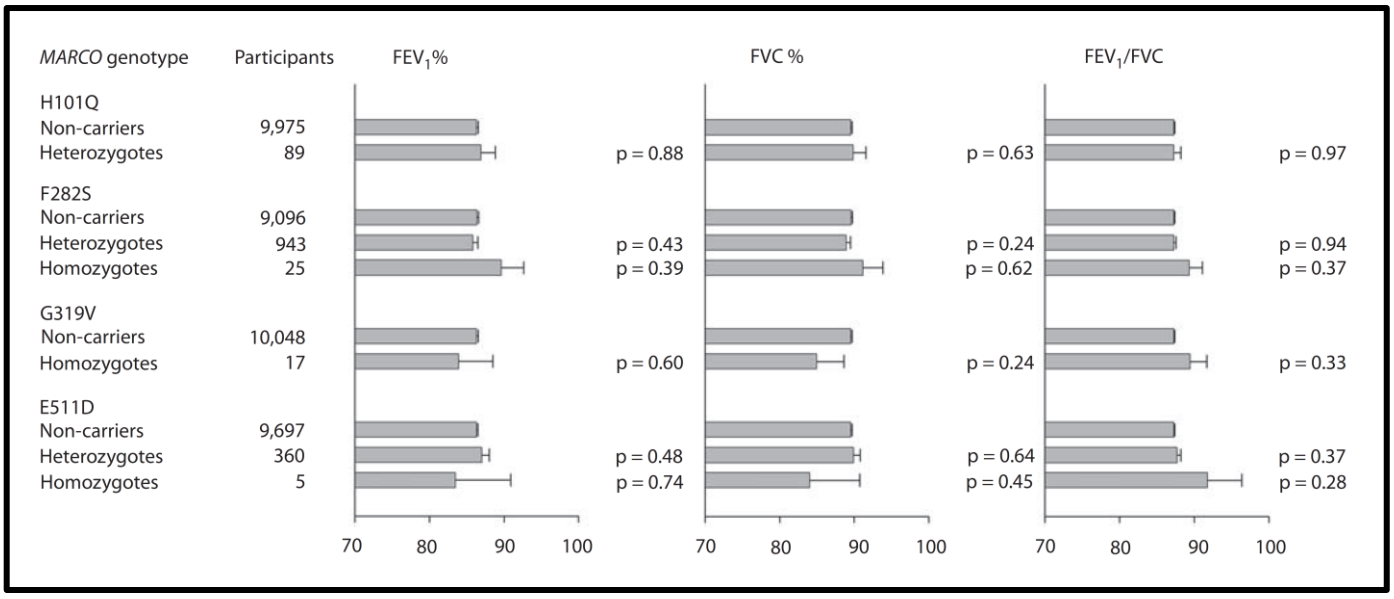
None of the genetic variants analyzed (H101Q, F282S, G319V, E511D) were associated with reduced lung function or risk of COPD (Figures 5 and 6). Because *MARCO* is upregulated in liver and spleen macrophages after infections [42,43] and is able to bind a high variety of ligands including lipopolysaccharides and lipoteichoic acid as well as intact bacteria [44], we also tested for associations with infectious endpoints. H101Q heterozygotes versus noncarriers had an increased odds ratio for sepsis hospitalization of 2.16 (95% confidence intervals, 1.05-4.41), while corresponding odds ratios for self-reported frequent pulmonary infection

FIGURE 4:



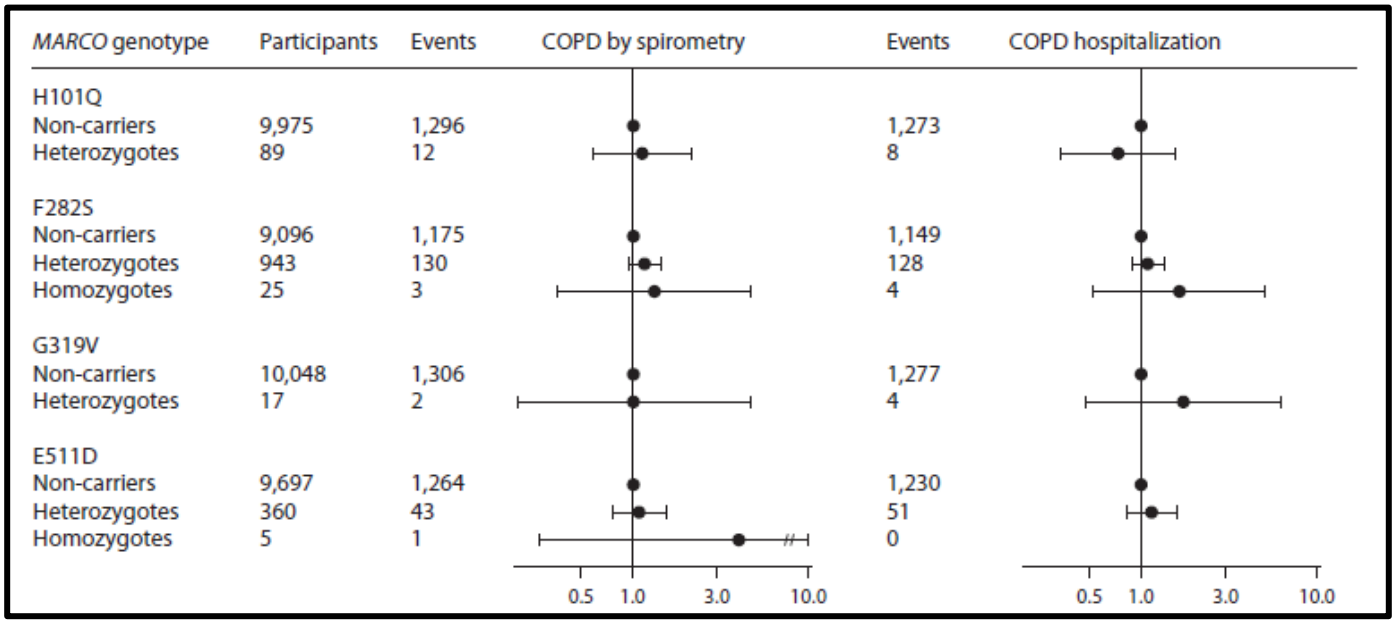
Structural model of macrophage receptor with collagenous structure (MARCO) and location of the 9 nonsynonymous variants identified.

FIGURE 5:



Lung function according to macrophage receptor with collagenous structure (MARCO) genotype. Values are mean and standard error. P-values are by Student's t-test for the comparison of heterozygotes and homozygotes with noncarriers.

FIGURE 6:



Risk of spirometry defined chronic obstructive pulmonary disease (COPD) and COPD hospitalization according to macrophage receptor with collagenous structure (MARCO) genotype. Spirometry-defined COPD was $FEV_1/FVC<0.7$ and $FEV_1<80\%$ predicted. Values represent odds ratios adjusted for age, sex, and packyears and 95% confidence intervals.

and pneumonia hospitalization were 1.04 (0.66-1.65) and 0.63 (0.33-1.21), respectively. None of the other three *MARCO* variants were associated with sepsis, self-reported frequent pulmonary infection, hospitalization for pneumonia, or a combination of these endpoints.

Current evidence indicating that reduced function of *MARCO* leads to impaired pathogen clearance and increased inflammation in the lungs comes from animal models [9,12,45]. These models use mice that are completely deficient in the expression of the gene, and this experimental setup does not necessarily mimic conditions found in humans. The genetic variants identified in this study might only be mildly deleterious or may not affect receptor function at all explaining our overall negative findings for lung disease. Another explanation could be that in spite of our large sample size we do not have statistical power enough to detect any potential association. We found H101Q heterozygotes to have an increased odds ratio for sepsis; however, the fact that this result was based on few numbers of individuals and the lack of association with the other infectious endpoints suggest that

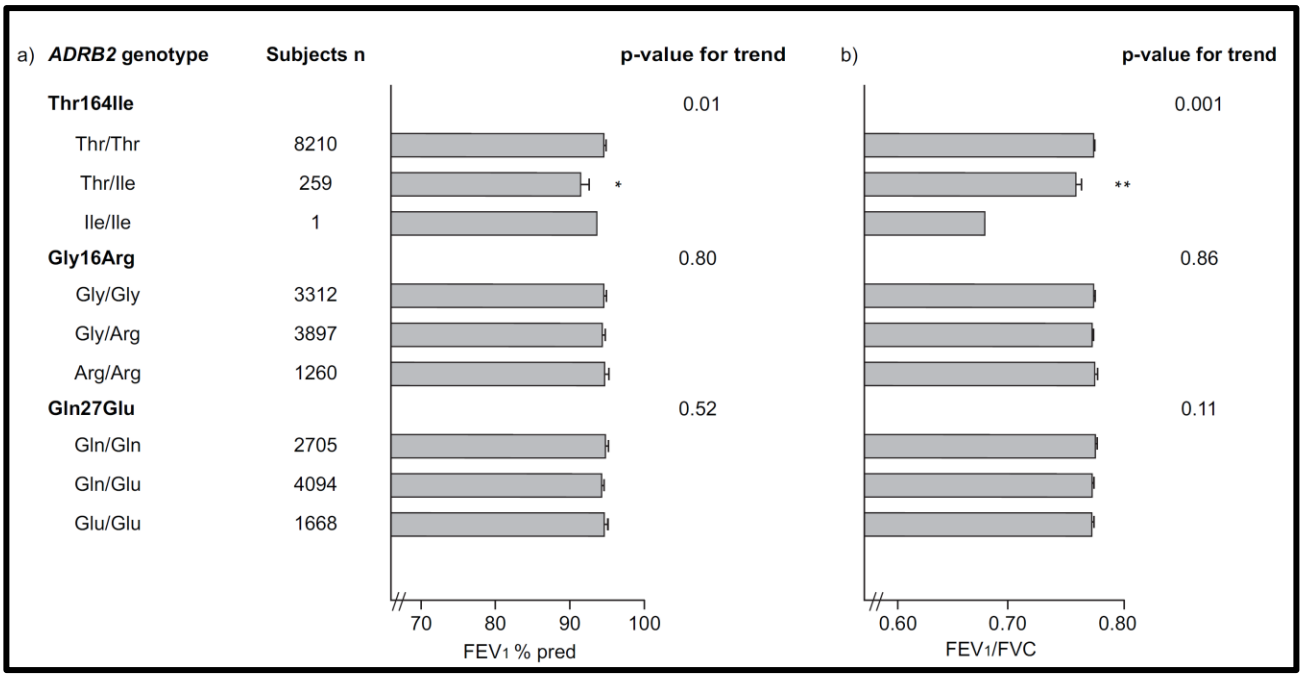
this result could represent a chance finding rather than a real phenomenon. Further research is therefore required to conclusively determine whether this mutation is associated with sepsis.

Genetic variation in *ADRB2*, lung function, risk of COPD and asthma

The Thr164Ile polymorphism was rare, as expected. We identified 60,910 Thr164Ile noncarriers, 1,822 heterozygotes and 16 homozygotes in the two studies combined. In the Copenhagen City Heart Study, Thr164Ile heterozygotes had 3% and 2% reduced FEV_1% predicted and FEV_1/FVC, respectively, compared with noncarriers (Figure 7). This relationship seemed independent of smoking and was thus possibly even present in never smokers (Figure 8). In accordance with the results on lung function, Thr164Ile heterozygotes also had an increased risk of spirometrically defined COPD with an age and sex adjusted odds ratio of 1.46 (95% confidence intervals,1.05-2.02) (Figure 9). There were no differences in lung function or risk of COPD with the more common Gly16Arg and Gln27Glu genotypes.We identified 60,910 Thr164Ile noncarriers, 1,822 heterozygotes and none of the three functional polymorphisms in *ADRB2* were associated with asthma. Aiming to replicate our findings for Thr164Ile, we genotyped participants from the Copenhagen General Population Study for this rare variant. In this cohort, Thr164Ile homozygotes and heterozygotes had 7% and 1% reduced FEV_1% predicted and 6% and 1% reduced FEV_1/FVC, respectively, compared with noncarriers (Figure 10). These relationships remained consistent when stratifying for smoking. In accordance with the results on lung function, Thr164Ile homozygotes and heterozygotes had odds ratios for COPD of 4.53 (1.54-13.28) and 1.07 (0.92-1.25), respectively, compared with noncarriers.

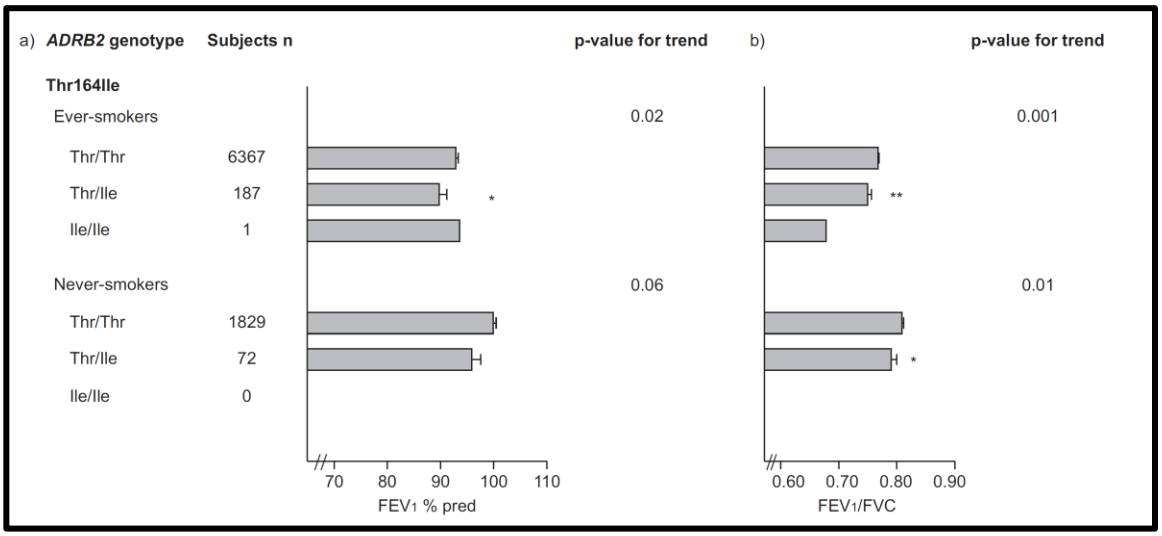
There are no previous studies on the rare Thr164Ile polymorphism in *ADRB2* and risk of COPD. However, the observed association in our study is biologically plausible, because Thr164Ile has the most profound functional consequences for the ADRB2 receptor out of the three variants studied and thus may modify the receptor's response to endogenous catecholamines. The substitution of isoleucine for threonine at position 164 lies next to a serine with predicted involvement in adrenergic ligand binding [20]. In concordance with this, studies using recombinant cells have found that the Thr164Ile allele has 4 times less ligand affinity and a 50% reduction in agonist-stimulated adenyl cyclase activity [46]. Similarly Thr164Ile heterozygotes had a 5-fold reduction in sensitivity to beta-2-receptor agonist-mediated vasodilatation, and they had increased vasoconstrictor sensitivity [47]. These profound effects of Thr164Ile on smooth muscle constriction could potentially influence lung function and risk of COPD. Taken together, our findings suggest that the Thr164Ile polymorphism may explain a small fraction of the missing heritability in COPD. However, due to the rarity of the mutation, these findings have to be replicated in even larger studies. Nevertheless, supporting that this is not a chance finding Thr164Ile homozygotes also had increased risks for obesity, hypertension, and ischemic heart disease, the latter two in women only [48,49]. These findings and the present finding for COPD are also plausible because the profound effects of Thr164Ile on ADRB2 are exerted in both the respiratory tract, in adipocytes, and in the vessel wall of arteries.

FIGURE 7:



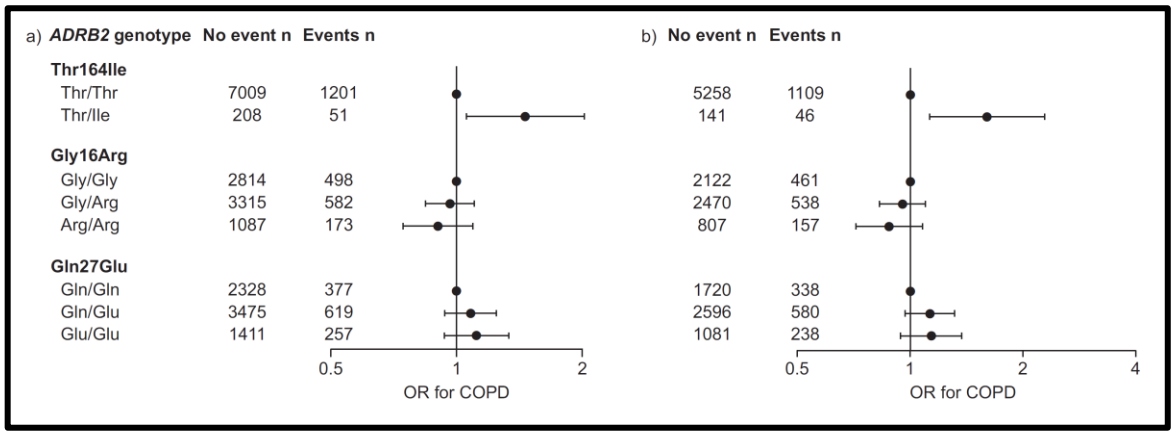
*a) Forced expiratory volume in 1 second in percent of predicted (FEV_1% pred) and b) FEV1/forced vital capacity (FVC) according to ADRB2 Thr164Ile, Gly16Arg and Gln27Glu genotype. Values represent mean ± SE. P for trend was determined by Cuzick's test for trend. *P<0.05 and **P<0.01 on Student's t-test comparing Thr164Ile heterozygotes with noncarriers.*

FIGURE 8:



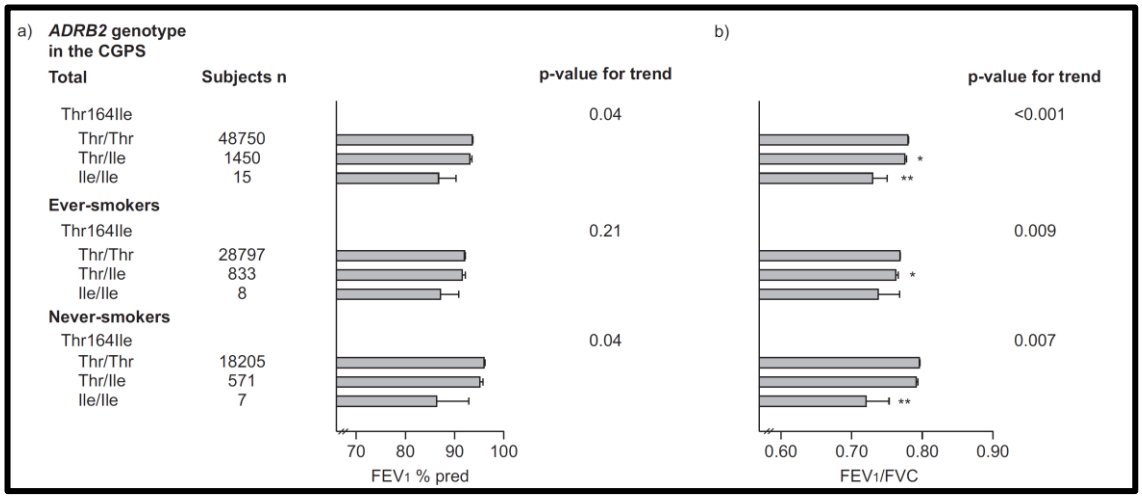
*a) Forced expiratory volume in 1 second in percent of predicted (FEV_1% pred) and b) FEV1/forced vital capacity (FVC) according to ADRB2 Thr164Ile genotype stratified by smoking status. Values represent mean ± SE. P for trend was determined by Cuzick's test for trend. *P<0.05 and **P<0.01 on Student's t-test comparing Thr164Ile heterozygotes with noncarriers.*

FIGURE 9:



Risk of chronic obstructive pulmonary disease (COPD) in a) all subjects and b) eversmokers according to ADRB2 Thr164Ile, Gly16Arg and Gln27Glu genotype. Values represent odds ratio and 95 % confidence interval. The adjusted logistic regression model allowed for age and gender. COPD was FEV_1/FVC<0.7 excluding individuals taking asthma medication.

FIGURE 10:



*a) Forced expiratory volume in 1 second in percent of predicted (FEV_1% pred) and b) FEV1/forced vital capacity (FVC) in ADRB2 Thr164Ile heterozygotes and homozygotes vs. noncarriers in the Copenhagen General Population Study, overall or stratified by smoking status. Values represent mean ± SE. P for trend was determined by Cuzick's test for trend. *P<0.05 and **P<0.01 on Student's t-test comparing Thr164Ile heterozygotes or homozygotes with noncarriers.*

CONCLUSIONS AND PERSPECTIVES

Rare functional variants in the candidate genes encoding scavenger and the β_2-adrenergic receptor may explain some of the missing heritability for risk of COPD. Using two large Danish general population studies we found that the rare Arg293X polymorphism truncating SRA-I/II was associated with reduced lung function and increased risk of COPD in men, as well as among individuals heterozygous for α_1-*antitrypsin* MZ and *superoxide dismutase-3* E1I1 genotypes. However, none of the nonsynonymous variants discovered by resequencing of the gene encoding the structurally similar MARCO were associated with lung function or risk of COPD. Furthermore, the Thr164Ile polymorphism with profound effect on ADRB2 function was associated with reduced lung function and increased risk of COPD in the general population.

Elucidating the genetic component behind reduced lung function and increased risk of COPD could lead to novel insights into pathogenesis and potentially new therapeutic targets; however, this has turned out to be a challenging task. The numerous loci identified through genome-wide association studies remain to be replicated. Also, due to the relatively small effect sizes and the difficulties in interpreting non-coding variations, the clinical value of these findings is unclear. Many more rare causal variants remain to be identified and for this purpose large-scale candidate gene studies seem to remain a valuable approach. In recent years, next-generation sequencing platforms have become widely available and are markedly reducing the costs of DNA sequencing. Accordingly, in the future exome sequencing is likely to increase the discovery of rare functional genetic variants associated with complex diseases such as COPD. Also, as our findings support the importance of exploring potential interactions between COPD susceptibility genes collaborations and coordination between centres in the field of COPD genetics is needed. Finally, translating our genetic knowledge to a general improvement of health in patients with COPD remains the ultimate challenge in the years to come.

SUMMARY

Chronic obstructive pulmonary disease (COPD) is a complex disease with many pathological components. Familial clustering indicates that genetic susceptibility may be important for the development of the disease. Genes encoding scavenger and β_2-adrenergic receptors are plausible candidate genes in COPD. Using data from two large Danish general population studies, we studied rare functional variants in the genes encoding scavenger receptor A-I/II (SRA-I/II), macrophage receptor with collagenous structure (MARCO), and the β_2--adrenergic receptor (ADRB2) for associations with lung function and risk of COPD. We found that the truncating variant Arg293X in the gene encoding SRA-I/II was associated with reduced lung function and with increased risk of COPD among men, as well as among α_1-antitrypsin MZ and superoxide dismutase-3 E1I1 heterozygotes. However, none of the nonsynonymous variants discovered by resequencing of the structurally similar MARCO were associated with lung function or risk of COPD. Finally, we found that the rare Thr164Ile polymorphism in ADRB2 was associated with reduced lung function and risk of COPD, whereas the more common Gly16Arg and Gln27Glu were not. Further studies are needed to confirm these relationships.

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