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# The Hedgehog Interacting Protein is a COPD Susceptibility Gene: The Rotterdam Study

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# Abstract

## *Rationale*

The hedgehog (Hh) signalling pathway plays an important role in lung morphogenesis and in cellular responses to lung injury. A genome-wide association (GWA) study demonstrated that two SNPs near the Hh interacting protein (Hip) gene, rs1828591 and rs13118928, are associated with the risk of COPD.

## *Objective*

The aim of this study was to validate the observed association between genetic variation near the *Hip* gene and COPD and to investigate if risk estimates were modified by smoking behaviour.

## *Methods*

The association between the *Hip* gene SNPs and COPD was studied in the Rotterdam Study by logistic regression analyses, adjusted for several covariates. In addition, an association meta-analysis was performed including data from the GWA study on COPD.

## *Results*

Both SNPs were significantly associated with the risk of COPD (OR 0.8; 95% CI, 0.72-0.91). Homozygosity for the minor G-allele resulted in a decreased risk of COPD of approximately 40% (95% CI, 0.47-0.78). There was a significant interaction with the amount of pack-years smoked (p=0.004). The meta-analysis yielded an odds ratio for COPD of 0.8 per additional G-allele (p=3.4x10-9).

## *Conclusions*

Genetic variation near the *Hip* gene is significantly associated with the risk of COPD, depending on the amount of pack-years smoked.

# Introduction

Chronic obstructive pulmonary disease (COPD) is worldwide a major and still increasing health problem (1). The disease is characterized by progressive airflow limitation, driven by an abnormal inflammatory response of the airways to inhaled particles and fumes (2;3). Smoking is the major etiologic factor for COPD in the Western world (4). However, only 15 to 25% of cigarette smokers seem to develop the disease, suggesting the presence of susceptibility genes for COPD (5;6). Previous studies have demonstrated familial aggregation of COPD and some twin studies on pulmonary function have indicated a genetic contribution to clinically relevant parameters such as FEV1 and FVC (7-9).

The evolutionary highly conserved Hedgehog (*Hh*)-signalling pathway is crucial in several developmental processes including lung organogenesis and is also implicated in the response of the airway epithelium to smoking (10-12). In the cells that respond to *Hh*, two proteins are up-regulated, i.e. the *Hh* receptor Patched (*Ptc*) protein and the vertebrate-specific *Hh*- interacting protein *Hip*. Both play an important role in the negative feedback regulation of *Hh* signalling. Loss of *Hip* function in mouse has already been associated with specific lung defects and results in neonatal lethality (13).Like in murines, dysfunction of the *Hh* pathway during foetal life in humans is responsible for severe lung malformations (14-16). In adult life, the *Hh* pathway plays a central role in the repair and regeneration of several tissues and aberrant activation of this pathway by epithelial stem cells has been linked to various malignancies including lung cancer (17;18).

Two recent genome-wide association (GWA) studies unexpectedly demonstrated that two SNPs near the *Hip* locus (rs13112928 and rs1828591) were associated with COPD and with lung function respectively, although genome-wide significance levels were not reached for the COPD phenotype (19;20).

The objective of the current study was to study the abovementioned association between SNPs near the *Hip* gene and COPD and to investigate whether smoking or gender modified this association in a large prospective population-based cohort study with approximately twenty years of follow-up.

# Methods

## *Study population*

The present study is part of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described elsewhere (21;22). In short, the Rotterdam Study cohort included 7983 participants aged ≥ 55 years, living in Ommoord, a well-defined suburb of the city of Rotterdam, the Netherlands. Almost all participants (99.8%) are of Caucasian descent. Baseline data were collected from 1989 until 1993. Participants were visited at home at the start of the study for a standardized interview on their health status. Since the start of the Rotterdam Study, each participant visited the research center every 2 to 3 years, during which several measurements were performed. In addition, participants were continuously monitored for the onset of major events which occurred during follow-up through automated linkage with files from general practitioners. Trained research assistants collected information from medical records of the general practitioners, hospitals and nursing homes. The medical ethics committee of the Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent, and permission to retrieve information from treating physicians.

### *Spirometry*

Spirometry was performed by using a SpiroPro® portable spirometer (Erich Jaeger GmbH, Hoechberg, Germany), according to the ATS/ERS guidelines by trained paramedical personnel (23). Forced expiratory volume in one second ($FEV_1$), forced vital capacity (FVC) and $FEV_1$/FVC ratio were measured; the spirogram (volume-time curve) and maximal expiratory flow-volume curve were also recorded. Spirometries that yielded results which did not meet ATS/ERS criteria for acceptability and reproducibility were classified as "not interpretable" (24).

### *Patient identification and validation*

For the validation of the COPD cases, we had access to hospital discharge letters, files from the general practitioners, spirometry reports and pharmacy dispensing data for patients participating in the Rotterdam Study, as previously described (24). Spirometry was performed in the context of the Rotterdam cohort study in 3550 participants. In addition, throughout the entire study period, spirometries were also performed on clinical indication by respiratory specialists and internists with a subspeciality in respiratory medicine. In the absence of spirometry, all medical information of subjects who used respiratory medication for at least 6 months (ATC-codes: R03AC, R03AK, R03BA, R03BB, R03CC, R03DA) (25) and all hospital discharge letters or mortality reports with a coded diagnosis of COPD (ICD10: J40-J44) (26) were reviewed. Drug use was only used for potential case finding but not for making the diagnosis of COPD. In case of confirmed COPD, the day of first drug use was used as an indicator of the date of onset of COPD.

The diagnosis of COPD was classified as definite or probable. Definite COPD was defined by a moderate-to-severe obstructive spirometry ($FEV_1/FVC < 0.7$ and $FEV_1 < 80\%$ predicted), and/or as COPD diagnosed by a specialist in internal medicine (mainly respiratory physicians or internists with a subspecialty in respiratory medicine) based upon the combination of clinical history, physical examination and spirometry. Probable COPD was defined by a mild obstructive spirometry ($FEV_1/FVC < 0.7$ and $FEV_1 \geq 80\%$ predicted) and/or as COPD diagnosed by a physician in another medical speciality (e.g. a general practitioner).

The index date was defined as the date of diagnosis of COPD found in the medical reports, or the date of a first prescription of COPD medication, or the date of the obstructive lung function examination, whichever came first.

### *Genotyping*

The Rotterdam Study used the Illumina Infinium II HumanHap550K BeadChip (version 3) for genotyping 6449 participants with available DNA from the Rotterdam Study, as previously described (27). Genotyping was performed at the Department of Internal Medicine, Erasmus Medical Center using BeadStudio software (version 0.3.10.14) for genotyping calling. Participants with call rate < 97.5%, excess autosomal heterozygosity, sex mismatch, or outlying identity-by-state clustering estimates were excluded. There were 5974 participants who were successfully genotyped and passed quality control. Genotype information for two specific SNPs near the *Hip* gene located on chromosome 4 (rs13118928 and rs1828591) was extracted from these data.

## *Statistical analyses*

Demographic and clinical characteristics of the study population are expressed as the median and the interquartile range (IQR) for description in the text.

First, we studied the association of the rs13118928 and rs1828591 with COPD within the Rotterdam Study. All COPD cases, prevalent and incident, were used for analyses, to obtain maximal power. Consequently, we used logistic regression analyses because a Cox proportional hazards analysis was not possible with prevalent cases at baseline. Both SNPs were tested for Hardy Weinberg Equilibrium with $\chi^2$ statistics. A power calculation using 'Quanto', based on the combined odds ratio of 0.7 of the Bergen Case Control and the NETT/NAS population (combined $\beta = -0.332$) from the study of Pillai et al. (19), showed that we were able to demonstrate an odds ratio for COPD of 0.7 with more than 99% power at a two-sided alpha of 0.05 (28). Additive and genotype-based logistic regression models, adjusted for age at baseline, gender, smoking behaviour (ever and never smoker) and the total amount of pack-years smoked, were used to calculate odds ratios (OR) and the 95% confidence intervals (CI). In the additive genetic model, we included a categorical variable with the number of variant alleles (so homozygous wild type [0 variant alleles]; heterozygous [1 variant allele]; homozygous variant allele [2 variant alleles]) as a continuous value in the logistic regression model in order to calculate trends.

The following covariates were considered as potential confounders or effect modifiers: age, gender, smoking behaviour and the total amount of pack-years smoked. Smoking status was self-reported and categorised as ever or never smoker. The category of ever smokers included both current and former smokers. Cigarette pack-years were computed as duration of smoking (years) multiplied by the number of smoked cigarettes, divided by 20. The amount of pack-years smoked by each study participant was calculated using prospectively collected data regarding the smoking behaviour of the cohort members, collected during the first, second and third follow-up visits of the Rotterdam Study.

Participants with asthma or a spirometry suggestive of restrictive lung disease were excluded from the analyses. Interaction terms and subanalyses were introduced to explore for potential effect modification by gender and tertiles of pack-years smoked. P-values below the conventional level of significance ($p< 0.05$) were considered as statistically significant.

Second, an inverse-variance weighted meta-analysis was performed. This analysis combined the obtained results with the recently published data by Pillai et al. (19). The regression coefficients and standard errors were calculated on the basis of the reported odds ratios and p-values from the 'Bergen Case Control Population' and the 'NETT/NAS' data from the recent GWA study by Pillai et al.(19).

The results of the association meta-analysis were calculated with Excel version 2007 (Microsoft Office). All other statistical analyses were performed using SPSS for Windows version 15 (SPSS Inc, Chicago, IL).

# Results

## *Baseline characteristics of the study population*

Within the Rotterdam Study source population of 7983 subjects, there were 928 patients with validated prevalent or incident COPD (24). Both SNPs, rs13118928 and rs1828591, were successfully genotyped in 5974 out of 7983 participants for whom DNA was available, including 742 COPD cases. We excluded 256 study participants with asthma or a spirometry suggestive of restrictive pulmonary disease (**Figure 1**). Overall, the median age of the study population at the start of the study was 68.4 years (interquartile range [IQR] 14 years) and 59% were women. Additional clinical characteristics are shown in **Table 1**.

*Association between variation near the Hip gene and COPD in the Rotterdam Study*

Both studied SNPs, rs13118928 and rs1828591, were in Hardy Weinberg equilibrium (rs13118928: $\chi^2 = 1.413$, p = 0.235; rs1828591: $\chi^2 = 1.215$, p = 0.270) and in perfect linkage disequilibrium ($r^2 = 1.00$ in HapMap CEU reference samples, [Utah residents of Northern and Western European descent]). Because of the high linkage between both SNPs, analyses yielded the same results and thus. For reasons of simplicity, only the results for the rs13118928 SNP are shown in the text and tables.

Under an additive genetic model, there was a significant association between both SNPs and the risk of COPD with an odds ratio (OR) of 0.8 per additional G-allele (95% CI, 0.72-0.91; p<0.001). Genotypic analysis showed that homozygosity for the minor G-allele was associated with a significantly decreased risk of COPD of approximately 40% (OR 0.6; 95% CI, 0.47-0.78; p<0.001) (**Table 2**). The decrease in risk remained significant when the analyses were restricted to those participants with an available spirometry examination during follow-up in the Rotterdam Study (OR 0.6; 95% CI, 0.43-0.89; p=0.011) (data not shown).

There was no interaction with gender (p=0.872), but a significant interaction effect was present with the total amount of pack-years smoked (p=0.004). Additional subanalyses demonstrated that the protective effect of having one or more variant alleles was most pronounced for smoking participants in the highest tertile of pack-years of smoking (> 36 pack-years) with an OR of 0.8 (95% CI, 0.62-0.93; p=0.007), versus an OR of 0.9 (95% CI, 0.67-1.09; p=0.209) in those who had never smoked (**Table 3**).

*Association between variation near the Hip gene and COPD, meta-analysis with published results*

The original p-value including the 'Bergen Case Control Population', the 'ICGN Population' and the 'NETT/NAS' data was $1.7x10^{-7}$. Since it was not possible to obtain beta estimates and standard errors for the 'ICGN Population', we also calculated the p-value for the combination of the 'Bergen' and the 'NETT/NAS' data. This resulted in a p-value of $1.0x10^{-6}$. The combination of the Rotterdam Study results with the 'Bergen' and the 'NETT/NAS' results yielded an overall OR of 0.8 per additional G-allele (95% CI, 0.70-0.84) with a p-value of $3.4x10^{-9}$ which is significant at a genome-wide level (**Table 4) (Figure 2**).

# Discussion

The results of this population-based prospective cohort study confirm the recently observed association between genetic variation around the *Hip* gene and COPD, as described by Pillai et al (19). We found that the minor G-allele of the rs13118928 and rs1828591 SNPs is associated with a significantly decreased risk of COPD. The risk seemed to be dependent on the smoking behaviour of the participants, since the decrease in risk was strongest in subjects that had smoked more than 30 pack-years at the start of the Rotterdam Study.

The *Hh*-signalling pathway is an evolutionary highly conserved signalling cascade which is crucial in several developmental processes (11;12). In mammals, there are three Hedgehog (*Hh)* proteins: Sonic, Indian, Desert. Sonic *Hh* specifically targets the lung and is expressed in the lung epithelium during embryogenesis (29). Signalling is initiated by binding of one of the *Hh* proteins to the 12 transmembrane protein receptor Patched (*Ptc*), which is required to inhibit the activity of Smoothened (*Smo*), a 7 transmembrane protein, responsible for regulation of the zinc finger-containing *GLi* transcriptional effectors *GLi1*, *GLi2* and *GLi3* (14;30). Upon binding of *Hh* to *Ptc*, the *Ptc*-mediated inhibition of *Smo* is relieved (31;32). This triggers a cascade which finally results in activation of the *GLi* family and transcription of *Hh* target genes including *Ptc* and *Gli* itself (33). The availability of the *Hh* ligands is regulated by expression of the *Hh*-interacting protein (*Hip)*. The *Hip* protein was initially discovered by screening murine cDNA expression for proteins that bound to Sonic *Hh.* The *Hip* gene, located on chromosome 4 (4q31.21 - 4q31.3), encodes for a transmembrane glycoprotein which binds all three *Hh* proteins with an equal affinity to that of *Ptc* and is upregulated upon *Hh* signalling (29). *Hip* is expressed in all *Hh* target tissues and acts by internalizing and degrading the *Hh* protein. Similar to *Ptc*, *Hip* thus serves as a natural antagonist of the *Hh* pathway.

In this way, gene alterations in the *Hip* gene can result in an altered gene expression of a functional protein or in a non-functional protein and an upregulation of the *Hh* signalling pathway.

Loss of *Hip* function in mice during foetal life has been associated with specific lung defects resulting in respiratory failure and neonatal lethality (13). In adult animals, down-regulation of *Hip* has been reported in murine lung cancer cell lines (34). Like in rodents, dysfunction of the *Hh* pathway during foetal life in humans is responsible for severe lung malformations (14-16). In adult life, the *Hh* pathway plays a central role in the repair and regeneration of several human tissues and aberrant activation of this pathway by epithelial stem cells has been linked to various malignancies including lung cancer (17;18). In addition, *Hip* seemed to be down-regulated in human non-small cell lung cancer tissues and endothelial cells during angiogenesis (34).

The results of this study and the two previous GWA reports demonstrate that variants near the *Hip* gene influence the risk of COPD. Although it is not clear which regulatory effect explains this association, several hypotheses are possible. First, Madison et al. demonstrated that ectopical expression of *Hip* in mice during foetal life was associated with expanded smooth muscle in the intestinal epithelium (32). If the same effect is present in the small airways, then it can be speculated that changes in *Hip* influence the thickness of the airway muscle layer and induce more or less airflow limitation. Second, a study of Pogach et al. demonstrated that several developmental pathways in the lungs of adult mice including *Hh* reactivate for tissue repair after hyperoxia (35). In addition, others showed that embryogenic signalling pathways such as *Hh* are activated in human bronchial epithelial cells exposed to cigarette smoke (36).

The prevalence, morbidity, and mortality of COPD in women are increasing (37). In addition, some reports suggest that women may be at greater risk to develop severe early-onset COPD due to sex differences in the metabolism of cigarette smoke (38). However, the results of these studies remain controversial since differences in lung development, smoking behaviour, diagnosis and treatment of COPD are possible confounders. Since gender can be of interest in the pathogenesis of COPD, we tested for a potential interaction effect of gender on the observed association between variation near the *Hip* gene and COPD. However, we found no significant influence (p= 0.872).

The results of these two studies raise the possibility that the *Hh* pathway is involved in the response of the airway epithelium to tissue injury and that alterations in Hh signalling can lead to susceptibility to COPD depending on the severity of the smoking behaviour. Thirdly, since a recent GWA study investigating genetic variants that influence adult height identified several relevant genes in the *Hh* signalling pathway (Indian *Hh*, *Hip* and *Ptc*), height could be considered as a potential confounder for the observed association between *Hip* and COPD (39). However, additional analyses including height as a covariate did not influence our estimates (data not shown).

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. A power analysis showed that this study was of a sufficient size to distinguish between the proposed effect and no effect. This study was conducted in a dataset independent from the original GWA study.

We demonstrated a similar significant protective risk with the same SNPs and the combined analysis led to a smaller p-value than in the original report below the stringent threshold frequently used to define genome-wide significance. Hence, this study satisfies all criteria as suggested by the NCI-NHGRI Working Group on Replication in Association Studies for establishing positive replication (40).

As in all other association studies no conclusions on causality or mechanism through which the associated SNPs influence COPD risk can be made. It is possible that the two studied SNPs are not the causal variants, but are in linkage disequilibrium with other polymorphisms that are responsible for the increased COPD risk. Since the SNPs are not situated within the *Hip* gene and do not seem to exhibit a strong linkage with other SNPs in the *Hip* gene itself, a regulatory function is possible. Additional functional and translational research examining the role of *Hip* in COPD is certainly warranted.

In conclusion, this study confirms the previously reported association between two SNPs near the *Hip* gene and COPD in a Caucasian population-based cohort study. In addition, we identified a potential gene-by-environment interaction, since the association was most apparent in the heavily smoking participants.

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# Disclosures

None of the authors has any conflicts of interest.

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# Table legends

**Table 1 :** Baseline characteristics of the study population.

**Table 2 :** Association of the rs13118928 and 1828591 SNPs with COPD in the Rotterdam Study.

**Table 3 :** Association of the rs13118928 and 1828591 SNPs with COPD in the Rotterdam Study, stratified according to the total amount of pack-years smoked at the start of the study.

**Table 4 :** Association of the rs13118928 and 1828591 SNPs with COPD, results of the meta-analysis.

**Table 1 Baseline characteristics of the study population**

| | Rotterdam Study | Cases | Controls |
|---|---|---|---|
| Subjects, No | 5718 | 742 | 4976 |
| Age, years (median, IQR) | 68.4 (14) | 67.0 (10) | 68.8 (14) |
| Female (%) | 3397 (59%) | 315 (43%) | 3082 (62%) |
| Smoking status | | | |
| Current smoker (%) | 1290 (23%) | 300 (40%) | 990 (20%) |
| Former smoker (%) | 2311 (40%) | 325 (44%) | 1986 (40%) |
| Never smoker (%) | 1960 (34%) | 111 (15%) | 1849 (37%) |
| Missing (%) | 157 (3%) | 6 (1%) | 151 (3%) |
| Pack-years smoked (median, IQR) | 9.0 (30) | 27.8 (32) | 6.8 (28) |
| rs13118928 | | | |
| AA | 2099 (37%) | 294 (40%) | 1805 (36%) |
| AG | 2698 (47%) | 359 (49%) | 2339 (47%) |
| GG | 921 (16%) | 89 (12%) | 832 (17%) |
| rs1828591 | | | |
| AA | 2092 (37%) | 293 (40%) | 1799 (36%) |
| AG | 2703 (47%) | 360 (49%) | 2343 (47%) |
| GG | 923 (16%) | 89 (12%) | 834 (17%) |

Abbreviations: IQR, interquartile range; No, number.

**Table 2 Association of the rs13118928 SNP with COPD**

| Model | Patients / cohort, No | Odds Ratio | 95% CI | p-value |
|---|---|---|---|---|
| Additive | 742 /5718 | **0.8** | **0.72-0.91** | **$2.0x10^{-4}$** |
| Genotype-based | | | | |
| AA | 294 / 2099 | 1.0 | Reference | |
| AG | 359 / 2698 | 0.9 | 0.75-1.05 | 0.169 |
| GG | 89 / 921 | **0.6** | **0.47-0.78** | **$1.0x10^{-4}$** |

NOTE : Additive and genotypic logistic regression models, adjusted for age, gender, smoking and the amount of pack-years smoked, were used for analyses.

Abbreviations : CI, confidence interval; COPD, chronic obstructive pulmonary disease; No, number; SNP, single nucleotide polymorphism.

**Table 3 Association between the rs13118928 & 1828591 SNPs near the *HIP* gene and COPD, stratified according to the total amount of pack-years* smoked**

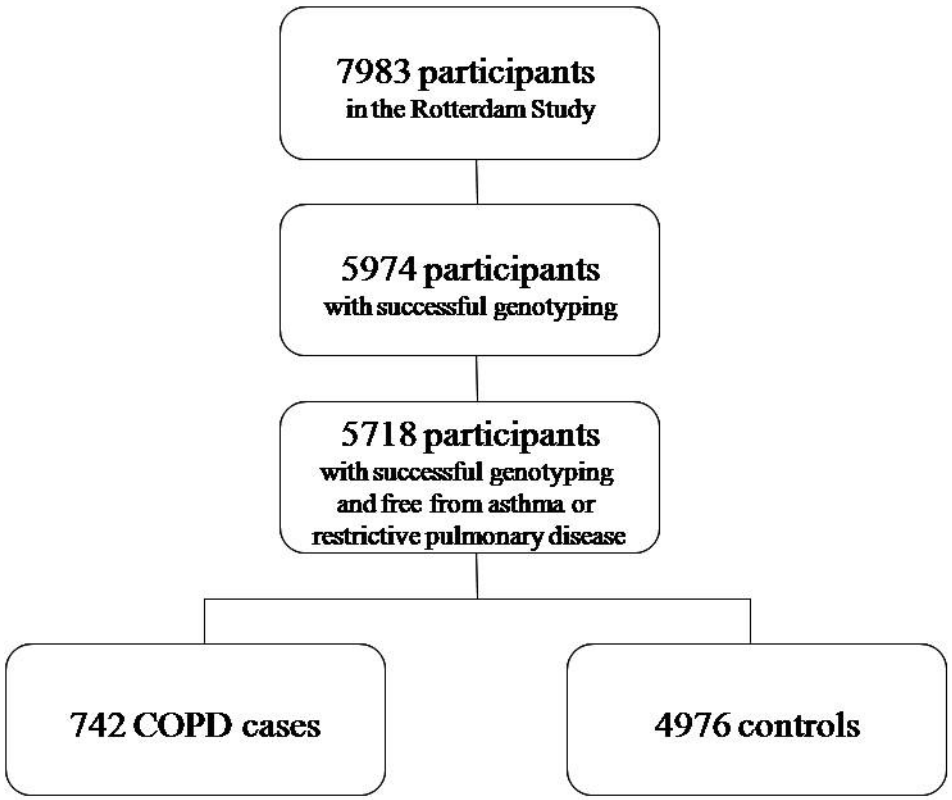
| Pack-yr, No † | Patients / cohort, No | Odds Ratio | 95% CI | p-value |
|---|---|---|---|---|
| 0 | 111 / 1960 | 0.9 | 0.67-1.09 | 0.209 |
| >0 - ≤15 | 114 / 1129 | 0.8 | 0.61-1.08 | 0.154 |
| >15 - ≤36 | 203 / 1082 | 0.9 | 0.70-1.09 | 0.224 |
| >36 | 277 / 1095 | **0.8** | **0.62-0.93** | **0.007** |

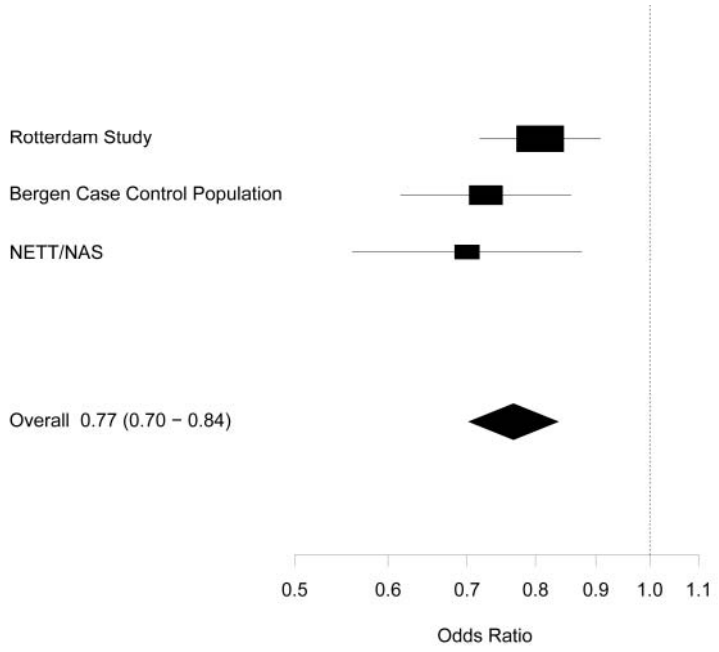
NOTE : Additive logistic regression models, adjusted for age and gender, were used for analyses.
Abbreviations : CI, confidence interval; COPD, chronic obstructive pulmonary disease;
HIP, Hedgehog interacting protein; No, number; SNP, single nucleotide polymorphism; yr, year.
† Pack-year categories were based on the 33rd percentile of the pack-year data in smokers.
* The amount of pack-years was calculated based on information regarding the smoking behaviour of each study participant, prospectively collected at each interview during the ERGO 1,2 and 3 follow-up visits.

**Table 4 Results of the combined analyses for the rs13118928 & 1828591 SNPs near the HIP gene and COPD**

| Sample | Odds Ratio | 95% CI | P value |
|---|---|---|---|
| Rotterdam Study | 0.81 | 0.72-0.91 | < 0.001 |
| Bergen Case Control Population † | 0.73 | 0.62-0.86 | < 0.001 |
| NETT/NAS † | 0.71 | 0.56-0.88 | 0.002 |
| **Combined** | **0.77** | **0.70-0.84** | **$3.43 \times 10^{-9}$** |

NOTE : logistic regression models, adjusted for age, gender, smoking behaviour and the amount of and the amount of pack-years smoked, were used for analyses.
Abbreviations : CI, confidence interval; No, number; SNP, single nucleotide polymorphism.
† Data originating from Pillai et al., *PLoS Genetics* 2009.




Rotterdam Study
Bergen Case Control Population
NETT/NAS
Overall 0.77 (0.70 − 0.84)
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Odds Ratio