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Superoxide dismutases, lung function and bronchial responsiveness in a general population

Authors: M Siedlinski[1], CC van Diemen[1], DS Postma[2], JM Vonk[1], and HM Boezen[1*]

Institutions: [1]Department of Epidemiology, [2]Pulmonology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

***Corresponding author:** Prof. HM Boezen, Department of Epidemiology, University Medical Center Groningen; E3.29, PO Box 30.001, 9700 RB Groningen; The Netherlands; email: H.M.Boezen@epi.umcg.nl; Telephone: + 31 50 3610899; Fax: + 31 50 3614493

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ABSTRACT

Oxidative stress is an important causative factor in the onset and progression of smoking-related lung diseases like chronic obstructive pulmonary disease (COPD). Superoxide Dismutases (SODs) can prevent an increase in oxidative burden.

1,390 subjects from the prospective Vlagtwedde–Vlaardingen cohort were genotyped for 2 Single Nucleotide Polymorphisms (SNPs) in *SOD2* and 4 SNPs in *SOD3,* that were further analyzed for associations with the presence of bronchial hyperresponsiveness (BHR; PC_{10}≤8 mg/ml of histamine), COPD (defined as GOLD stage ≥ II), lung function level and the longitudinal course of FEV_1.

The intronic C5774T SNP of *SOD2* was significantly associated with the presence of COPD and BHR in the total population. The T/T genotype for this polymorphism and the Val/Val genotype for the *SOD2* Ala16Val substitution were risk factors for BHR in individuals without COPD. The *SOD3* Arg213Gly substitution was associated with slower FEV_1 decline in never smokers exclusively, and the *SOD3* G(-4466)T SNP was associated with a lower Vital Capacity level.

Both *SOD2* polymorphisms are associated with BHR, a risk factor for COPD, while *SOD2* C5774T additionally confers a risk for COPD in the total population. We furthermore confirm previously reported associations of *SOD3* SNPs with lung function in the general population.

INTRODUCTION

An imbalance between oxidants and antioxidants is considered to be an important causative factor in the onset and progression of chronic obstructive pulmonary disease (COPD) [1, 2]. A key molecule in the regulatory process of oxidative stress is superoxide anion, which is inhaled with cigarette smoke and additionally generated during numerous cellular reactions [3]. Free superoxide anion is a substrate for the synthesis of highly reactive oxygen species which can damage epithelial cells, impair epithelial ciliary function, alter expression of cellular adhesion molecules, and increase airway smooth muscle contraction in response to histamine and other stimuli in *in vitro* and in *in vivo* models [4]. Therefore, impaired superoxide metabolism may contribute to the development of bronchial hyperresponsiveness (BHR) and the subsequent onset of COPD [5, 6]. Indeed, we have previously shown that patients with COPD have increased production of superoxide anion by blood leukocytes, which was associated with more severe hyperresponsiveness [7]. However, this cross-sectional study did not elucidate whether the increased production of superoxide anion was the cause or consequence of BHR and COPD. So far, studies on the relation between superoxide anion and both the development of COPD and BHR in the general population are lacking.

The family of superoxide dismutases (SOD) is the sole unique enzymatic system able to degrade a superoxide anion [8]. Impairment of the mitochondrial superoxide dismutase (SOD2) activity was related to asthma pathophysiology [9], and extracellular superoxide dismutase (SOD3) was shown to protect lungs against oxidant-mediated injury [10, 11].

Therefore, it is of interest to study whether Single Nucleotide Polymorphisms (SNPs) in these *SOD* genes contribute to the development of BHR and/or COPD. *SOD2* contains one prevalent nonsynonymous SNP (nsSNP), i.e. Ala16Val, which is localized in a protein signal sequence, thus possessing a possible functional role [12-14]. This substitution has been associated with a higher lung cancer risk in Caucasians [15-17] but not with the presence of COPD in Caucasian and Chinese smokers [18, 19]. *SOD3* contains three nonsynonymous SNPs i.e. Ala40Thr, Phe131Cys and Arg213Gly. The Arg213Gly substitution has been associated with elevated SOD3 levels in human plasma [20] and with protection against COPD development in Caucasians [18, 21], whereas Ala40Thr and Phe131Cys have not yet been studied in this context. Recently it has been shown that the rs8192288 SNP in the *SOD3* 5' untranslated region is associated with lower Forced Vital Capacity in two large Danish cohorts and constitutes a risk for more frequent COPD hospitalization in the Copenhagen City Heart Study [22].

This study links the well-known risk factors for COPD development, i.e. BHR and smoking, with polymorphisms located in genes involved in response to oxidative stress, a key manifestation in COPD pathogenesis. Furthermore it aims to broaden the knowledge on candidate *SOD3* SNPs with respect to (the course of) lung function and it puts a new insight into the *SOD2* role in this context.

METHODS

Subjects

A total of 1,390 subjects of the Vlagtwedde-Vlaardingen cohort who participated in the last survey (1989–1990) were included in the study. This general population-based cohort of Caucasian individuals of Dutch descent started in 1965 and has been followed-up for 25 years [23-25]. Standardized Forced Expiratory Volume in 1 second (FEV_1) and Vital Capacity (VC) measurements were performed every 3 years (see online supplement for details) [26]. The study protocol was approved by the local university hospital medical ethics committee and all participants gave their written informed consent.

DNA extraction and genotyping

See the online supplement for a description of DNA extraction and the genotyping protocol. Six SNPs in two *SOD* genes were genotyped, i.e.: Ala16Val (rs4880) and C5774T (rs2842958) in *SOD2*, and Ala40Thr (rs2536512), Arg213Gly (rs1799895), G(-4466)T (rs8192288), and Phe131Cys [27] in *SOD3*.

The C5774T SNP was included to be able to tag *SOD2* haplotypes, as described previously [28].

Presence of COPD and bronchial hyperresponsiveness (BHR) phenotype

We identified subjects with COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria, i.e. an $FEV_1/VC<70\%$ and an $FEV_1<80\%$ predicted (GOLD stage II or higher) [29] at the last survey.

Bronchial responsiveness to histamine (30 seconds method) was assessed in a random subsample of the total cohort as described previously [25]. BHR at the last survey was expressed dichotomously, i.e. as a $PC_{10} \leqslant 8$ mg/ml histamine.

Statistics

Differences in prevalence of rare alleles of SNPs between subjects with and without COPD or BHR were tested with X^2 test and logistic regression. Additionally, we performed all mentioned analyses stratified according to smoking habits (never and ever smokers), since we expected the SOD effects to depend on smoking habits. For 2 *SOD3* SNPs we were not able to estimate the odds ratios for COPD and BHR for the homozygous mutant genotype due to the lack of (Arg213Gly), or low (G(-4466)T, n=3) numbers of subjects.

In order to disentangle the phenotypes COPD and BHR, subjects were classified into 4 phenotypic groups according to the presence of COPD and/or BHR (i.e.: 'COPD-/BHR-', 'COPD-/BHR+', 'COPD+/BHR-' and 'COPD+/BHR+'). Multinomial logistic regression, adjusted for packyears of smoking, was performed for all SNPs separately, with the 4 phenotypic groups as dependent variable. These analyses allowed us to predict if

subjects with certain genotypes were more likely to develop a COPD phenotype or a BHR phenotype.

Linear regression analysis (adjusted for sex, height, age and packyears of smoking) was used to test whether the FEV_1 or VC level differs between *SOD* genotypes at the last survey. Linear Mixed Effect models from the statistical package R (version 1.9.1) [30] were used to estimate longitudinal changes in lung function in the total cohort as well as in the ever and never smokers subgroups (see online supplement for details).

Haplotype analysis was performed with HAPSTAT (version 3.0) software [31, 32] (see online supplement for details). Remaining statistical analyses were performed using SPSS (version 12.0.1 for Windows; SPSS, Chicago, IL). P values<0.05 were considered to be significant (tested 2-sided).

RESULTS

Study population and distribution of *SOD* SNPs

The study population characteristics and genotype frequencies are shown in table 1. All SNPs were distributed according to Hardy Weinberg Equilibrium in the total population and were not correlated with each other (r^2<0.27 for any SNP pair). The Phe131Cys substitution in *SOD3* was not present in our cohort. We found no homozygote mutants for the Arg213Gly SNP in *SOD3* in our population. We identified respectively 3 and 5 haplotypes in *SOD2* and *SOD3* occurring with a frequency >1% in the total population (table S4 in the online supplement).

SOD SNPs and the presence of COPD

The *SOD2* C5774T SNP was significantly associated with COPD in the total population and in ever smokers (table 2 and table S2 in supplementary data), but not in never smokers (data not shown). No significant associations were found for the other investigated SNPs (table 2). The haplotype containing the *SOD2* C5774T SNP was associated with a significant higher odds for the presence of COPD (recessive effect; OR=2.1 [95% CI=1.1-4.0], p=0.02), compared to the wild type haplotype (table S4 in the online supplement).

SOD SNPs and the presence of BHR

The *SOD2* C5774T SNP was significantly associated with the presence of BHR in the random sub-group of the total population (n=409/1,390; table 2 and table S3 in supplementary data). Mutant homozygotes had a significantly higher risk of having BHR compared to wild types. The Ala16Val substitution in SOD2 was borderline significantly associated with BHR (p=0.07 for mutant vs. wild type homozygotes, table 2). Stratified analyses according to smoking showed no significant associations for these two SNPs. The haplotype containing the mutant allele of C5774T SNP was associated with an increased risk for BHR as compared to the wild type haplotype (additive effect; OR=1.7 [1.1-2.6], p=0.01; table S4 in the online supplement).

The Ala40Thr SNP was significantly associated with BHR only in never smokers (OR for the presence of BHR Ala/Thr vs. Ala/Ala=2.84 [1.08-7.48]; and Thr/Thr vs. Ala/Ala 14.40

[2.92-71.08] adjusted for FEV_1% predicted), but not in the total population and in ever smokers (table 2).

SOD SNPs and the presence of COPD and/or BHR phenotype

Two hundred and thirteen subjects had neither COPD nor BHR (COPD-/BHR-), 154 subjects had COPD-/BHR+, 6 subjects COPD+/BHR-, and 36 subjects had COPD+/BHR+. Subjects with the homozygous Ala16Val substitution in *SOD2* were most likely to be COPD-/BHR+ (OR=2.1 [1.1-3.8] for the Val/Val variant compared to Ala/Ala), indicating that the Ala16Val substitution was predictive for the presence of BHR and not for COPD or their combination. Likewise, subjects with the homozygous mutant *SOD2* C5774T SNP were most likely to have COPD-/BHR+ (OR=3.1 [1.03-9.51] for the homozygous mutant variant compared to the wild type). Haplotype analysis revealed that the only haplotype containing mutant alleles of both *SOD2* SNPs showed significantly increased odds ratio (OR for an additive effect=1.7 [1.2–2.6], p=0.01) for BHR in non-COPD subjects as compared to wild type haplotype. The polymorphisms in the *SOD3* gene were not associated with any of the 4 phenotypic outcome groups.

SOD SNPs and the level of FEV_1 or VC

No significant differences between the *SOD* genotypes in relation to the level of FEV_1 or VC were observed in the total population (see table S5 in the online supplement) or in the never or ever smokers subgroups (data not shown). Subjects homozygous for the G(-4466)T SNP in *SOD3* had borderline significantly lower VC (B (SE)= -532.2 ml (292.0), p=0.068), but not FEV_1, compared to the homozygous wild type group (fig. 1).

There were no homozygous *SOD3* G(-4466)T SNP subjects in the never smokers. In ever smokers that were homozygous for the *SOD3* G(-4466)T SNP we observed an effect size (i.e. -533.2 ml (301.2), p=0.077) that was similar to that in the total cohort.

SOD SNPs and longitudinal change in FEV_1

The annual FEV_1 decline was similar in all genotype subgroups of the investigated SNPs (see table S6 in the online supplement). Stratification according to smoking habits revealed that the *SOD3* Arg213Gly substitution was associated with slower FEV_1 decline in never smokers, e.g. 9.3 ml less decline per year in carriers of the Arg/Gly genotype while compared to the wild type (fig. 2). The other SNPs were not associated with excess FEV_1 decline in stratified analyses.

DISCUSSION

Of all human tissues, the lungs are most directly and particularly exposed to noxious free radicals. Tobacco smoke and environmental air pollution are the major sources of these particles. Therefore, the redox balance regulation can be an important factor for the development of bronchial hyperresponsiveness, excess decline in lung function, and development of COPD. We showed that *SOD2*, containing the mutant allele of the C5774T SNP, is a risk factor for bronchial hyperresponsiveness in the general population. Exclusively in subjects who had smoked, COPD was more prevalent in carriers of two copies of this mutant allele compared to wild type carriers. The *SOD3* Arg213Gly substitution, that has previously been shown to be protective for COPD [18, 21], was associated with slower FEV_1 decline in never smokers only. Additionally our study confirms a recently observed negative association of the G(-4466)T *SOD3* SNP with lung VC level in the general population [22].

Our finding is of interest since SOD2 dysfunction and inactivation has been recently described *ex vivo* in bronchial epithelial cells derived from airways of asthmatic patients [9]. This confirms the importance of SOD2 in lung homeostasis where reactive oxygen species can impair epithelial ciliary function and increase airway smooth muscle contraction in response to histamine [4], which may contribute to the development of BHR. Our data suggest that this may also be the case in COPD.

We found the mutant homozygosity of the *SOD2* SNP (C5774T), located in intron 3, to be a risk factor for the presence of COPD and BHR in the total population at the last survey. Although the role of SNPs localized in introns is not clear yet, it is known that

they may affect alternative splicing processes [33, 34] which result in different aminoacid sequences in mature proteins. The main difference between the *SOD2* isoforms is the presence of an additional exon within the third intron [35], but the implication with respect to putative differences in activity between both isoforms is yet unknown. It is indeed confirmed that SOD2 isoforms formation may depend on genetic variations as this has been shown *in vitro* for a polymorphism (single base pair deletion) that is prevalent in African-Americans [36]. This study concluded however that other (cis-acting) factors are necessary for switching between two isoforms *in vivo*. Thus it is also possible that C5774T SNP is in high linkage disequilibrium with another (functional) SNP, which is actually responsible for the associations that we observed.

Theoretically, the Ala16Val substitution could be of importance as well, since *in vitro* studies have shown impaired transport of Val16-SOD2 to the mitochondrion [12, 13], and lower mitochondrial SOD2 activity in leukocytes for the Val/Val genotype as compared to the other genotypes [14]. We did not find an association of the Ala16Val SNP with either the presence of COPD or with lung function decline, which is in concordance with results of two previous studies performed [18, 19].

Bronchial responsiveness may be modulated by superoxide anion production [7] and BHR is a known risk factor for COPD development [5, 6]. Therefore impaired superoxide radical detoxification, caused by loss of SOD2 function, may increase the level of BHR and affect the development of COPD. Remarkably, subjects with homozygous variants of the *SOD2* Ala16Val substitution or the *SOD2* C5774T SNP showed increased odds ratios for having 'pure BHR', which suggests it is indeed the phenotype BHR that is associated with these *SOD2* SNPs, and the relation that we have found between

C5774T SNP and COPD is driven by the association with BHR. Out of 2 mutant *SOD2* haplotypes only the one containing the mutant allele of the C5774T SNP was associated with increased risk for BHR as compared to the wild type haplotype, which implies that this SNP, rather than Ala16Val substitution, is associated with the presence of BHR.

Juul and colleagues [21] have shown prospectively and cross-sectionally a lower risk for COPD for the *SOD3* Arg213Gly SNP heterozygotes while compared to the wild type. This has been confirmed by another cross-sectional study [18]. The underlying mechanism of these associations may be explained by reduced proteolytic processing of mutated SOD3 protein [37] and higher SOD3 level in blood plasma due to lower affinity to heparan sulphate in the extracellular matrix [20, 38]. We additionally found that the protective effect of this SNP on longitudinal FEV_1 change appears in never smokers. Therefore we hypothesize that there are other antioxidant-related genetic factors that additionally contribute to the FEV_1 change in smokers. Recently Dahl and colleagues reported that the G(-4466)T (rs8192288) SNP, and particularly the homozygote mutant genotype, in *SOD3* constitutes a risk factor for a low level of Forced Vital Capacity and more frequent COPD hospitalization in the Copenhagen City Heart Study [22]. Interestingly in our study the same SNP was borderline significantly associated with low lung VC level, with a relatively large effect size. However we identified only 3 subjects carrying the risk (homozygous mutant) genotype and therefore we were not able to detect possible associations with FEV_1 decline or estimate the odds ratio for COPD presence.

There are some limitations to the study. It is conceivable that the low prevalence of the C5774T SNP in *SOD2* and especially of G(-4466)T and Arg213Gly in *SOD3* may have

negatively affected the power of our study. Therefore, we may have been unable to detect associations with BHR, FEV_1 decline or with COPD in the general population or in ever smokers. Another study limitation is the fact that the Vlagtwedde-Vlaardingen cohort is a unique prospective study with measurements of both BHR and FEV_1 at several time points over 25 years. Therefore we are not able to check for replication of our findings in another prospective cohort in order to reduce type I error (false positives). We additionally did not correct our p values for multiple comparisons.

In summary, we conclude that SOD2 is an antioxidant enzyme in which variations in DNA sequence can play a role in the development of BHR. By showing the protective effect of the Arg213Gly SNP in *SOD3* on the course of FEV_1 in never smokers we provide another piece of the puzzle on the role of this substitution in pulmonary disease. Additionally, we provide supportive evidence for an association of G(-4466)T (rs8192288) *SOD3* SNP with the level of VC in the general population. Further studies are needed to confirm the possible role of *SOD* SNPs in COPD and BHR, and additional functional studies are warranted.

Competing interests: All authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Adjusted means of the level of FEV_1 and VC according to the *SOD3* G(-4466)T genotypes in the total population

FEV_1 = Forced Expiratory Volume in 1 second

SOD = Superoxide Dismutase

VC = Vital Capacity

CI = Confidence Interval

Figure 1.

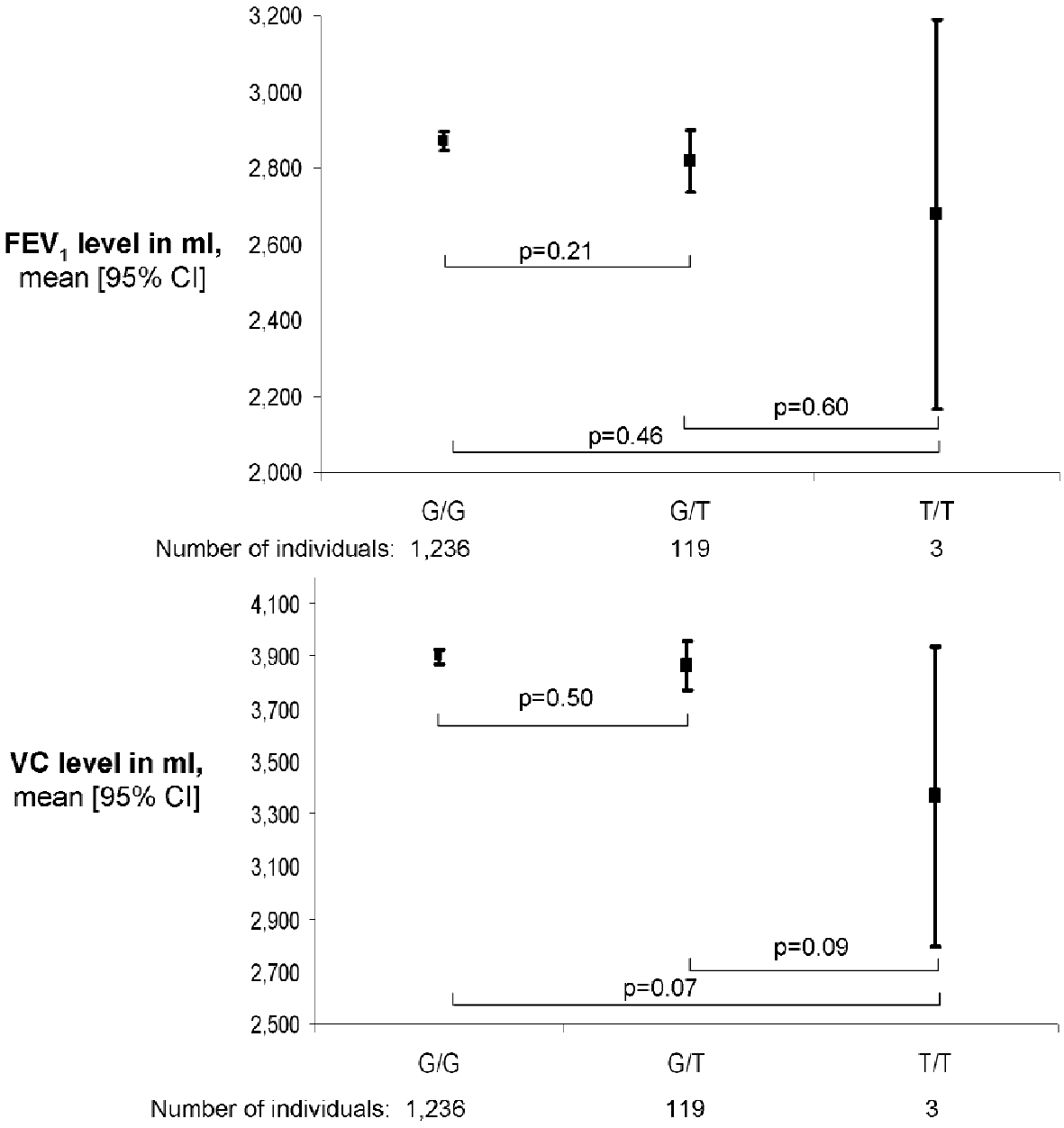


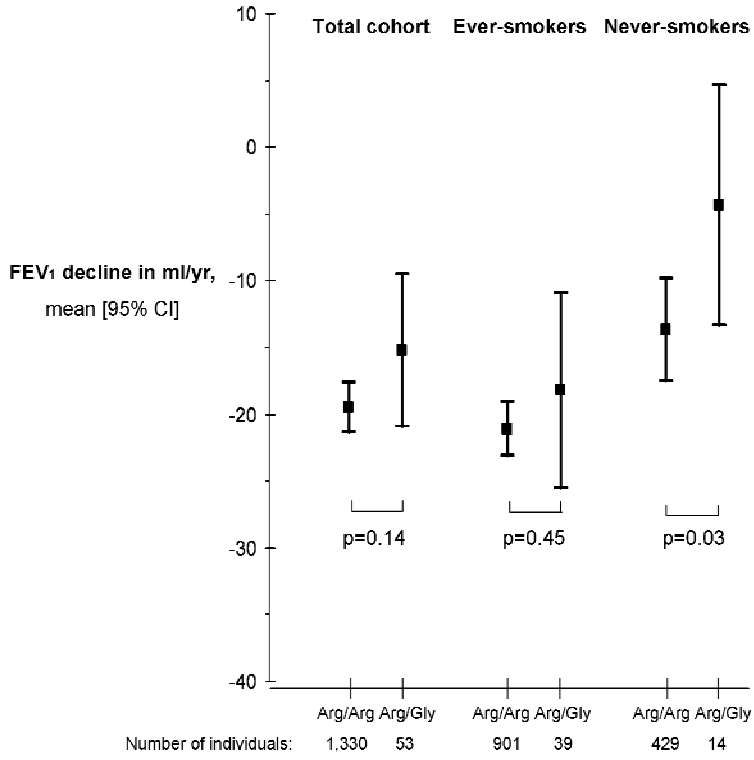
Figure 2. Adjusted means for the annual change in FEV_1 for the *SOD3* Arg213Gly genotypes in the total population and according to smoking habits

FEV_1 = Forced Expiratory Volume in 1 second

SOD = Superoxide Dismutase

CI = Confidence Interval

Figure 2.



TABLES

Table 1. Characteristics of the sample of the Vlagtwedde–Vlaardingen cohort at the last survey

	Total population n=1,390	*Ever smokers* n=945*
Males, n (%)	714 (51.4)	610 (64.6)
Age in years, median (range)	52 (35–79)	50 (35-79)
Packyears of smoking, median (range)	8.0 (0–262.2)	18.8 (0-262.2)
FEV_1 % predicted, mean (SD)	92.6 (15.3)	90.6 (15.5)
VC % predicted, mean (SD)	101.8 (14.5)	100.2 (14.0)
FEV_1/VC %, mean (SD)	73.9 (8.7)	72.4 (9.2)
COPD – GOLD stage II or higher, n (%)	167 (12.4)	140 (15.3)
BHR – $PC_{10} \leqslant 8$ mg/ml histamine, n (%)[§]	190 (46.5)	141 (50.4)
Genotype frequency (%heterozygotes / %homozygotes mutant):		
SOD2 Ala16Val:	49.6/23.8	49.8/23.6
SOD2 C5774T:	29.3/4.5	28.8/5.2
SOD3 Ala40Thr:	43.5/11.9	42.0/12.1
SOD3 Arg213Gly:	3.8/0.0	4.1/0.0
SOD3 G(-4466)T:	8.6/0.2	8.9/0.3

*sub-population of the total population

[§]BHR test performed on a random sub-group (n=409) of the total population (n=1,390)

SD = standard deviation; BHR = bronchial hyperresponsiveness; COPD = Chronic Obstructive Pulmonary Disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease; FEV_1 = Forced Expiratory Volume in 1 second; VC = Vital Capacity

PC_{10} = the histamine concentration causing an FEV_1 decrease of 10% or more from baseline

Table 2. Odds ratios (95% CI) for the presence of COPD respectively BHR, in the total population and in ever smokers, according to *SOD* SNPs

SNP	MAF	OR [CI] (heterozygotes vs. wild type)	OR [CI] (homozygotes vs. wild type)
Arg213Gly	0.01	0.48 [0.09-2.64]	
G(-4466)T	0.04	0.64 [0.25-1.63]	
SOD2		**COPD in total population (n=1,390)**	
Ala16Val	0.49	1.05 [0.66-1.67]	0.94 [0.62-1.41]
C5774T	0.21	0.74 [0.50-1.11]	**1.98 [1.04-3.78]***
SOD3			
Ala40Thr	0.34	1.03 [0.72-1.49]	0.59 [0.31-1.13]
Arg213Gly	0.02	1.66 [0.77-3.58]	-
G(-4466)T	0.05	1.25 [0.72-2.14]	-
SOD2		**COPD in ever smokers (n=945)**	
Ala16Val	0.48	0.93 [0.59-1.46]	1.19 [0.71-1.98]
C5774T	0.21	0.75 [0.48-1.12]	**2.37 [1.20-4.67]***
SOD3			
Ala40Thr	0.33	1.18 [0.70-1.78]	0.68 [0.34-1.36]
Arg213Gly	0.02	1.96 [0.89-4.36]	-
G(-4466)T	0.05	1.45 [0.82-2.58]	-
SOD2		**BHR in total population (n=409)**	
Ala16Val	0.45	1.24 [0.74-2.08]	1.80 [0.97-3.35]¥
C5774T	0.20	1.14 [0.61-2.13]	**3.21 [1.03-10.03]***
SOD3			
Ala40Thr	0.32	1.01 [0.63-1.62]	1.31 [0.63-2.71]
Arg213Gly	0.01	0.33 [0.07-1.52]	-
G(-4466)T	0.04	0.80 [0.36-1.80]	-
SOD2		**BHR in ever smokers (n=280)**	
Ala16Val	0.46	1.62 [0.87-3.03]	1.61 [0.76-3.41]
C5774T	0.19	0.95 [0.54-1.69]	2.97 [0.81-10.98]§
SOD3			
Ala40Thr	0.32	0.67 [0.38-1.21]	0.57 [0.24-1.36]

Table 2 legend:

Logistic regression analysis with OR for COPD adjusted for packyears, and OR for BHR adjusted for packyears and FEV_1% predicted. Significant associations are depicted in bold.

*p<0.05

¥p=0.07

§p=0.10

BHR = bronchial hyperresponsiveness

SNP = single nucleotide polymorphism

MAF = minor allele frequency in the investigated group

OR = odds ratio

CI = confidence interval

COPD = Chronic Obstructive Pulmonary Disease

SOD = Superoxide Dismutase