Published in final edited form as:

COPD. 2010 August ; 7(4): 262–268. doi:10.3109/15412555.2010.496821.

POLYMORPHISMS IN THE SUPEROXIDE DISMUTASE-3 GENE ARE ASSOCIATED WITH EMPHYSEMA IN COPD

IC Sørheim^{1,3}, DL DeMeo^{1,2}, G Washko^{1,2}, A Litonjua^{1,2}, D Sparrow⁴, R Bowler⁵, P Bakke^{3,6}, SG Pillai⁷, HO Coxson^{8,9}, DA Lomas^{10,*}, EK Silverman^{1,2,*}, and CP Hersh^{1,2}

¹ Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

² Pulmonary and Critical Care Division, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

³ University of Bergen, Bergen, Norway

⁴ Veterans Affairs Boston Healthcare System and Boston University School of Medicine, Boston, MA

⁵ National Jewish Health, Denver, CO

⁶ Department of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway

⁷ GlaxoSmithKline, Research Triangle Park, North Carolina

⁸ Division of Respiratory Medicine and the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, University of British Columbia, St. Paul's Hospital, Vancouver, Canada

⁹ Department of Radiology, University of British Columbia and Vancouver General Hospital, Vancouver, Canada

¹⁰ Department of Medicine, University of Cambridge, Cambridge, United Kingdom

Abstract

Superoxide dismutase-3 (SOD3) is a major extracellular antioxidant enzyme, and previous studies have indicated a possible role of this gene in chronic obstructive pulmonary disease (COPD). We hypothesized that polymorphisms in the SOD3 gene would be associated with COPD and COPD-related phenotypes.

Correspondence: Craig P. Hersh, M.D., M.P.H., Channing Laboratory, 181 Longwood Avenue, Boston, MA 02115, Phone: 617-525-0729, Fax: 617-525-0958, craig.hersh@channing.harvard.edu.

*on behalf of the International COPD Genetics Network Investigators

DECLARATION OF INTEREST:

ICS received lecture fees from AstraZeneca in 2008, and lecture fees and a travel grant from GlaxoSmithKline in 2009. DLD has no conflicts of interest to disclose. GW has no conflicts of interest to disclose. AL has no conflicts of interest to disclose. DS has no conflicts of interest to disclose. RB has no conflicts of interest to disclose. PB has received speaking fees from GSK and AstraZeneca, and is a principle investigator in a GSK sponsored study. SGP holds stocks in GlaxoSmithKline and is an employee of Roche Pharmaceuticals. HOC has served on an advisory board for GSK in 2006–2009. In addition HOC is the co-investigator on two multi-center studies sponsored by GSK and has received travel expenses to attend meetings related to the project. HOC has three contract service agreements with GSK to quantify the CT scans in subjects with COPD and a service agreement with Spiration Inc to measure changes in lung volume in subjects with severe emphysema. HOC is the co-investigator (D Sin PI) on a Canadian Institutes of Health – Industry (Wyeth) partnership grant. There is no financial relationship between any industry and the current study. DAL receives grant support and lecture and consultancy fees from GlaxoSmithKline. EKS received an honorarium for a talk on COPD genetics in 2006, grant support for two studies of COPD genetics, and consulting fees from GlaxoSmithKline. He also received honoraria in 2007 and 2008 and consulting fees from AstraZeneca. CPH has no conflicts of interest to disclose.

We genotyped three *SOD3* polymorphisms (rs8192287 (E1), rs8192288 (I1) and rs1799895 (R213G)) in a case-control cohort, with severe COPD cases from the National Emphysema Treatment Trial (NETT, n=389) and smoking controls from the Normative Aging Study (NAS, n=472). We examined whether the SNPs were associated with COPD status, lung function variables, and quantitative CT measurements of emphysema and airway wall thickness. Further, we tried to replicate our initial findings in two family-based studies, the International COPD Genetics Network (ICGN, n=3061) and the Boston Early-Onset COPD Study (EOCOPD, n=949).

In NETT COPD cases, the minor alleles of SNPs E1 and I1 were associated with a higher percentage of emphysema (%LAA950) on chest CT scan ($p=0.029$ and $p=0.0058$). The association with E1 was replicated in the ICGN family study, where the minor allele was associated with more emphysema ($p=0.048$). Airway wall thickness was positively associated with the E1 SNP in ICGN; however, this finding was not confirmed in NETT. Quantitative CT data were not available in EOCOPD. The SNPs were not associated with lung function variables or COPD status in any of the populations.

In conclusion, polymorphisms in the *SOD3* gene were associated with CT emphysema but not COPD susceptibility, highlighting the importance of phenotype definition in COPD genetics studies.

INTRODUCTION

The lungs are exposed to a variety of oxidants that potentially can lead to tissue damage and lung disease. Several antioxidants are present in the lung to counteract the damaging effects from oxidants. Increased oxidative stress in the lung can either result from an increase in oxidants or a decrease in antioxidants (1). Chronic obstructive pulmonary disease (COPD) is largely caused by environmental risk factors like cigarette smoking and occupational exposures, but disease susceptibility also has a substantial genetic component. There is evidence for increased oxidative stress in the lungs of individuals with COPD (2), and genes affecting the oxidant/antioxidant balance are believed to be important determinants of COPD risk.

Some of the most important antioxidants in the lungs are the superoxide dismutase (SOD) enzymes ((3, which catalyze the conversion of superoxide anions to hydrogen peroxide and oxygen. Superoxide dismutase-3 (SOD3) is the extracellular form of the enzyme, and this antioxidant is highly expressed in airways and lung parenchyma (4). The mechanism of SOD3 protection in the lung is not fully understood, but it has been suggested that SOD3 activity may reduce inflammation by inhibiting oxidative fragmentation of lung matrix components (5–7). As these matrix fragments are chemotactic, inhibition of fragmentation may subsequently prevent an inflammatory response.

Several studies have indicated a possible role for *SOD3* as an important candidate gene in COPD. The *SOD3* gene is a small (5.4 kB) gene consisting of 3 exons and 2 introns, located on chromosome 4p (4,8). R213G (rs1799895) is a functional single nucleotide polymorphism (SNP) causing a substitution of Arginine for Glycine; heterozygotes for the minor allele have substantially increased plasma SOD3 levels (approximately 10-fold), while homozygotes for the minor allele have dramatically increased plasma SOD3 levels (approximately 40-fold) (9, 10). Several studies have suggested that R213G may protect against the development of COPD in smokers (10–12). Dahl and colleagues identified two novel polymorphisms in the *SOD3* gene, the SNPs E1 (rs8192287) and I1 (rs8192288), and showed that individuals homozygous for the minor alleles had reduced FVC in two large, population-based studies (8).

We hypothesized that polymorphisms in the *SOD3* gene would be associated with COPD or COPD-related phenotypes. We genotyped the three SNPs E1, I1 and R213G in a well-characterized case-control cohort of ex- or current smokers, and examined whether these

SOD3 polymorphisms were associated with COPD status, lung function variables, and quantitative CT measurements of emphysema and airway wall thickness. Further, we tried to replicate our initial findings in two family-based studies. To our knowledge, this is the first study to investigate the relationship between *SOD3* and quantitative CT measurements.

MATERIAL AND METHODS

Study populations

The case-control study included severe COPD cases from the National Emphysema Treatment Trial (NETT) and smoking control subjects from the Normative Aging Study (NAS). NETT is a multicenter clinical trial where COPD subjects with emphysema and severe airflow obstruction were randomized to either lung volume reduction surgery or conventional treatment.(13) Participants were required to have $FEV_1 \leq 45\%$ predicted and emphysema on chest CT scan at the time of inclusion. For this analysis, we included 389 non-Hispanic white subjects from the NETT Genetics Ancillary Study. All subjects were ex-smokers.

The NAS is a longitudinal study of aging in healthy men, performed by the Veterans Administration in Greater Boston. 472 white male participants were included as control subjects in this analysis. Inclusion criteria were at least 10 pack-years of smoking and no airflow obstruction at the most recent visit (FEV_1/FVC ratio $>90\%$ predicted and $FEV_1 >80\%$ predicted). Chest CT scans were not available for the control subjects.

The family-based International COPD Genetics Network (ICGN) study included participants from ten study centers in Europe and North America ((15,14. COPD probands were 45–65 years old, with a smoking history of ≥ 5 pack-years, and had at least one sibling who had smoked ≥ 5 pack-years. Of the 1946 relatives included in this paper, 54 were parents and 1892 were siblings of their respective probands. Probands were required to have post-bronchodilator $FEV_1 < 60\%$ predicted and $FEV_1/VC < 90\%$ predicted, whereas relatives were included irrespective of lung function.

The Boston Early-Onset COPD Study (EOCOPD) has been previously described (16,17). This study recruited probands with severe early-onset COPD. All probands were < 53 years of age, had $FEV_1 < 40\%$ predicted and no severe alpha 1-antitrypsin deficiency. Members of the extended pedigrees of these probands were also invited to participate.

Written informed consent was obtained from all participants, and the studies were approved by the respective Institutional Review Boards.

Chest CT Scans

High-resolution chest computed tomography (CT) scans were performed in NETT cases and in a subset of the ICGN family study. The CT protocols for NETT (18–20) and ICGN (14) have been described previously. In brief, CT scans in NETT were performed with 2–8 mm slice thickness for the emphysema measurements and 1–2 mm slice thickness for the airway measurements. CT scans in ICGN were performed with 1 mm slice thickness. No quantitative CT measurements were available for the EOCOPD study.

Quantitative assessment of emphysema was performed using density mask analysis with a threshold of -950 Hounsfield units (HU), to determine the percentage of lung voxels with attenuation lower than -950 HU (%LAA950) (21). Airway wall thickness was assessed by plotting the airway lumen perimeter (P_i) against the square root of wall area for airways with a $P_i > 6$ mm, and then using this regression to estimate the square root of the wall area at an internal perimeter of 10 mm (SRWA- P_i10) (22).

SNPs and genotyping

We genotyped the three *SOD3* SNPs E1 (rs8192287), I1 (rs8192288) and R213G (rs1799895) in NETT-NAS and EOCOPD. These SNPs were chosen due to significant associations with COPD-related phenotypes in previous studies (8,10,12). E1 is located in the non-coding 5' untranslated region of exon 1, and I1 is located in the first intron of the *SOD3* gene. R213G is a functional coding variant in exon 3. In ICGN, only one SNP (E1) was genotyped - due to high linkage disequilibrium between E1 and I1 and lack of significant associations with R213G in NETT-NAS. Genotyping was performed using Taqman assays (Applied Biosystems, Foster City, CA).

Statistical analysis

In the case-control study, genotype-phenotype associations were tested with the Cochran-Armitage test for trend for the binary outcome of COPD status and with linear regression models for the quantitative outcomes, using SAS 9.1 (SAS Institute, Cary, NC). In the family-based studies, analyses of all genotype-phenotype associations were performed using the extended pedigree family-based association test, implemented in PBAT software ((23).

In all three populations, quantitative traits were analyzed under additive genetic models, adjusting for relevant covariates. Lung function variables were adjusted for sex, age, pack-years and height (and ever-smoking in EOCOPD). CT measurements were adjusted for sex, age, pack-years and weight. In addition, clinical centers were added as covariates in separate models when analyzing CT measurements. The analyses of COPD status did not include any covariates.

RESULTS

The characteristics of the NETT-NAS case-control study are shown in Table 1A. All control subjects were male, and there was a male predominance among cases (64.0 %). Control subjects were somewhat older than the cases, whereas the cases had a significantly higher smoking exposure than controls (66.4 vs. 40.3 pack-years, $p<.0001$). The NETT cases had severe lung function reduction, with mean FEV₁ being 28 % predicted.

The characteristics of the participants in the ICGN and EOCOPD family studies are shown in Tables 1B and 1C. Statistical comparisons between probands and relatives were not performed due to relatedness. There were more male subjects among probands as compared to relatives in ICGN, whereas the probands in EOCOPD were predominantly female. Probands had severe airflow obstruction both in ICGN (FEV₁ 36.1 % predicted) and in EOCOPD (FEV₁ 21.9 % predicted).

The *SOD3* SNP allele frequencies in all study participants are shown in Table 2. E1 and I1 had allele frequencies between 4.8 and 6.6 % in our study populations. The coding variant R213G is a rare SNP, with minor allele frequencies varying between 0.7 and 1.5 % in this study. All SNPs were in Hardy-Weinberg equilibrium in NAS controls and in founders from the two family-based studies. E1 and I1 were in tight linkage disequilibrium in NETT-NAS ($r^2=0.96$) and in EOCOPD ($r^2=1.0$). Only E1 was genotyped in ICGN.

In NETT cases, the minor alleles of SNPs E1 and I1 were associated with a higher percentage of emphysema (%LAA950) on chest CT scan ($p=0.029$ and $p=0.0058$, respectively) (Table 3). When adjusting for clinical center in addition to the other covariates, the results were similar ($p=0.0095$ and $p=0.0029$ respectively). The association with E1 was replicated in the ICGN family study, where the minor allele of E1 was associated with increased emphysema ($p=0.048$). However, when adding clinical center as a covariate to the model, the association was no longer significant in ICGN ($p=0.17$). Airway wall thickness was positively associated

with E1 in ICGN (Table 3), however this finding was not confirmed in NETT. Quantitative CT data were not available in EOCOPD.

COPD case-control status as a binary outcome showed no significant associations with any of the *SOD3* SNPs in any of the three populations. Similarly, lung function variables FEV₁, FVC and FEV₁/FVC ratio were not significantly associated with any of the SNPs in this study.

DISCUSSION

We examined whether polymorphisms in the *SOD3* gene were associated with COPD susceptibility and the specific COPD-related phenotypes of lung function and quantitative CT measurements in a well-characterized case-control cohort, and we tried to replicate our findings in two large family-based studies. We were not able to replicate previously published associations with COPD status and lung function. However, we found that polymorphisms in the *SOD3* gene were associated with CT emphysema in two independent populations, suggesting that *SOD3* may particularly be important to the emphysema subtype of COPD. To our knowledge, this is the first study to assess the associations between *SOD3* and quantitative CT measurements. Our results also demonstrate the relevance of accurate phenotyping, for instance using quantitative CT analysis, in genetic studies of heterogeneous lung disorders such as COPD.

Our findings add to and expand the current knowledge on *SOD3* and its role in COPD susceptibility. Several previous studies have identified associations between variations in *SOD3* and COPD-related phenotypes. The most studied *SOD3* SNP in COPD is the rare functional variant R213G (9). This SNP has a marked impact on plasma levels of *SOD3*, and Juul et al showed that heterozygous carriers of R213G had reduced risk of COPD in the Danish general population (10). The protective effect was demonstrated in smokers, but not in non-smokers. Another study observed that the R213G polymorphism was more common among smokers who did not develop COPD, so-called “resistant smokers”, also suggesting that this variant may have a protective effect against COPD development (12). Recently, a Dutch study demonstrated a slower decline in FEV₁ in R213G carriers, but only among never-smokers (11). Dahl and colleagues identified the two novel polymorphisms E1 and I1, and showed that homozygous individuals had reduced FVC % predicted in two studies from the general population in Denmark (8). In the Dutch study, a trend for lower VC among individuals homozygous for I1 was found (11).

COPD is a heterogeneous disease, with several disease processes contributing to the clinical manifestations of the disease. It has long been observed that there are clinical subtypes of COPD, such as airway- or emphysema-predominant disease. Quantitative measurements of emphysema severity and distribution on chest CT scan, as well as determinations of airway wall thickness, have made better phenotyping of COPD subjects possible. Our results demonstrate that using more specific and well-defined phenotypes in COPD studies can improve our ability to detect genetic associations, and possibly also suggest which disease process the genes are most likely to affect. We found that polymorphisms in the *SOD3* gene were determinants of emphysema severity in COPD, but they were not significantly associated with lung function variables or COPD status. Our results suggest that *SOD3* may be important in the emphysema subtype of COPD.

The CT protocols for NETT and ICGN were not uniform, and the difference in slice thickness limits our ability to compare the %LAA950 values between the two studies. We did not directly compare %LAA950 values across studies in any analysis, but this methodological difference should be noted when interpreting characteristics of the study subjects. Even though ICGN relatives have a higher %LAA950 value than NETT cases, it is unlikely that they have more

severe emphysema. Rather, this is a consequence of different CT protocols being used in the two studies.

The limitations of our study should be acknowledged. First of all, the low minor allele frequencies gave us limited power to detect associations, especially for the rare coding variant. Also, the NETT cases are a highly selected group of COPD subjects, with a narrow range of lung function. This may have contributed to the lack of replication of previous associations with lung function variables in the NETT study. The replication of the association between E1 and emphysema in ICGN was not robust to adjustment for clinical center. Although there was a non-significant trend in the same direction, we cannot fully exclude the possibility that clinical site differences may have biased our results. The functional relevance of E1 and I1 is unclear. These SNPs may be functionally relevant, or they may be in LD with other functional variants. By testing only 3 SNPs, we are not capturing the total genetic variation in the *SOD3* gene, and other polymorphisms that we did not genotype may influence COPD-related phenotypes.

In summary, we demonstrated an association between polymorphisms in the *SOD3* gene and emphysema severity on chest CT scan in two independent populations. However, we were not able to replicate previous prior associations between *SOD3* variants and COPD susceptibility or lung function. Our findings suggest a role for *SOD3* in the emphysema subtype of COPD, and also emphasize the importance of precise phenotyping in clinical COPD studies. Our study shows that well-defined phenotypes like quantitative CT measurements may enable us to detect genetic associations that otherwise may have gone unnoticed.

Acknowledgments

Sources of financial support:

This work was supported by the National Institutes of Health [K08HL080242, R01HL094635, R01HL075478, R01HL084323, P01HL083069] and a grant from the Alpha-1 Foundation.

The ICGN study was supported by GlaxoSmithKline.

The National Emphysema Treatment Trial was supported by the National Heart, Lung, and Blood Institute [N01HR76101, N01HR76102, N01HR76103, N01HR76104, N01HR76105, N01HR76106, N01HR76107, N01HR76108, N01HR76109, N01HR76110, N01HR76111, N01HR76112, N01HR76113, N01HR76114, N01HR76115, N01HR76116, N01HR76118, N01HR76119], the Centers for Medicare and Medicaid Services, and the Agency for Healthcare Research and Quality.

The Normative Aging Study is supported by the Cooperative Studies Program/Epidemiology Research and Information Center of the US Department of Veterans Affairs and is a component of the Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC), Boston, MA.

The authors thank Scott Weiss, Frank Speizer, Jeffrey Drazen, Hal Chapman, Leo Ginns and Steven Mentzer for their roles in developing the Boston Early-Onset COPD Study.

Co-investigators in the NETT Genetics Ancillary Study include Joshua Benditt, Gerard Criner, Malcolm DeCamp, Philip Diaz, Mark Ginsburg, Larry Kaiser, Marcia Katz, Mark Krasna, Neil MacIntyre, Barry Make, Rob McKenna, Fernando Martinez, Zab Mosenifar, Andrew Ries, Paul Scanlon, Frank Sciurba, and James Utz. International COPD Genetics Network (ICGN) Investigators (in addition to Drs. Lomas and Silverman): AlvarAgusti, Son Dureta Hospital and Fundación Caubet-Cimera, Palma de Mallorca, Spain; Peter M. A. Calverley, University of Liverpool, Liverpool, UK; Claudio F. Donner, Division of Pulmonary Disease, S. Maugeri Foundation, Veruno (NO), Italy; Robert D. Levy, University of British Columbia, Vancouver, Canada; Barry J. Make, National Jewish Medical and Research Centre, Denver, Colorado; Peter D. Paré, University of British Columbia, Vancouver, Canada; Stephen I. Rennard, University of Nebraska, Omaha, Nebraska; Jørgen Vestbo, Department of Cardiology and Respiratory Medicine, Hvidovre Hospital, Copenhagen, Denmark; Emiel F. M. Wouters, University Hospital Maastricht, The Netherlands.

LIST OF ABBREVIATIONS

COPD	chronic obstructive pulmonary disease

COPD. Author manuscript; available in PMC 2011 August 1.

CT	computed tomography
EOCOPD	Boston Early-Onset COPD Study
FEV ₁	forced expiratory volume in 1 second
FVC	forced vital capacity
HU	Hounsfield units
ICGN	The International COPD Genetics Network Study
%LAA950	percentage low attenuation area below −950 Hounsfield units
NETT	National Emphysema Treatment Trial
NAS	Normative Aging Study
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SOD3	superoxide dismutase-3
SRWA-Pi10	square root of wall area of an airway with internal perimeter 10 mm
VC	vital capacity

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TABLE 1
Characteristics of the study subjects

Unless otherwise noted, values are shown as mean (SD). P-values not shown for ICGN and EOCOPD due to relatedness between groups.

A. National Emphysema Treatment Trial (NETT) – Normative Aging Study (NAS) case control study			
	NETT cases (n=389)	NAS controls (n=472)	p
Male, n (%)	249 (64.0)	472 (100)	<.0001
Age	67.4 (5.8)	69.8 (7.5)	<.0001
Pack-years	66.4 (30.4)	40.3 (27.6)	<.0001
FEV ₁ % predicted	28.0 (7.4)	100.0 (13.1) *	<.0001
FEV ₁ /FVC ratio	0.32 (0.06)	0.79 (0.05) *	<.0001
%LAA950	17.0 (10.9)	-	-
SRWA-Pi10, cm	0.46 (0.05)	-	-

B. The International COPD Genetics Network (ICGN)		
	Probands (n=1115)	Relatives (n=1946)
Male, n (%)	670 (60.1)	1009 (51.9)
Age	58.3 (5.4)	57.8 (9.4)
Pack-years	52.3 (29.2)	39.0 (25.0)
FEV ₁ % predicted	36.1 (12.9)	83.2 (26.1)
FEV ₁ /FVC ratio	0.37 (0.12)	0.64 (0.1)
%LAA950 **	26.3 (14.8)	18.7 (11.6)
SRWA-Pi10, cm **	0.49 (0.04)	0.48 (0.04)

C. The Boston Early-Onset COPD Study (EOCOPD)		
	Probands (n=127)	Family members (n=822)
Male, n (%)	32 (25.2)	364 (44.3)
Age	48.1 (4.7)	46.2 (18.8)
Ever-smokers, n (%)	124 (97.6)	526 (64.0)
Pack-years	38.9 (21.9)	19.0 (25.0)
FEV ₁ % predicted	21.9 (8.4)	87.2 (20.3)
FEV ₁ /FVC ratio	0.31 (0.10)	0.73 (0.13)

* Pre-bronchodilator

** Quantitative emphysema data were available for 402 probands and 569 relatives, and data on airway wall thickness were available for 339 probands and 480 relatives

Abbreviations:

%LAA950, % low attenuation areas < -950 Hounsfield units; SD, standard deviation; SRWA-Pi10, square root of wall area at internal perimeter of 10mm

TABLE 2

Allele frequencies (%) in the study populations

	E1 (rs8192287)		I1 (rs8192288)		R213G (rs1799895)	
	G	T	G	T	C	G
NETT-NAS						
Cases	94.0	6.0	94.2	5.8	98.8	1.2
Controls	94.4	5.6	94.6	5.5	98.5	1.5
ICGN						
Probands	93.4	6.6	-	-	-	-
Relatives	94.0	6.0	-	-	-	-
EOCOPD						
Probands	95.2	4.8	95.2	4.8	99.2	0.8
Relatives	94.6	5.4	94.7	5.4	99.3	0.7

Abbreviations:
EOCOPD, The Boston Early-Onset COPD Study; ICGN, The International COPD Genetics Network; NETT-NAS, National Emphysema Treatment Trial – Normative Aging Study

TABLE 3

Genotype-phenotype associations for CT measurements

Associations are shown for the National Emphysema Treatment Trial (NETT) and International COPD Genetics Network (ICGN) - with and without adjustment for clinical center.

	E1 (rs8192287)		I1 (rs8192288)		R213G (rs1799895)	
	NETT		NETT		NETT	
	β (SE)	p	Informative families	**p	β (SE)	p
%LAA950						
- adj. for center	3.6 (1.6)	0.029	90	0.048	4.9 (1.8)	0.0058
+adj. for center	3.4 (1.3)	0.0095	90	0.17	4.2 (1.4)	0.0029
SRWA-P10						
- adj. for center	0.05 (0.08)	ns	87	0.025	0.08 (0.09)	ns
+adj. for center	0.09 (0.07)	ns	87	0.016	0.12 (0.08)	ns

* only E1 was genotyped in ICGN

** p-values based on PBAT (additive model)

Abbreviations:

%LAA950, % low attenuation areas < -950 Hounsfield units; ns, not significant; SE, standard error; SRWA-P10, square root of wall area at internal perimeter of 10mm