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Candidate Genes for Respiratory Disease Associated with Markers of Inflammation and Endothelial Dysfunction in Elderly Men

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Abstract

Background—Inflammation and endothelial dysfunction are important risk factors for cardiovascular disease (CVD). We hypothesized that candidate genes selected for a study of asthma and chronic obstructive pulmonary disorder (COPD) are associated with markers of systemic inflammation and endothelial dysfunction in an aging population.

Methods—Plasma levels of circulating C-reactive protein (CRP), fibrinogen, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were obtained from 679 elderly male participants in the Normative Aging Study. Blood samples were analyzed for 202 SNPs in 25 candidate genes and included both haplotype tagSNPs and functional SNPs based on literature review. Data were stratified into discovery and replication cohorts for 2-stage analysis. In the discovery cohort, the relationship between biomarker level and genotype was analyzed using linear mixed effects with random intercepts for each subject and models were adjusted for age and BMI. A positive outcome in the discovery cohort was defined as a p-value <0.1 for the SNP. SNPs that met this criterion were analyzed in the replication cohort and confirmed for those which met a criterion of significance ($p<0.025$).

Results—In our analyses, SNPs in the *CRHR1, ITPR2*, and *VDR* genes met criteria of significant effects.

Conclusions—Our results suggest that genes thought to play a role in the pathogenesis of asthma and COPD may influence levels of serum markers of inflammation and endothelial dysfunction via novel SNP associations which have not previously been associated with cardiovascular disease.

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Disclosures

There are no competing financial interests that might affect the conduct or reporting of this research.

Keywords

biomarkers; cardiovascular disease; SNPs; inflammation; endothelial dysfunction

Background

The etiology of cardiovascular disease (CVD) involves a confluence of genetic, environmental and lifestyle components which is not fully understood. Studies have also established a link between genes and levels of biomarkers related to inflammation and endothelial function [1–3], suggesting that polymorphisms in the genetic sequence may alter the functionality of genes and the proteins that they encode. Long-term exposure to even low levels of inflammation triggers a cascade of events promoting lipid dysregulation, atherosclerosis and endothelial dysfunction, which are precursors of CVD [4,5]. Because serum markers of inflammation and endothelial function such as C-reactive protein (CRP), fibrinogen, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) have been closely linked to CVD and established as consistent predictors of cardiovascular morbidity and mortality [6–8], identifying factors which influence levels of these biomarkers is critical to increasing our understanding of the pathogenesis of inflammation.

We investigated the relationship between markers of systemic inflammation and endothelial dysfunction and single nucleotide polymorphisms (SNPs) in order to identify novel candidate genes which may elucidate the etiology and pathogenesis of CVD. SNPs were selected from a study of chronic obstructive pulmonary disease (COPD) and asthma in a population of elderly men. Both asthma and COPD have been reported to be independent risk factors of CVD [9, 10] and are diseases of chronic airway inflammation with systemic effects, though they involve different inflammatory mechanisms and mediators [11]. Because of the overlap in pathways involved with respiratory diseases and CVD, we hypothesized that candidate genes for asthma and COPD may be associated with biomarkers of CVD as well and therefore SNPs from these candidate genes were analyzed in our study.

Methods

Study Population

This study utilizes data from the Normative Aging Study (NAS) details of which have been published previously [12]. Briefly, the NAS is an on-going longitudinal closed-cohort study of aging established by the Veterans Administration in 1963. A total of 2,280 men from the Boston area (21–81 years of age) confirmed to be free of known chronic medical conditions were enrolled and were asked to return for examinations every 3–5 years. Study center visits took place in the morning, after an overnight fast and abstention from smoking. Information on medication use and smoking habits including smoking status [never, current, former] and pack-years smoked was also collected by questionnaire, with responses confirmed by a trained interviewer. Participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of all participating institutions.

Biomarkers

The serum markers of inflammation and endothelial dysfunction measured for this study were CRP, fibrinogen, ICAM-1 and VCAM-1. CRP concentrations were measured using immunoturbidimetric assay on the Hitachi 917 analyzer (Roche Diagnostics—Indianapolis, IN) with reagents and calibrators from Denka Seiken (Niigata, Japan) [13]. Fibrinogen was assayed using the MDA Fibriquick method [14]. ICAM-1 and VCAM-1 were measured with ELISA (R & D Systems, Minneapolis, MN) using the quantitative sandwich enzyme immunoassay method. Sensitivity of the assay for ICAM-1 was 0.35 ng/mL and the day-to-

day variabilities of the assay at concentrations of 64.2, 117, 290 and 453 ng/mL were 10.1, 7.4, 6.0 and 6.1%, respectively. Sensitivity of the assay for VCAM-1 was 2.0 ng/mL and day-to-day variabilities of the assay at concentrations of 9.8, 24.9 and 49.6 ng/mL were 10.2, 8.5 and 8.9%, respectively. Technicians were blinded to genotype status of study participants.

SNP Selection and Genotyping

Candidate genes were chosen based on their known or suspected roles in the etiology of COPD and asthma from previous positional cloning and candidate gene studies. A total of 202 SNPs in 25 genes were identified for our analysis. SNPs were chosen if they were located within genes associated with lung inflammation, protease and anti-protease imbalance, oxidative stress, and extracellular matrix synthesis or destruction. We selected tagSNPs with r^2 >0.80 and minor allele frequency (MAF) > 5% located within 5kb upstream and downstream of the first and last exons of each gene, non-synonymous amino acid changes with MAF >1% as well as known variants associated with asthma and related phenotypes.

Genotyping was performed using Illumina BeadStation 500G (San Diego, CA USA) in the discovery cohort and replicated using either Sequenom MassArray MALDI-TOF mass spectrometer (Sequonom, CA, USA) with semiautomated primer design (SpectroDESIGNER, Sequenom) and implementation of the very short extension method [15] or TaqMan 5' exonuclease assays (Applied Biosystems, CA, USA) with primers from Applied Biosystems (ABI) using radioactive labeled probes detected using ABI Prism 7900 Sequence Detector System.

Population stratification was assessed using 101 unlinked SNPs chosen from the Celera dataset on the SNP Consortium (TSC) website (http://snp.cshl.org/). Identity-by-descent clustering was tested using PLINK [17]. SNPs included in the stratification panel had Illumina scores ≥0.65, indicating a high likelihood of successful genotyping, a minor allele frequency ≥0.25 in Caucasians, and did not map to a gene in SNPper (http://snpper.chip.org/). SNPs fewer than 100kb apart, located on X-chromosome or in regions of COPD linkage (2q, 8p, 12p, 19q) were excluded.

Statistical Analysis

Markers of inflammation and endothelial dysfunction were log-transformed in our analysis in order to improve the normality of the data distribution. In order to address the issue of multiple testing we used a two-stage strategy where the study population was divided into a discovery cohort and a replication cohort. The rationale for this approach is that random chance can create false associations in either cohort, but since it is random there is no reason to expect the same SNPs to have the same false associations in both cohorts. In the discovery dataset we analyzed the data using linear mixed effect models with random intercepts for individual subjects and included terms in each model for an individual SNP and adjusted for age and BMI.

Genotypes (wild-type, heterozygous and homozygous variant) were coded using categorical variables [levels 0,1,2] for testing under an additive model, and as binary variables [0,1] for testing under dominant and recessive models. These 3 models were assessed in order to avoid assumptions about the model of inheritance for each SNP. The entire SNP set was analyzed in the discovery dataset and SNPs associated with an inflammatory markers at the p <0.1 level were selected for replication in the replication dataset. The same linear mixed effects models were fitted in the replication dataset and a SNP association was confirmed if it was observed to be in the same direction as in the discovery dataset with a p-value of <0.025. Beta coefficients were then examined in final models using the full cohort data and these models were adjusted for age, BMI, smoking status (ever, current, former), diagnosis of coronary heart disease and

2 or more drinks per day. In addition, analyses for CRP were adjusted for statin use, which has been associated with lower levels of CRP [18].

The use of confirmation in the replication dataset provides protection against false positives. Because of the correlations in the data among outcomes, repeated measures, and SNPs, the exact false discovery rate (FDR) cannot be computed theoretically. Instead, false positive predictive rates were examined empirically in a simulation study that preserved the correlation structure among SNPs and outcomes present in the data. The genotype data for each subject was randomly permuted, and then merged back into the repeated measures of phenotype data. 10 new simulation datasets were created in which any associations observed in each individual simulation should be random. We then identified all SNPs within each of the 10 discovery simulations which met our criteria of a p-value <.1 and analyzed them in the simulated replication dataset. We defined the probability of observing each of the associations we saw in the true dataset as the probability of observing a greater absolute value t-statistic in the replication arm of the simulation study.

Results

Population Characteristics

679 participants provided at least 1 blood sample during the study period. 499 participants had 2 biomarker marker measurements available. Table 1 describes the baseline characteristics of the discovery dataset and replication dataset. The mean age was 73.9 in the discovery dataset and 74.3 in the replication dataset. The majority of study participants were former smokers (65% in the discovery dataset and 66.8% in the replication dataset) and the mean and distributions for biomarkers, anthropometric data and medical history were similar in both datasets. Table 2 describes the correlations between the 4 biomarkers. The correlations between these four markers ranged from 0.036 to 0.5. Only the correlation between fibrinogen and VCAM-1 was not statistically significant at the p<.05 level.

Under dominant, recessive and additive models, 202 SNPs were genotyped. The 25 genes associated with these SNPs are listed in Table 3. No evidence of population stratification was detected when a total of 101 unlinked SNPs were tested for identity-by-descent clustering using PLINK [17]. 6 associations in 5 SNPs met our significance criteria. The allele frequencies and Hardy-Weinberg Equilibrium p-values are presented in Table 4 and the percent changes for models using full cohort data are in Table 5.

46 associations in 31 SNPs were associated with CRP at the 0.1 level. Of these, 3 associations were replicated in the same direction and with a $p < 0.025$. One intronic SNP in the inositol triphosphate receptor 2 (ITPR2) (rs2122268) was associated with 48.3% higher CRP levels (95% CI: 19.0, 84.9). 2 SNPs in the corticotropin releasing hormone receptor 1 gene (CRHR1) were associated with 16–18% lower CRP (rs7209436 95% CI:−28.12,−3.7, rs110402 95%C: −29.3,−5.0) both under dominant models of inheritance. We also observed 56 associations in 40 SNPs which were associated with fibrinogen levels at p-value <0.1 in the discovery dataset. 3 SNP associations were confirmed in the replication dataset. Two SNPs in an intronic region of ITPR2 (rs16930912 and rs16930911) were associated with 6.3% (95%CI: −11.6,−0.6) and 6.9% (95% CI: −11.3,−1.3) lower fibrinogen levels. An intronic SNP in the VDR gene (rs2239179) was associated with 7.1% (95% CI: 1.0, 13.6) higher fibrinogen levels under a recessive model of inheritance.

In our analysis, 49 associations in 27 SNPS were associated with ICAM-1 levels at p-value <0.1 in the discovery dataset. 2 SNP associations were confirmed in the replication dataset in the *CRHR1* (rs17689824 and rs16904065) and were found to be associated with 4% lower ICAM-1 levels (rs17689824 95% CI: −7.7,−0.2, rs16940665 95% CI: −6.7,−1.1) both under

an additive model of inheritance. 61 associations in 37 SNPS were associated with VCAM-1 levels. 1 SNP within a coding exon in *ITPR2* met criteria in the replication dataset for VCAM-1 (rs2230376) and was associated with 5% lower VCAM-1 levels (95% CI: −8.7, −1.2).

Discussion

We have investigated the association between genetic polymorphisms in candidate genes for respiratory disease and serum markers of systemic inflammation and endothelial dysfunction. A key strength of this analysis is that we have utilized a split sample approach which adjusts for multiple testing within the context of a repeated measures analysis. We also evaluated associations with four outcomes to examine interrelated mechanisms of CVD. CRP is a widely used marker of inflammation and a consistent predictor of CVD and CHD [19]. Fibrinogen, a measure of blood viscosity which moderates coagulation [20] may be important in platelet aggregation characteristic of atherogenesis [21]. ICAM-1 is thought to be associated with non-endothelial cell inflammation, while VCAM-1 is expressed more locally within vascular system. Our results suggest that candidate genes thought to play a role in the pathogenesis of asthma and COPD may also influence levels of serum markers of inflammation and endothelial dysfunction via several novel SNP associations which have not previously been associated with cardiovascular disease. In our analyses, SNPs in the *CRHR1, ITPR2,* and *VDR* genes met criteria for significant inflammatory effects.

The *CRHR1* gene, for which SNPs were associated with significantly lower levels of and ICAM-1 and CRP, is activated by stress within the hypothalamic-pituitary-adrenal (HPA) axis. The rs16940665 SNP which codes for a synonomous change within the coding exon region and the intronic SNP rs17689824 polymorphisms in *CRHR1* were both associated with approximately 4% lower levels of ICAM-1. These SNPs were found to be in linkage disequilibrium (LD) with an r^2 close to 1. We also observed that 2 intronic SNPs in *CRHR1*, rs7209436 and rs110402, were associated with 16–18% lower levels in CRP. *CRHR1* acts in HPA axis disregulation as CRH is released from hypothalamus and upregulates cortisol production, leading to downstream effects including deposition of visceral fat, CVD and atherosclerosis. The consistent directionality we observed across outcomes suggests that *CRHR1* polymorphisms may alter expression of markers of inflammation and endothelial dysfunction. *CRHR1* has been shown to induce inflammation via Th2 response [22] which may be an important mechanism for inducing systemic autoinflammatory effects and this gene is the primary receptor for CRH in the pituitary gland, controlling release of ACTH through a feedback mechanism [23]. Because the *CRHR1* receptors are also expressed on endothelial cells, these receptors may promote endothelial dysfunction via this pathway.

In this analysis, modestly lower levels of approximately 6% were observed for intronic SNPs in *ITPR2* SNPs predictive of fibrinogen (rs16930911) and VCAM-1 (rs2230376). We also observed a significant association between *ITPR2* intronic SNP rs2122268 and 45% higher CRP levels (95% CI: 17.16 – 80.32%). The *ITPR2* gene is central to calcium regulation, an important process for cellular stability and second messenger activity, as well as a key factor in promoting cellular adhesion [24]. In addition, this pathway is also central to the mechanism of T-cell activation as well as subsequent B-cell and cytokine production for inflammation response [25]. As a result, *ITPR2* is an important regulator of vascular function, particularly with regard to smooth muscle and endothelial function through upregulation of endothelial cellular adhesion molecules. It is also essential to the activation of angiotensin II and the renin-angiotensin system.

We found one intronic SNP in *VDR* (rs2239179) which was associated with approximately 7% higher levels of fibrinogen. *VDR* is known to be an important calcium regulator and the hormonally active form of vitamin D exerts its control predominately through transcription of

target genes. It is expressed in monocytes and endothelial cells [26] and therefore may be a critical regulator in diseases of calcium imbalance due to *VDR* interaction with vitamin D, which regulates transcription in immunological function. There has been some suggestion in the literature that *VDR* is associated with CVD outcomes via activation of vascular smooth muscle [27]. Positive associations have been observed between the *BsmI* polymorphism in the *VDR* gene with MI [28] and carotid intimal-medial thickness [29].

In this study, we adopted stringent criteria for replication and report adjusted p-values using a permutation approach, allowing us to preserve the correlation structure within the data. We have adopted this strategy rather than the classic Bonferroni correction method because the latter is not appropriate for testing associations within a complex correlation structure, overlooking some true associations at the expense of identifying a spurious association. Other procedures include the widely used Benjamini and Hochberg FDR approach [30], which utilizes a modified Bonferroni approach to be less conservative. However even these tests generally assume independence or only weak correlation. In this analysis, not only is there correlation between the 4 biomarker outcomes associated with inflammation and endothelial dysfunction in this study, and between the repeated measures of each biomarker within each subject, the density of SNPs selected within candidate genes for this analysis suggests high probability of linkage disequilibrium among SNPs located in close proximity to one another. Furthermore, correlation also exists because we have conducted tests under additive, dominant and recessive models of inheritance. The use of a simulation approach provides a simple yet comprehensive approach to addressing the problem of multiple testing.

Limitations

We recognize that this study was performed on a limited subset of genes and not using genome-wide association techniques, however we believe that the genes chosen for asthma and COPD are plausible candidates for our markers of inflammation and endothelial function. In order to account for the possibility of false positives, we divided the cohort into discovery and replication cohorts. We also calculated adjusted p-values where we defined the probability of observing each of the associations we saw in the true dataset as the probability of observing a greater absolute value t-statistic in the replication arm of the simulation study.

We measured biomarkers in subjects only 1 or 2 times during the study, thus our data may be subject to some measurement error, however this bias is most likely non-differential in nature and would be expected to decrease the likelihood of statistical significance, therefore we may have missed some true associations between SNPs and markers of inflammation. We obtained repeated measures on 499 individuals and used random intercepts to adjust for inter-individual variation. The extent of confounding in this study is likely to be limited because we are examining a genetic exposure of interest, and our approach offers a kind of sensitivity analysis as well; while we only adjusted for age and BMI in the initial stage of our analysis, we examined whether the addition of covariates in the final model significantly changed the associations in our results and we found that this was not the case.

Conclusions

We believe that the pathways of asthma and COPD represent important potential candidate genes for CVD outcomes and have allowed us to identify new candidate genes for future work, extending the scope of our study outside of genes previously targeted. In our analyses we observed novel associations with *ITPR2*, *CRHR1* and *VDR* not previously associated with CVD. By examining four different continuous biomarkers of inflammation and endothelial function, we have greater power to detect associations with SNPs in regions of interest. These results suggest that SNPs in several non-traditional cardiovascular candidate genes may be associated with levels of important inflammatory markers and cardiovascular disease in an

aging population. Comprehensive work involving larger case-control or cohort studies to determine the associations between SNPs in these genes and clinical CVD outcomes merit further investigation.

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Table 1

Characteristics [mean ± SD or *n* (%)] of the study subjects

	Discovery						Replicate					
	#obs[a]	N[b]	Mean	SD	n	(%)	#obs	N	Mean	SD	n	(%)
BMI (kg/m^2)	603	341	28.2	(4)			574	337	27.9	(4)		
AGE (years)	604	342	73.9	(6.7)			574	337	74.3	(7)		
ICAM-1 (ng/mL)$^+$	600	340	293	(1.3)			564	332	301	(1.3)		
VCAM-1 (ng/mL)$^+$	600	340	1004	(1.4)			566	333	1028	(1.4)		
Fibrinogen (mg/dL)$^+$	554	294	329	(1.3)			516	284	330	(1.3)		
CRP (mg/L)$^+$	600	340	1.5	(2.8)			565	332	1.6	(3)		
SBP (mmHg)	600	342	128	(17)			574	337	128	(16)		
DBP (mmHg)	600	342	73	(10)			574	337	73	(10)		
PM2.5 (ug/m^3)	592	342	12.4	(6)			568	337	12.4	(60)		
pack-years	602	341	20	(25)			570	337	20	(23)		
Smoking status		340						337				
never					104	(31)					95	(28.2)
current					15	(4.4)					17	(5)
former					221	(65)					225	(66.8)
Statin use		342						337				
no					208	(61)					225	(66.7)
yes					134	(39)					112	(33.3)
Diabetes diagnosis		337						334				
no					291	(86)					285	(85.3)
yes					46	(14)					49	(14.7)
Hypertension		337						334				
no					94	(28)					99	(29.6)
yes					243	(72)					235	(70.4)
Coronary Heart Disease		342						337				

	Discovery						Replicate					
	#obs[a]	N[b]	Mean	SD	n	(%)	#obs	N	Mean	SD	n	(%)
no					245	(72)					234	(69.4)
yes					97	(28)					103	(30.6)

[a] # obs indicates the total number of observations available for study participants.

[b] N indicates number of study participants who provided data for covariates.

[+] geometric mean (SD)

Table 2
Spearman rank-correlations between log-transformed biomarkers

The biomarkers were not highly correlated. The highest correlation was observed between CRP and fibrinogen with a correlation coefficient of 0.512.

	CRP	Fibrinogen	ICAM-1	VCAM-1
CRP	1			
	0.512[a]			
Fibrinogen		1		
ICAM-1	0.264 [a]	0.106 [a]	1	
VCAM	0.098 [a]	0.036	0.431 [a]	1

[a] indicated p<.05

Table 3

Candidate Genes included in this analysis

25 genes were selected for analysis in association with biomarkers of inflammation and endothelial dysfunction.

Gene	Full name	Position	Gene	Full name	Position
ITGB6	integrin, beta 6	chr2:q24.2	PHF11	PHD finger protein 11	chr13:q14.3
ITGAV	integrin alpha-V precursor	chr2:q32.1	SERPINA3	serpin peptidase inhibitor, clade A, member 3	chr14:q32.13
IL6	interleukin 6 (interferon, beta 2)	chr7:p15.3	SERPINA1	serine (or cysteine) proteinase inhibitor, clade	chr14:q32.13
DEFB1	defensin, beta 1 preproprotein	chr8:p23.1	IL4R	interleukin 4 receptor alpha chain isoform b	chr16:p12.1
TLR4	toll-like receptor 4 precursor	chr9:q33.1	CRHR1	corticotropin releasing hormone receptor 1	chr17:q21.31
MBL2	soluble mannose-binding lectin precursor	chr10:q21.1	AZU1	azurocidin 1 preproprotein	chr19:p13.3
SFTPD	pulmonary surfactant-associated protein D	chr10:q22.3	ELA2	elastase 2, neutrophil preproprotein	chr19:p13.3
GSTP1	glutathione transferase	chr11:q13.2	TGFB1	transforming growth factor, beta 1	chr19:q13.2
MMP1	matrix metalloproteinase 1 preproprotein	chr11:q22.2	IL11	interleukin 11 precursor	chr19:q13.42
MMP12	matrix metalloproteinase 12 preproprotein	chr11:q22.2	ADAM33	ADAM metallopeptidase domain 33 isoform alpha	chr20:p13
ITPR2	inositol 1,4,5-triphosphate receptor, type 2	chr12:p11.23	MMP9	matrix metalloproteinase 9 preproprotein	chr20:q13.12
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor	chr12:q13.11	SLPI	secretory leukocyte peptidase inhibitor	chr20:q13.12
DCN	decorin isoform b precursor	chr12:q21.33			

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Table 4

Allele frequencies for SNPs meeting significance criteria

All SNPs which met criteria in the discovery and replication analysis were in Hardy-Weinberg Equilibrium (HWE).

	rs number	alleles	wild-type	heterozygote	homozygote	N	χ^2	HWE p-val
CRHR1	rs16949665	A/G	376	239	40	655	0.06	0.81
CRHR1	rs17689824	C/T	373	237	39	649	0.03	0.87
ITPR2	rs16930911	C/T	589	67	2	658	0.004	0.95
ITPR2	rs2230376	C/T	298	292	67	657	0.13	0.71
VDR	rs2239179	A/G	195	347	128	670	1.44	0.23

Table 5

Percent changes for SNPs that met significance criteria

Model	rs Number	Gene	% change	95%CI	Adjusted p-value	Role
***CRP*[a,b]**						
dominant	rs2122268	*ITPR2*	48.3	(19.0,84.9)	0.01	intron
dominant	rs7209436	*CRHR1*	−16.8	(−28.12,−3.7)	.004	intron
dominant	rs110402	*CRHR1*	−18.1	(−29.3,−5.0)	<.002	intron
***Fibrinogen*[a]**						
additive	rs16930912	*ITPR2*	−6.3	(−11.6,−0.6)	.02	intron
dominant	rs16930911	*ITPR2*	−6.9	(−11.3,−1.3)	.02	intron
recessive	rs2239179	*VDR*	7.1	(1.0, 13.6)	.006	Intron
***ICAM-1*[a]**						
additive	rs17689824	*CRHR1*	−4.0	(−7.7 , −0.2)	.02	intron
additive	rs16940665	*CRHR1*	−3.9	(−6.7, −1.1)	.015	Coding exon
***VCAM-1*[a]**						
additive	rs2230376	*ITPR2*	−5.0	(−8.7,−1.2)	.016	Coding exon

[a]Models adjusted for age, BMI, CHD, >2 drinks per day and smoking status.

[b]Models also adjusted for statin use.