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Genetic analysis of the soluble epoxide hydrolase gene, *EPHX2*, in subclinical cardiovascular disease in the Diabetes Heart Study

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Conflicts of interest statement

None declared.

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Abstract

Epoxide hydrolase is involved in metabolism of vasoactive and anti-inflammatory epoxyeicosatrienoic acids to their corresponding diols. Consequently, epoxide hydrolase 2 (*EPHX2*) is a candidate cardiovascular disease (CVD) gene. We investigated *EPHX2* for association with subclinical CVD in European American (EA) and African American (AA) families from the Diabetes Heart Study. The R287Q polymorphism was associated with carotid artery calcified plaque (CarCP) in EAs. Other *EPHX2* polymorphisms were associated with coronary artery calcified plaque (CorCP), CarCP or carotid artery intima-media thickness (IMT). Polymorphism rs7837347 was associated with all traits in the AAs (p=0.003, 0.001 and 0.017, respectively). Polymorphism rs7003694 displayed association with IMT (p=0.017) and, along with rs747276, a trend towards association with CorCP in diabetic EAs (p=0.057 and 0.080, respectively). These results provide additional evidence that *EPHX2* contributes to the risk of subclinical CVD, although the true trait defining polymorphisms may not be identified and the effect size could be small.

Keywords

calcified plaque; cardiovascular disease; gene polymorphisms; type 2 diabetes

Introduction

Cardiovascular disease (CVD) is a complex disorder well known to have both environmental and genetic susceptibility components. Many studies have investigated the contribution of candidate genes to the risk of CVD. Recently, a polymorphism in epoxide hydrolase 2 (*EPHX2*), the gene encoding soluble epoxide hydrolase (sEH), was found to be associated with subclinical CVD as measured by coronary artery calcified plaque (CorCP).[1,2] Epoxide hydrolases catalyze degradation of vasoactive epoxyeicosatrienoic acids (EETs), produced from arachidonic acid by cytochrome p450 2J2 (CYP2J2),[3] into their corresponding dihydroxyeicosatrienoic acids (DHETs).[4] EETs and DHETs have been shown to promote vasorelaxation of small arteries,[5,6] and display anti-inflammatory properties through the inhibition of nuclear factor kappa B (NF-κB) activation.[7] These data have led to speculation that variations in the abundance or function of sEH may contribute to the development of pathogenic states in the vasculature through the regulation of these vasoactive and anti-inflammatory compounds. Soluble EH has also been shown to modulate vascular smooth muscle (VSM) cell proliferation,[8] providing another potential avenue for the contribution of this enzyme to CVD. Nevertheless, the precise role of sEH in the pathogenesis of calcified atherosclerotic plaque is unclear.

In order to assess further the contribution of this gene to the risk of CVD, we have assessed several variants of the *EPHX2* gene in a sample of type 2 diabetes mellitus (T2DM) patients from the Diabetes Heart Study (DHS) and their available unaffected siblings. T2DM increases significantly the risk of CVD. Mortality from CVD is 2–5-fold greater in diabetic

versus non-diabetic subjects, with almost 60% of diabetic individuals having CVD complications.[9–12] The majority of participants in this study have detectable CorCP. This enrichment of the study population with subjects having extensive subclinical CVD should provide increased power to detect association of *EPHX2* with CVD.

Methods

Patients and phenotyping

Recruitment and phenotyping of DHS participants have been previously described.[13,14] Briefly, siblings concordant for T2DM were recruited from internal medicine clinics, endocrinology clinics and community advertising. T2DM was defined as a clinical diagnosis of diabetes after the age of 34 years, in the absence of historical evidence of diabetic ketoacidosis, and active treatment at the time of examination. Unaffected siblings, similar in age to the siblings with T2DM, were also invited to participate, as were any additional diabetes-affected siblings. The sample includes European-American (EA) and African-American (AA; approximately 15% of the total) participants.

All protocols were approved by the institutional review board of Wake Forest University School of Medicine and all participants gave their informed consent. Participant examinations were conducted in the General Clinical Research Center of the Wake Forest University Baptist Medical Center. They included interviews for medical history and health behaviours, anthropometric measures, resting blood pressure, fasting blood sampling and spot urine collection. Laboratory assays included urine albumin and creatinine, total cholesterol, high-density lipoprotein cholesterol (HDL), triglycerides, glycosylated haemoglobin (HbA_{1C}), fasting glucose and blood chemistries.

Intima-media thickness (IMT) of the common carotid artery was measured by high-resolution B-mode ultrasonography with a 7.5-MHz transducer and a Biosound Esaote (AU5) ultrasound machine, as previously described.[13] Coronary artery (CorCP) and carotid artery (CarCP) calcified plaque were measured using fast-gated helical computerised tomography (CT) scanners; calcium scores were calculated as described previously.[14,15]

Genetic analysis

Total genomic DNA was purified from whole blood samples obtained from subjects using the PUREGENE® DNA isolation kit (Gentra, Inc., Minneapolis, MN). DNA was quantitated using standardised fluorometric readings on a Hoefer DyNA® Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Each sample was diluted to a final concentration of 5 ng/μL.

Six single nucleotide polymorphisms (SNPs) were chosen from the HapMap database (http://www.hapmap.org)[16] to cover the 63.84 kb genomic region containing *EPHX2* comprehensively, including 5 kb upstream and downstream of the *EPHX2* transcript. Four of the SNPs evaluated in this study had a minor allele frequency (MAF) more than or equal to 0.05 in both the Centre d'Etude du Polymorphisme Humain (CEPH) Utah residents with ancestry from northern and western Europe (CEU population) and the Yoruba population from Ibadan, Nigeria (YRI population). Of the remaining two SNPs, one was polymorphic

in the YRI population but monomorphic in the CEU population (rs7837347); the other was polymorphic in the CEU population and had no data available for the YRI population (rs7003694). The R287Q polymorphism (rs751141) was selected based on an *a priori* hypothesis of association with vascular calcified plaque.[1] The remaining five SNPs were selected in an effort to maintain consistent SNP spacing (average spacing of 1 SNP / 9 kb). SNP rs1042064 in the 3′ untranslated region of the gene failed to genotype and was removed from the analysis.

SNP genotypes were determined using a MassARRAY® SNP genotyping system (Sequenom, Inc., San Diego, CA), as previously described.[17] This genotyping system uses single base extension reactions to create allele-specific products that differ in mass and can be separated and automatically scored in a matrix-assisted laser desorption ionisation-time-of-flight (MALDI-TOF) mass spectrometer. Primers for PCR amplification and extension reactions were designed using the Assay Design software (Sequenom Inc., San Diego, CA).

Statistical analyses

Clinical characteristics were compared between the two ethnic groups using generalised estimating equations (GEE1) as described below. Ethnic-specific allele and genotype frequencies were calculated from unrelated probands and tested for departure from Hardy–Weinberg Equilibrium (HWE) using a chi-squared test. Linkage disequilibrium (LD) was assessed through calculation of D′ and r^2 values. To test for an association between each SNP and each phenotype, a series of GEE1[18] models was evaluated. The correlation between subjects within a family was adjusted for in the analyses by assuming exchangeable correlation among siblings within a pedigree and computing the sandwich estimator of the variance.[19] The sandwich estimator is also denoted the robust or empirical estimator of the variance as it is robust to misspecification of the correlation matrix because it estimates the within-pedigree correlation matrix from the first two moments of the data.[19]

A two degrees of freedom overall test of genotypic association was performed for all SNPs with IMT, CorCP and CarCP. For phenotypes that demonstrated a significant association, the three individual contrasts defined by the *a priori* genetic models (i.e. dominant, additive and recessive) were computed. This is consistent with the Fisher's protected least significant difference multiple comparison procedure. The test of the dominant model compares the phenotypic means of the combined "1/2" and "2/2" genotypic class versus the "1/1" genotypic class (i.e. a difference in mean based upon presence versus absence of allele "2"). The additive model tests for a cumulative effect of allele 2. The test of the recessive model compares the phenotypic mean of the "2/2" genotypic class versus the mean of the combined "1/1" and "1/2" genotypic class (i.e. two copies of allele "2"). Within each trait, a sequential Bonferroni multiple comparison adjustment was computed.[20] This conservative multiple testing adjustment rank-orders the observed p values, divides the *a priori* threshold for statistical significance by the p value rank and declares significance if the observed p value is less than the rank-adjusted threshold for significance. Because this investigation contains a strong *a priori* hypothesis, we report the unadjusted p value and indicate which SNPs retain statistical significance after adjustment by the conservative sequential Bonferroni.

All analyses were conducted incorporating effects of known risk factors (age, gender, diabetes status, smoking status and use of lipid-lowering medication) in the models. IMT, CorCP+1 and CarCP+1 values were natural log-transformed to best approximate the distributional assumptions (e.g. approximate conditional normality, homogeneity of variance) of these tests.

Results

DNA was collected from 982 EA individuals from 367 families and 176 AA individuals from 73 families. Clinical characteristics of participants are summarised in table 1. Obesity, measured through body mass index (BMI), was significantly different between the two ethnic groups (31.8±6.6 kg/m^2 in EA versus 33.8±7.2 kg/m^2 in AA, p=0.0003). Subclinical CVD, defined as the presence of CorCP and/or CarCP, was prevalent in both EA and AA subjects. EA subjects had greater levels of CorCP (1,254± 2,445 versus 727±1,604, p=0.0091) and CarCP (341±695 versus 192±614, p=0.0096) than AA subjects, and had more prevalent CVD. In general, the participants are representative of a diabetes-enriched collection of older, obese subjects with significant CVD burden.

DNA from these participants was genotyped for six SNPs selected to cover the 73.84 kb genomic region containing *EPHX2* completely (table 2). One SNP (rs1042064) failed to genotype using the Sequenom platform, and was therefore excluded from subsequent analysis. The ability of the five remaining SNPs to capture genotypic variation was assessed using the greedy pair-wise tagging algorithm implemented in the Tagger program[21] of Haploview.[16] In the CEU population, the four polymorphic SNPs (rs7003694, rs751141, rs721619 and rs747276) captured a very high proportion of the genetic variation within this region (mean r^2=0.82). In the YRI population, the four polymorphic SNPs (rs7837347, rs751141, rs721619 and rs747276) captured less genetic variation (mean r^2=0.37). No data were available on rs7003694 in the YRI population. In each ethnic group, allele and genotype frequencies were consistent with those expected under HWE. SNP rs7837347 was not variable in the EA subjects and had a minor allele frequency of only 0.06 in the AA group. The extent of LD between SNPs was assessed with the D′ statistic implemented in Haploview.[22] The four polymorphic SNPs in EAs were contained in a single block of high LD (figure 1a) whereas the four polymorphic SNPs in AAs did not conform to any block structure (figure 1b).

Each SNP in *EPHX2* was assessed for association with IMT, CorCP and CarCP in each ethnic group (table 3). Additionally, EAs with diabetes were analysed as a separate subgroup. Due to the small number of AAs available for study, only those with diabetes were included in the analysis in order to maximise the homogeneity of the sample.

SNP rs751141 (coding for R287Q), previously reported to be associated with CorCP in AAs but not EAs,[1] was not associated with CorCP in either ethnic group. There was evidence of association between rs751141 and CarCP (p=0.048) in the EA sample. Although not statistically significant after adjusting for all polymorphisms using the sequential Bonferroni adjustment, this association merits consideration given the biological similarity to CorCP. The geometric mean for CarCP by genotype is shown in table 4, along with the p values for

association under the three *a priori* genetic models. The strongest evidence of association was observed with the additive model, consistent with the stepwise increase in mean CarCP with the addition of each copy of allele 2.

The strongest evidence for association was found with SNP rs7837347. This SNP was significantly associated with all three traits in AAs (table 3) and remained statistically significant even after applying the conservative sequential Bonferroni multiple comparison correction. As previously noted, this polymorphism is uncommon among the current AA sample and is not present in the current EA sample. Thus, these results are interesting but should be viewed as exploratory in nature. The association with IMT appears to be driven by a reduction in IMT among the heterozygous individuals (table 4). The dominant genetic model provides the evidence of statistical association (p=0.05) but the two individuals homozygous for the 2 (T) allele have the largest standard deviation. The association between rs7837347 and CorCP is most consistent with either a dominant (p=0.008) or additive (p=0.002) genetic model as demonstrated by an increasing mean as a function of the number of copies of allele 2. In contrast, the association between rs7837347 and CarCP appear to be most consistent with a recessive genetic model. However, this result is suspect given that only three individuals were homozygous for the 2 allele.

SNP rs7003694 is also associated with IMT, particularly in the EA diabetic subset, although the strongest evidence comes from a recessive model and is highly influenced by only five homozygotes (table 4). This SNP, as well as rs747276, displays suggestive evidence of association with CorCP in the diabetic EA group (p=0.057 and 0.080, respectively).

Discussion

While the evidence for association of the *EPHX2* gene with any of the three subclinical CVD measures is limited, the results of this study are consistent with a role for the *EPHX2* gene in CVD risk, when interpreted in context with the previous reports of association.[1,2,23,24]

Fornage *et al.*[1] and Wei *et al.*[2] investigated the contribution of the *EPHX2* R287Q variant and haplotypes containing this variant to CorCP in the CARDIA cohort of young, largely asymptomatic adults. They were able to include a larger sample than the current study but only 11% of EAs and 7% of AAs had detectable CorCP, which translates to approximately 172 and 89 individuals, respectively. The risk factor profile of the current study is quite different from that of the CARDIA cohort in that the DHS population consists of older, obese, diabetic subjects with a significant CVD burden. Approximately 76% of our sample had detectable CorCP, namely 737 EA and 147 AA individuals, providing substantially increased power to detect an association with this trait, particularly in the EA samples.

Fornage *et al.*[1] were unable to detect association in the Caucasian component of the CARDIA study using single SNP analysis, although a variant in the gene was associated with CorCP in AAs. The DHS[15] and other studies[25,26] have indicated ethnic differences in arterial calcification. Fornage *et al.*[1] recognised that the *EPHX2* gene may account for some of this difference, but also pointed out that other factors whose prevalence differs between

races may impact their observed association and contribute to the observed ethnic differences. Our study of *EPHX2* suggests that variation within the gene does not account for ethnic differences in arterial calcification. A small effect was detected in the EA individuals with associations of both IMT and calcified plaque in the current study. This difference may be due to the improved power of the current study, or to the specific SNPs selected. As with the CARDIA study, we failed to detect any association of the R287Q polymorphism (rs751141) in EAs. However, we also failed to detect an effect of this SNP in AAs, suggesting that any effect this gene is having in the CARDIA cohort is not necessarily mediated through this variant.

Since the initial report of Fornage *et al.*,[1] more detailed investigations of the association of sequence variation in *EPHX2* with cardiovascular risk have been conducted. In the CARDIA cohort, a haplotype tagged by the R287Q polymorphism and a different haplotype tagged by an intron 11 SNP (rs721619) were associated with increased risk for CorCP in AAs and Caucasians, respectively.[2] Each of these polymorphisms was also significantly associated with CorCP in the single SNP analyses. The Atherosclerosis Risk in Communities (ARIC) study evaluated *EPHX2* polymorphisms for association with ischaemic stroke[24] and coronary heart disease (CHD) risk.[23] Haplotypes associated with risk for ischaemic stroke were identified in both AAs and Caucasians.[24] Interestingly, the haplotype spanning the intron 11 region that was associated with increased CorCP in the Caucasians of the CARDIA cohort[2] was also associated with lower ischaemic stroke risk in Caucasians from the ARIC study.[24] The ARIC study also identified the K55R variant, and haplotypes containing this variant, as being associated with increased risk for CHD in Caucasians; the R287Q polymorphism was not significantly associated with CHD in either the AAs or the Caucasians.[23] In the present study, we have evaluated both the R287Q polymorphism and the intron 11 polymorphism (rs721619) for association with subclinical measures of CVD; however, neither was associated with CorCP, CarCP or carotid IMT in either the EA or AA ethnic groups.

Firm conclusions about association are not possible due to the low allele frequency for most of the SNPs. This increases the likelihood of observing a false positive association, particularly under the recessive model. If there is an association between these polymorphisms and CVD measures, the effect size is likely to be small. Thus, much larger sample sizes will be required to delineate the putative relationships fully. It should be noted that the statistical power in the AA sample of the current study (n=176 individuals) is modest and therefore, results in the AA sample should be interpreted with caution. Specifically, under an additive genetic model and for the minor allele frequencies in table 2, the AA sample has 0.70 and 0.80 power to detect a third to a half of a standard deviation of the respective vascular calcium measures reported in table 1. Additional studies may also benefit from complete resequencing of the gene, including intronic regions, in order to identify additional genetic variation, allowing selection of more informative SNPs and a more thorough description of the LD pattern.

This study consists of an evaluation of several *EPHX2* SNPs with three measures of subclinical atherosclerosis and, therefore, includes multiple statistical comparisons. This study was undertaken with an *a priori* hypothesis for the association of *EPHX2* SNPs with

the reported phenotypes, based on previous reports from the CARDIA[1,2] and ARIC[23,24] studies, and should therefore be considered as a confirmation study for the R287Q polymorphism. It should be acknowledged that there is no generally accepted method of application of corrections in studies of this kind where the SNPs are in LD with each other and the traits are also correlated. We do not believe the genomic structure of the region or the interdependence among the traits supports a highly conservative approach such as a Bonferroni correction for multiple comparisons. However, even if we make a conservative sequential Bonferroni adjustment across all traits and polymorphisms, the intron 2 SNP rs7837347 remains significantly associated with CarCP and CorCP.

In summary, the Diabetes Heart Study adds to the evidence that the *EPHX2* gene is involved in CVD risk. However, studies in larger at-risk cohorts incorporating complete resequencing of the gene are required to confirm the association of both single SNPs and haplotypes.

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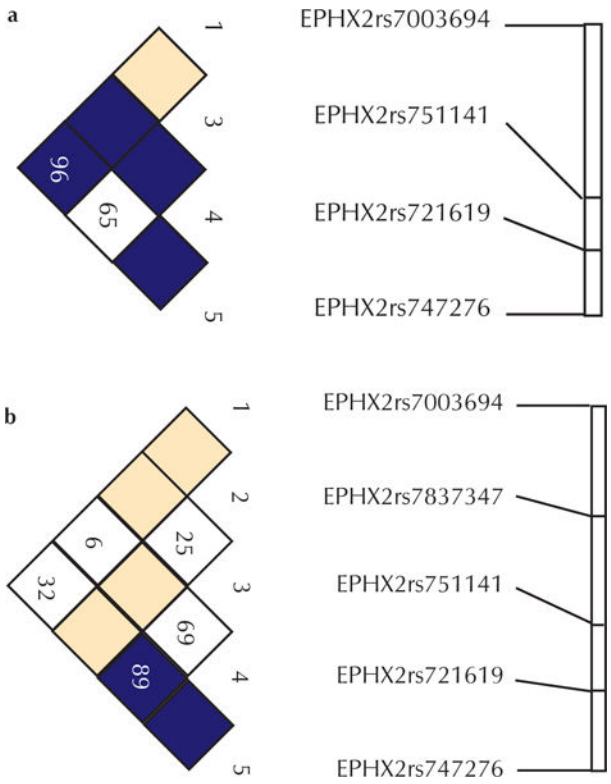

Figure 1.
LD between the *EPHX2* polymorphic SNPs genotyped in (a) European-Americans and (b) African-Americans

Table 1

Clinical characteristics of all participants by ethnicity. Mean ± standard deviation or % (n)

	European-American	African-American
N	982	176
Age (years)	61.6.±9.4	58.8+9.2
Female (%)	53.5 (525)	67.1 (118)
Diabetes diagnosis (%)	83.6 (821)	89.8 (158)
Diabetes duration (years)	10.3±7.0	10.7±7.9
BMI (kg/m^2)	31.8±6.6	33.8±7.2
Lipids		
Cholesterol (mg/dL) *	188±42	191±37
HDL (mg/dL) *	43±13	50±15
LDL (mg/dL) *	106±32	114±31
Smoking (%) current or past	59.1 (578)	61.5 (107)
Lipid-lowering medication (%)	42.7 (415)	33.1 (59)
Prevalent CVD (%)		
Myocardial infarction	19.7 (191)	8.6 (15)
Angina	18.2 (166)	16.3 (26)
Stroke	9.5 (92)	6.4 (11)
Coronary Artery Bypass Graft	14.1 (138)	5.7 (10)
Angioplasty	15.2 (148)	7.4 (13)
Endarterectomy	2.2 (21)	0.6 (1)
Vascular imaging		
CorCP>0	92.9 (737)	91.9 (147)
Untransformed CorCP score	1,254±2,445 (793)	727±1604 (160)
Log CorCP [1]	5.2±2.6 (793)	4.4±2.5 (160)
Untransformed CarCP score	341±695 (897)	192±614 (168)
Log CarCP [2]	3.6±2.7 (897)	2.6±2.5 (168)
Untransformed IMT (mm)	0.67±0.13 (952)	0.69±0.13 (163)
Log IMT [3]	0.51±0.08 (952)	0.52±0.08 (163)

Key: BMI = body mass index; HDL = high-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol; CVD = cardiovascular disease; CorCP = coronary artery calcified plaque; CarCP = carotid artery calcified plaque; IMT = intima-media thickness;

* to convert mg/dL to mmol/L. multiply by 0.0259;

[1] EA skewness=−0.290; EA kurtosis=−0.106; AA skewness=−0.032; AA kurtosis=−0.351;

[2] EA skewness=−0.216; EA kurtosis=−0.692; AA skewness=0.232; AA kurtosis=−0.787;

[3] EA skewness=1.038; EA kurtosis=2.346; AA skewness=0.961; AA kurtosis=1.410

Table 2

SNPs genotyped in *EPHX2*. The frequency of allele 2 and the number of observations of each genotype in unrelated probands by ethnicity is shown

			European-Americans				African-Americans			
SNP	**Position in gene**	**Alleles 1/2**	**Frequency**	**1/1**	**1/2**	**2/2**	**Frequency**	**1/1**	**1/2**	**2/2**
rs7003694	Promoter	T/C	0.092	277	58	2	0.174	44	22	0
rs7837347	Intron 2	C/T	0.000	–	–	–	0.063	63	8	1
rs751141	R287Q	G/A	0.101	282	61	6	0.146	49	22	1
rs721619	Intron 11	C/G	0.320	165	145	41	0.102	53	16	1
rs747276	Intron 12	G/C	0.139	259	81	9	0.667	14	29	26

Key: SNP = single nucleotide polymorphism

Table 3

P values* for association of *EPHX2* SNPs with measures of subclinical atherosclerosis

	IMT			CorCP			CarCP		
SNP	**EA**	**dEA**	**AA**	**EA**	**dEA**	**AA**	**EA**	**dEA**	**AA**
rs7003694	0.058	0.017	0.560	0.266	0.057	0.374	0.791	0.700	0.569
rs7837347	–	–	0.017	–	–	0.003	–	–	0.001
rs751141	0.446	0.448	0.579	0.188	0.757	0.194	0.048	0.077	0.184
rs721619	0.331	0.193	0.850	0.284	0.348	0.648	0.932	0.610	0.498
rs747276	0.312	0.261	0.559	0.244	0.080	0.722	0.209	0.342	0.246

Key: EA = European-American; dEA = diabetic European-American; AA = African-American; SNP = single nucleotide polymorphism; IMT = intima-media thickness; CorCP = coronary artery calcified plaque; CarCP = carotid artery calcified plaque;

*based on 2 degrees-of-freedom test

Table 4

Untransformed mean trait values by genotype and model-specific p values for association

		Mean ± Standard Deviation (N)*			P value		
SNP (Sample)	**Trait**	**1/1**	**1/2**	**2/2**	**Dominant**	**Additive**	**Recessive**
rs7003694 (dEA)	IMT	0.68±0.13 (608)	0.68±0.15 (126)	0.60±0.04 (5)	0.874	0.654	0.004
rs7837347 (AA)	IMT	0.70±0.13 (139)	0.63±0.11 (17)	0.75±0.15 (3)	0.050	0.277	0.683
	CorCP	659±1612 (137)	698±1235 (15)	1021±622 (4)	0.008	0.002	0.002
	CarCP	159±501 (143)	188±483 (17)	313±337 (4)	0.819	0.419	0.0002
rs751141 (EA)	CarCP	324±648 (688)	426±882 (156)	486±749 (14)	0.083	0.042	0.046

*Numbers reported reflect the data from individuals with both genotype and phenotype information available.

Key: dEA = diabetic European-Americans; AA = African-Americans; IMT = intima-media thickness; CorCP = coronary artery calcified plaque; CarCP = carotid artery calcified plaque; SNP = single nucleotide polymorphism