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Carotid plaque and candidate genes related to inflammation and endothelial function in Hispanics from northern Manhattan

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Abstract

BACKGROUND AND PURPOSE—The genetic influence on carotid atherosclerotic plaque is mostly unknown. This study examines the association between carotid plaque and single nucleotide polymorphisms (SNPs) in selected genes implicated in inflammation and endothelial function.

METHODS—A total of 43 genes (197 SNPs) involved in inflammation and endothelial function were interrogated in 287 Dominicans from the Northern Manhattan Study (mean age 64±7 years, 58% women) who had undergone high-resolution B-mode ultrasound for examination of carotid plaque. Using an additive genetic model, multiple logistic regression analyses were conducted, a within gene haplotype analysis was performed and interactions between genes were examined. Results were validated in an independent set of 301 Dominicans.

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RESULTS—Carotid plaque was present in 143(47%) participants. Nine genes had at least one SNP associated (p≤0.01) with carotid plaque phenotypes: TNF, NOS2A, IL6R, TNFSF4, PPARA, IL1A, TLR4, ITGA2, HABP2. SNPs in TNFSF4, PPARA, TLR4, ITGA2, and *HABP2* were also implicated with the same carotid phenotype in the validation analysis. Haplotype analysis revealed an additional gene of interest, *VCAM1*.

CONCLUSIONS—We report novel associations between variations in ten genes involved in inflammation and endothelial function and carotid plaque phenotypes in a Dominican sample, with replication for five genes in an independent Dominican sample.

Keywords

candidate gene; single nucleotide polymorphisms; carotid plaque; inflammation; Caribbean Hispanic

Introduction

Carotid plaque, a distinct phenotype of subclinical atherosclerosis, is an inflammatory lesion associated with a significantly increased risk of stroke. Identification of novel risk factors for carotid plaque is important for prevention of atherosclerosis and stroke (1,2).

Atherosclerosis is a complex inflammatory disorder that involves several different mechanisms including endothelial injury, activation and recruitment of immuno-inflammatory cells, smooth muscle cell proliferation, and influx of lipoproteins through the vessel injury space (3,4). The heritability of subclinical carotid atherosclerosis is substantial and estimated to be about 50% for presence of plaque (5,6).

Differential risk of atherosclerosis in the population may reflect variation in genes that modulate the inflammatory response to oxidized lipids in the arterial walls (3). A potential role for genes involved in inflammatory processes has been suggested in the pathogenesis of atherosclerosis (7-11). However, information about specific genetic determinants of plaque is lacking, particularly among Hispanic populations. The goal of this study was to examine the association between candidate genes implicated in inflammation and endothelial function with carotid plaque among individuals of Dominican Republic descent.

Materials and Methods

Study population

Samples in this study were drawn from the Genetic Determinants of Subclinical Carotid Disease (GDSCD) study, a substudy of the prospective community-based Northern Manhattan Study (NOMAS) cohort, which was stroke-free at baseline (1).

Discovery Sample—Individuals were enrolled if they: (1) self-identified to be of Dominican origin, (2) had carotid ultrasound, and (3) had available DNA (N=287).

Validation Sample—Subjects from NOMAS were enrolled if they (1) were not previously included in the GDSCD substudy, (2) had carotid ultrasound, (3) had DNA collected, and (4) self-identified to be of Dominican origin (N=301).

All vascular risk factors were collected at baseline and included body mass index, body weight, hypertension, diabetes, smoking, and dislipidemia. Cigarette smoking was assessed by self-report at baseline and categorized as ever versus never smoking. Pack-years of smoking was also computed.

Carotid Ultrasonography

Carotid ultrasound was performed according to standard scanning and reading protocols (1). Internal and common carotid arteries and the bifurcations were examined for the presence of atherosclerotic plaque, defined as an area of focal wall thickening more than 50% greater than surrounding wall thickness. Maximal carotid plaque thickness (MCPT, in mm) was measured at the highest plaque prominence in any of the carotid arteries. Thick plaques were defined as an MCPT > 1.9mm as these plaques were significantly associated with increased stroke risk in NOMAS (1). Calcified plaque was defined by the presence of any acoustic shadowing, a reduction in amplitude of echoes caused by intervening structures with high attenuation. Irregular plaque surface characteristics were recorded. Intra- and inter-rater agreement for plaque calcification and surface characteristics were greater than 0.90 (2).

The primary outcome of interest was presence of plaque, and secondary outcomes were (1) MCPT >1.9mm, (2) irregular plaque, and (3) calcified plaque. Each of these outcomes was compared to the lack of plaque as the reference.

Gene and SNP Selection

SNPs from 147 genes with functional relevance to atherosclerosis were selected for the GDSCD which met any one of the following criteria:(1) SNPs with the minor allele frequency (MAF)>0.05 submitted to the dbSNP by more than one source (http://www.ncbi.nlm.nih.gov/SNP) and examined previously, (2) SNPs located at evolutionarily conserved sequence homology (http://genome.lbl.gov/vista/index.shtml), (3) tagging SNPs across different human populations (http://pga.gs.washington.edu), (4) functional SNPs, or (5) SNPs leading to amino acid changes. An attempt was made to select SNPs with a distance of more than 3000 base pairs.

Genotyping was performed using the GoldenGate® Assay (Illumina Inc., San Diego, USA) (12). The final set included 694 SNPs. From the 147 candidate genes, 43 genes (197 SNPs) implicated in inflammation and endothelial function were included in this study. The genes (chromosome, number of SNPs) for inflammatory function included IL10 (1,5), IL6R (1,3), TGFB2 (1,7), TNFSF4 (1,4), IL1A (2,5), IL1B (2,5), IL1RN (2,3), TFPI (2,5), IL8 (4,2), LTA (6,3), LTB (6,3), TNF (6,2), IL6 (7,5), TLR4 (9,5), CXCL12 (10,4), TGFB3 (14,3), NOS2A (17,4), TGFB1 (19,3), CEBPB (20,4), THBD (20,5), and PPARA (22,11). The genes for endothelial function included GJA4 (1,4), MTHFR (1,5), SELE (1,4), SELL (1,4), SELP (1,7), VCAM1 (1,5), DES (2,5), ITGA2 (5,7), EDN1 (6,4), VEGF (6,2), NOS3 (7,6), HABP2 (10,5), ITGA8 (10,6), CD9 (12,5), NOS1 (12,7), SELPLG (12,3), ITGA2B (17,5), ITGB3 (17,6), and ICAM1 (19,4). Three genes mostly implicated in anti-oxidation were also included: SOD3 (4,3), PON1 (7,6), and XRCC1 (19,7). The median number of SNPs in each gene was 5 (range 2-11) and the average gene size was 32 Kb.

Statistical analysis

Using an additive genetic model for single SNP analyses, a multiple logistic regression was performed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). Associations between the 197 SNPs and plaque phenotypes were tested while controlling for age, sex, ever smoking (current or former smoking of more than 100 cigarettes, cigars, or pipefulls in a lifetime), hypertension, dislipidemia, diabetes, and the time span between baseline assessments and carotid ultrasound. The major allele was the reference. SNPs were considered to be of interest if the association p-value was ≤0.01. This was based on the assumption that each gene had enough a priori evidence to be considered an independent hypothesis, while there was no a priori evidence for the SNPs chosen within each gene. Therefore, multiple testing corrections were done based on a Bonferonni correction of an average of five SNPs per candidate gene. Assuming a minor allele frequency of 0.20, we

had at least 80% power to detect an odds ratio of 1.31 for our primary outcome of interest, plaque presence.

A validation study of genes implicated in the single SNP analysis ($p \leq 0.01$) was performed in an independent set of 301 NOMAS Dominican participants with data genotyped on the Affymetrix 6.0 platform. The first principal component from Eigenstrat was used as a covariate in addition to the covariates mentioned above (13). Multiple logistic regression, using an additive genetic model, was conducted in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) with the major allele as the reference (14). A subset of the samples in the Dominican discovery set (153 of 287), also genotyped on the Affymetrix 6.0 platform, were used to compute the D' and r^2 between SNPs from the GDSCD substudy and the validation study.

In an exploratory analysis, we examined haplotypes within the 43 genes first using Multifactor Dimensionality Reduction (MDR) as a filter (http://www.epistasis.org/software.html) (15). For each carotid plaque phenotype and for each gene, up to a five-SNP model was tested to find the best fit. These SNPs were then examined as a haplotype using the haplo.glm (16) function of the haplo.stats package in R. (Mayo Foundation for Medical Education and Research. http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm). To evaluate the effect of haplotype combination and interaction between two genes for each carotid plaque phenotype, the FAMHAP software v16 was used (17). Genes were chosen for interaction analysis if a SNP in the gene had $p \leq 0.01$ or a haplotype in the gene had $p \leq 0.05$. A gene region was defined as the single SNP or haplotype from a gene as chosen above (18).

Results

Table 1 summarizes the characteristics of the study population. Carotid plaques were present in 134(47%) participants. Among those with plaque, 54(40%) had thick plaques, 31(23%) irregular plaques, and 20(15%) calcified plaque.

Nine genes involved in inflammation and endothelial function had SNPs strongly associated ($p \leq 0.01$) with any of the carotid plaque phenotypes in the single SNP analysis: TNF, NOS2A, IL6R, TNFSF4, PPARA, IL1A, TLR4, ITGA2, and HABP2 (Table 2, full data in Supplementary Table S1). Two NOS2A SNPs (rs2297518, rs8081248) and TNF/rs1799724 were significantly associated with plaque presence; the same two NOS2A SNPs as well as an IL6R SNP (rs1386821), a PPARA SNP (rs4253655), and a TNFSF4 SNP (rs1234313) with thick plaque; the same PPARA SNP, a TLR4 SNP (rs752998), and an IL1A SNP (rs1800587) with irregular plaque; and the PPARA SNP (rs4253655), an ITGA2 SNP (rs1991013), and a HABP2 SNP (rs932650) with calcified plaque.

In the validation sample (Table 1), data were available on 672 SNPs from these nine genes, TNF (1), NOS2A (54), IL6R (21), TNFSF4 (102), PPARA (26), IL1A (19), TLR4 (268), ITGA2 (57), and HABP2 (124), which were in HWE, had MAF>0.05, and genotyping efficiency >95%. The validation analysis independently implicated association between SNPs ($p \leq 0.05$) with the same carotid phenotypes in TNFSF4, PPARA, TLR4, ITGA2, and HABP2 (Table 3, full data in Supplementary Table S2). In addition, three SNPs in NOS2A were trending towards significance for thick plaque in the validation analysis (p=0.06-0.08, Supplementary Table S2). The TNFSF4 SNP, rs1234313, which was associated with thick plaque in discovery, was in strong LD with five of the TNFSF4 SNPs implicated in the validation analysis (D'=1) and in moderate LD with one additional SNP (D'=0.77) (Table 3). The PPARA SNP, rs4253655, which was associated with thick and irregular plaque in the discovery sample, available as a validation SNP, was associated with thick plaque in

validation and was also in strong LD with rs4253617 (D'=1), associated with thick and irregular plaque in validation. The TLR4 SNP, rs752998, which was associated with irregular plaque in discovery, was in moderate LD with one of the SNPs (rs1927910) implicated in validation (D'=0.69). The HABP2 SNP, rs932650, which was associated with calcified plaque, was in strong LD with rs10885476 (D'=0.94), implicated in validation.

Examination of haplotypes within genes revealed an additional gene of interest, VCAM1, and reinforced the importance of TLR4, NOS2A, PPARA, and HABP2 (Table 4, full data in Supplementary Table S3). For VCAM1, three SNPs (rs3783615, rs3783613, rs3181092) were associated as a haplotype with calcified plaque (p=0.0059). A two-SNP haplotype from TLR4 was associated with calcified plaque (p=0.00075). A two-SNP NOS2A haplotype was associated with thick (p=0.000070) and calcified plaque (p=0.0053). A three-SNP PPARA haplotype was associated with thick plaque (p=0.0092), and a three-SNP HABP2 haplotype was also associated with thick plaque (p=0.0032).

The interaction analysis of unlinked regions (Table 5, full data in Supplementary Table S4) revealed ($p < 0.001$) two region interactions between haplotypes in combinations of IL6R, TNFSF4, NOS2A, and PPARA (p= 0.0000088 to 0.00086) for thick plaque. For irregular plaque, an interaction between haplotypes in PPARA and IL1A was seen (p=0.00049). Interactions were seen between haplotypes for combinations of ITGA2 with PPARA (p = 0.00038 and TLR4 (p = 0.0008) for calcified plaque.

Discussion

We observed significant associations between genetic variants in seven genes related to inflammation (TNF, NOS2A, IL6R, TNFSF4, PPARA, IL1A, TLR4) and two genes related to endothelial function (ITGA2, HABP2) with carotid plaque phenotypes in people of Dominican descent. The validation analysis in an independent sample confirmed associations for TNFSF4, PPARA, TLR4, ITGA2, and HABP2. In addition, a haplotype within the VCAM1 gene was associated with calcified plaque. Statistical interactions were observed between associated SNPs and haplotypes and the carotid plaque phenotypes.

A number of studies have explored the association between inflammatory genes and ischemic heart disease, but few have focused on subclinical atherosclerosis. Certain plaque phenotypes, such as irregular plaques and maximal carotid plaque thickness, may be important markers of vulnerable plaques susceptible to rupture leading to stroke (1,2). Our study is among the first to investigate the association between variations in genes related to inflammatory processes and endothelial function and possible markers of vulnerable plaque in a multi-ethnic population.

NOS2A was associated with plaque presence and in particular thick plaque, and was marginally significant in validation ($p<0.10$, Supplementary Table S2). NOS2A (or iNOS) produces nitric oxide and is involved in vascular tone regulation, neurotransmission and immune response. iNOS has been found in the deep portions of plaques in humans, and its inhibition slowed down the progression of atherosclerosis in rabbits (10). In a histochemistry study from endarterectomy specimens, most of the nonruptured plaques had iNOS mRNA and protein, while the ruptured plaques did not, implying that iNOS might contribute to the plaque rupture (19).

PPARA is a nuclear receptor that, when activated, triggers the transcription of acyl-CoA oxidase, which catalyzes the fatty acid beta-oxidation pathway. While PPARA has been shown to affect atherosclerosis, lipid metabolism, and oxidative stress, studies on the potential relationship between PPARA and plaque and plaque rupture are lacking. The PPARA rs4253655 SNP implicated in our study has not been reported, although associations

with other PPARA SNPs have been found (7,8). PPARA polymorphisms have been associated with the progression of atherosclerosis in a cohort of Finnish men (9). In our study the PPARA gene was associated with thick plaque, irregular plaque, and calcified plaque, indicating its important role in various stages of atherosclerotic plaque.

TNFSF4 encodes a cytokine in the tumor necrosis factor ligand family which regulates adhesion of activated T cells to endothelial cells. While the current study is the first to show an association with thick plaque, polymorphisms in TNFSF4 have been associated with the size of atherosclerotic lesions in mice, and with the risk of MI in humans (11).

TLR4 (toll-like receptor 4), which is involved in innate immune response, was associated with irregular plaque in our study. An association between global gene variation in TLR4 and risk of MI was previously observed, but no association was reported with stroke (7) or carotid IMT (20-23).

ITGA2 regulates cell adhesion and cell-surface mediated signaling. Its variants, particularly the C807T polymorphism, have been associated with risk of ischemic stroke in some but not all studies (24,25), and the T allele has been protective against carotid IMT and plaque in patients with type 2 diabetes (26). In our study, an ITGA2 SNP was associated with calcified plaque, a surrogate measure of an increased risk of general atherosclerosis, but also a marker of protection against rupture. A recent large case-control study found evidence suggesting associations between ITGA2 tagging SNPs and risk of ischemic stroke (24). Although the SNP implicated in our study (rs1991013) was not evaluated in these other studies, it was located close to a few of these significant SNPs.

Vascular integrity is also regulated by HABP2, which encodes a protein involved in cell adhesion. HABP2 is believed to affect vascular smooth muscle cell proliferation and atherosclerotic plaque instability (27). A HABP2 polymorphism has been associated with inhibited activation of prourokinase and progression of carotid stenosis (28), and HABP2 variants have been associated with venous thromboembolic disease (29).

We identified associations between HABP2, TLR4, NOS2A, PPARA, and VCAM1 haplotypes and thick and calcified plaque. This analysis underscores the complex nature of the genetic effects and the potentially synergistic role of SNPs in conferring an increased risk of plaque. Furthermore, while some previous studies have also examined the role of various genes in plaque etiology, ours is the first to elucidate the role of these genes in relation to one another. We observed several statistical interactions between unlinked regions of our significant genes in relation to thick, irregular, and calcified plaque, and in particular, a very strong interaction between SNPs in NOS2A and TNSF4 in relation to thick plaque. As it has become more evident that many common phenotypes may be polygenic in nature, it is necessary to explore gene-gene interactions when studying the genetic influence on plaque phenotypes (30,31).

None of the inflammatory genes associated with plaque phenotypes in the current study were associated with carotid IMT in the same population in our previous report (32). This finding supports the hypothesis that carotid IMT and carotid plaque are distinct phenotypes of subclinical atherosclerosis (33).

Strengths of this study include the examination of novel SNPs, use of standardized technology and restriction to one ethnic group to reduce heterogeneity. However, several limitations are also noted. Although this study focused on one ethnic group, the Dominican American population living in northern Manhattan is, in fact, a rather racially and ethnically diverse group in itself and therefore a small potential for bias due to population stratification persists. Dominican Americans are a rapidly growing population in the US and population-

based studies in this group are important. Although this study examined the role of several known important genes involved in inflammation and endothelial function, other known and unknown genes were not captured. Despite our validation study, further exploration of these genes in relation to carotid atherosclerosis is needed in both Hispanic and non-Hispanic populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of the study populations: Demographics, vascular risk factors, and carotid plaque phenotypes

	Discovery N = 287		Validation N = 301	
	n	%	n	%
Hypertension	228	79.4	216	71.8
Diabetes	53	18.5	71	23.6
Ever smoking	120	41.8	141	46.8
Sex				
Female	167	58.2	210	69.8
Male	120	41.8	91	30.2
Plaque Prevalence	134	46.7	139	46.2
% Among those with Plaque				
Thick Plaque	54	40.3	70	50.4
Plaque Irregularity	31	23.1	61	43.9
Calcified Plaque	20	14.9	12	8.6

	Mean ± SD	Mean ± SD
Age	64.08 ± 7.20	62.53 ± 8.19
BMI (kg/m2)	28.05 ± 4.36	28.91 ± 4.81
Weight (pounds)	161.82 ± 27.78	163.19 ± 29.59
Height (inches)	63.78 ± 3.54	63.04 ± 3.46
Total cholesterol (mg/dl)	198.16 ± 37.37	203.26 ± 38.54
LDL (mg/dl)	127.76 ± 33.10	130.79 ± 35.34
HDL (mg/dl)	42.10 ± 12.04	45.24 ± 12.51
TG (mg/dl)	142.48 ± 72.10	136.15 ± 68.41
SBP (mmHg)	144.08 ± 20.50	139.36 ± 19.88
DBP (mmHg)	86.92 ± 11.62	83.46 ± 9.86
Years between Plaque measured and Baseline	0.53 ± 1.36	2.10 ± 3.47

Table 2

Top SNP Results in Genes related to Inflammatory and Endothelial Function (p≤0.01)

Phenotype	Chr	Gene	SNP	Position (MB)	Function	Minor Allele	MAF	Major Allele	P Value*	Odds ratio (95% CI) †
Plaque	6	TNF	rs1799724	31.650	near-gene-3	A	0.09	G	7.10E-03	0.39 (0.19-0.77)
	17	NOS2A	rs8081248	23.106	downstream	A	0.28	G	2.90E-03	0.54 (0.36-0.81)
			rs2297518	23.121	missense	A	0.19	G	9.10E-03	1.85 (1.16-2.93)
Thick	1	IL6R	rs1386821	152.649	intron	C	0.12	A	6.60E-03	2.62 (1.31-5.23)
	1	TNFSF4	rs1234313	171.433	intron	A	0.23	G	7.60E-03	2.11 (1.22-3.65)
	17	NOS2A	rs2297518	23.121	missense	A	0.18	G	3.00E-04	3.18 (1.70-5.95)
			rs8081248	23.106	downstream	A	0.28	G	4.00E-04	0.29 (0.14-0.58)
	22	PPARA	rs4253655	44.948	intron	A	0.12	G	4.20E-03	2.83 (1.39-5.76)
Irregular	2	IL1A	rs1800587	113.259	untranslated-5	A	0.32	G	6.50E-03	0.30 (0.13-0.71)
	9	TLR4	rs752998	119.524	upstream	A	0.43	C	9.60E-03	2.33 (1.23-4.43)
	22	PPARA	rs4253655	44.948	intron	A	0.12	G	1.00E-03	4.92 (1.91-12.66)
Calcified	5	ITGA2	rs1991013	52.396	intron	A	0.49	G	9.40E-03	2.97 (1.31-6.78)
	10	HABP2	rs932650	115.337	intron	G	0.36	A	9.30E-03	2.83 (1.29-6.18)
	22	PPARA	rs4253655	44.948	intron	A	0.11	G	3.60E-03	5.24 (1.72-15.98)

*adjusted for baseline age, sex, smoking, diabetes, dislipidemia, hypertension, and years between phenotype and risk factor collection

†Odds ratio is measuring the risk of the minor allele

Full Results are in Supplementary Table S1

MAF = Minor Allele Frequency

Table 3

Validation of Genes in Independent NOMAS Cohort of 301 Dominicans

Chr	Gene	Discovery SNP	Validation SNP	Function	Position (MB)	D'	Minor Allele	MAF	P Value*	OR (95% CI)†
Thick Plaque										
2	TNFSF4	rs1234313	rs10489269	intron	171.427	1.00	A	0.08	0.049	2.26 (1.00-5.11)
			rs2840317	upstream	171.493	0.08	A	0.22	0.015	2.01 (1.14-3.54)
			rs2901716	upstream	171.494	0.06	A	0.21	0.021	1.97 (1.11-3.48)
			rs2022449	upstream	171.505	0.12	T	0.25	6.46E-03	2.15 (1.24-3.72)
			rs844665	upstream	171.516	0.57	T	0.13	0.035	0.44 (0.20-0.94)
			rs10912580	upstream	171.523	0.08	C	0.20	9.49E-03	2.17 (1.21-3.90)
			rs947505	upstream	171.569	0.77	A	0.18	0.014	2.20 (1.17-4.14)
			rs1539259	upstream	171.579	0.13	C	0.19	0.024	1.96 (1.10-3.52)
			rs1539256	upstream	171.579	1.00	G	0.07	7.88E-03	3.20 (1.36-7.55)
			rs16845685	upstream	171.581	1.00	A	0.08	5.37E-04	4.49 (1.92-10.51)
			rs7550607	upstream	171.582	1.00	G	0.07	7.88E-03	3.20 (1.36-7.55)
			rs16845722	upstream	171.594	1.00	T	0.07	7.88E-03	3.20 (1.36-7.55)
			rs6701066	upstream	171.619	0.07	C	0.32	9.21E-05	0.32 (0.18-0.57)
			rs4916219	upstream	171.640	0.19	T	0.29	0.015	1.87 (1.13-3.11)
			rs10798277	upstream	171.640	0.35	C	0.35	1.74E-03	2.14 (1.33-3.45)
			rs7526628	upstream	171.644	0.05	T	0.48	0.012	1.84 (1.15-2.96)
			rs3934575	upstream	171.665	0.14	A	0.15	0.044	0.46 (0.22-0.98)
22	PPARA	**rs4253655**	rs4253617	intron	44.927	1	G	0.11	4.22E-03	0.27 (0.11-0.66)
			rs4253655	intron	44.948	1	A	0.08	0.047	2.37 (1.01-5.56)
Irregular Plaque										
9	TLR4	rs752998	rs1930706	downstream	119.709	0.32	G	0.25	0.011	0.40 (0.20-0.81)
			rs2151370	downstream	119.765	0.27	C	0.28	0.018	2.02 (1.13-3.62)
			rs12335791	downstream	119.560	0.04	G	0.13	0.018	2.28 (1.15-4.50)
			rs2780244	downstream	119.768	0.27	T	0.29	0.018	2.02 (1.13-3.62)
			rs10983777	downstream	119.566	0.06	C	0.14	0.022	2.19 (1.12-4.27)
			rs6478338	downstream	119.748	0.47	G	0.25	0.028	0.51 (0.28-0.93)

Chr	Gene	Discovery SNP	Validation SNP	Function	Position (MB)	D'	Minor Allele	MAF	P Value*	OR (95% CI)†
			rs488101	downstream	119.747	0.40	C	0.24	0.031	1.93 (1.06-3.51)
			rs4837527	downstream	119.718	0.06	C	0.37	0.032	1.88 (1.06-3.35)
			rs1927910	intron	119.510	0.69	A	0.06	0.044	0.18 (0.04-0.95)
22	PPARA	rs4253655	rs4253617	intron	44.927	1.00	G	0.11	4.30E-03	0.20 (0.07-0.60)
				Calcified Plaque						
5	ITGA2	rs1991013	rs4865756	intron	52.349	0.44	A	0.36	0.031	0.22 (0.05-0.87)
			rs3797497	intron	52.370	0.00	T	0.36	0.049	0.28 (0.08-1.00)
10	HABP2	rs932650	rs4918818	upstream	115.159	0.01	A	0.11	0.046	5.09 (1.03-25.17)
			rs11196327	upstream	115.169	0.02	A	0.11	0.013	4.81 (1.39-16.60)
			rs1416131	upstream	115.183	0.02	C	0.36	0.046	0.27 (0.07-0.97)
			rs10509977	upstream	115.184	0.06	T	0.12	0.018	4.39 (1.29-14.96)
			rs11196335	upstream	115.200	0.03	A	0.19	0.022	3.42 (1.20-9.73)
			rs10885459	upstream	115.200	0.05	C	0.19	0.021	3.43 (1.21-9.74)
			rs8181320	upstream	115.207	0.01	A	0.14	5.94E-03	5.01 (1.59-15.80)
			rs7909440	upstream	115.270	0.15	C	0.16	0.040	3.29 (1.05-10.29)
			rs10885476	intron	115.321	0.94	T	0.13	0.035	3.06 (1.08-8.62)
			rs3121494	downstream	115.419	0.13	C	0.34	0.034	0.23 (0.06-0.89)
22	PPARA	rs4253655	rs5768939	upstream	44.913	0.03	G	0.11	0.024	3.94 (1.20-13.00)

*adjusted for baseline age, sex, and smoking, hypertension, dislipidemia, diabetes, and number of years between baseline and phenotype measurement

**Odds ratio is measuring the risk of the minor allele

MAF = Minor Allele Frequency

D' and R2 are the measures of linkage disequilibrium between the SNP from the discovery analysis and the SNP from the validation analysis

Table 4

Haplotype Results ($p \leq 0.01$) for Global Test

Phenotype	Gene	Haplotype Candidate from MDR	Freq Control	Freq Case	Coef	SE	P*
Thick	**HABP2**	**SNP1=rs7923349;SNP2=rs2302373;SNP3=rs2240878**					**3.24E-03** *
		c a c (reference)	0.38	0.35			
		c g c	0.11	0.03	1.84	1.01	0.069
		c g a	0.02	0.10	1.59	0.75	0.035
		c a a	0.19	0.14	0.20	0.49	0.685
		a g c	0.07	0.17	1.47	0.58	0.013
		a g a	0.07	0.07	0.13	0.68	0.846
		a a c	0.13	0.04	0.70	0.74	0.344
		a a a	0.03	0.10	1.82	0.73	0.013
	NOS2A	**SNP1=rs3730017;SNP2=rs2297518**					**6.96E-05** *
		g g (reference)	0.73	0.53			
		g a	0.14	0.30	1.31	0.33	1.15E-04
		a g	0.13	0.17	0.73	0.36	0.046
	PPARA	**SNP1=rs6007662;SNP2=rs4253681;SNP3=rs11090819**					**9.20E-03** *
		a a g (reference)	0.49	0.51			
		g g g	0.03	0.12	2.10	0.78	7.29E-03
		g g a	0.08	0.05	1.21	0.72	0.095
		g a g	0.11	0.07	1.21	0.62	0.051
		g a a	0.15	0.11	0.56	0.43	0.188
		a g g	0.12	0.10	0.30	0.48	0.534
		a a a	0.03	0.01	0.56	0.84	0.504
		Rare Haplotypes	0.00	0.02	does not converge		
Calcified	**VCAM1**	**SNP1=rs3783615;SNP2=rs3783613;SNP3=rs3181092**					**5.92E-03** *
		t c a (reference)	0.49	0.26			
		t g a	0.04	0.11	1.09	0.80	0.173
		t c g	0.45	0.55	0.86	0.48	0.076
		Rare Haplotypes	0.02	0.08	2.61	0.99	8.80E-03

Phenotype	Gene	Haplotype Candidate from MDR	Freq Control	Freq Case	Coef	SE	p*
	TLR4	**SNP1=rs752998;SNP2=rs1927911**					**7.49E-04** *
		c g (reference)	0.51	0.28			
		c a	0.09	0.14	1.48	0.67	0.029
		a g	0.05	0.17	2.13	0.75	5.07E-03
		a a	0.36	0.41	0.85	0.46	0.064
	NOS2A	**SNP1=rs3730017;SNP2=rs2297518**					**5.26E-03** *
		g g (reference)	0.73	0.53			
		g a	0.14	0.29	1.39	0.53	9.66E-03
		a g	0.13	0.18	0.95	0.51	0.065

* p-value is the global haplotype significance computed using Fisher's Method

p-values are all adjusted for age, sex, smoking, diabetes, dislipidemia, hypertension, and years between phenotype and risk factor collection - from haplo.glm and represents the significance of the specified haplotype relative to the reference haplotype

Haplotypes with a frequency < 10 were grouped as 'Rare Haplotypes'

Full results are in Supplementary Table S3.

Table 5

Interactions between Unlinked Regions ($p \leq 0.001$)

Phenotype	Gene	P-value*
Thick	IL6R, TNFSF4	4.61E-04
	IL6R, NOS2A-1	1.03E-04
	IL6R, NOS2A-2	1.62E-04
	TNFSF4, NOS2A-1	**8.80E-06**
	TNFSF4, NOS2A-2	1.18E-04
	TNFSF4, PPARA-1	6.06E-04
	NOS2A-1, NOS2A-2	1.34E-04
	NOS2A-1, PPARA-1	1.63E-04
	NOS2A-1, PPARA-2	3.65E-04
	NOS2A-2, PPARA-1	4.42E-04
	NOS2A-2, PPARA-2	8.61E-04
Irregular	IL1A, PPARA	4.91E-04
Calcified	ITGA2, PPARA	3.81E-04
	ITGA2, TLR4	7.99E-04

* p-value from FAMHAP. multiple testing correction has been applied via the minP approach

For **thick** plaque, analyzed SNPs included rs1386821 for IL6R ; rs1234313 for TNFSF4; rs2297518 and rs3730017 for NOS2A-1; rs8081248 for NOS2A-2; rs4253655 for PPARA-1; and rs6007662, rs4253681, and rs11090819 for PPARA-2. For **irregular** plaque, analyzed SNPs included rs1800587 for IL1A and rs4253655 for PPARA. For **calcifie**d plaque, analyzed SNPs included rs1991013 for ITGA2; rs4253655 for PPARA; and rs752998 and rs1927911 for TLR4.

Full results are in Supplementary Table S4