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Common Genetic Variants and Subclinical Atherosclerosis: The Multi-Ethnic Study of Atherosclerosis (MESA).

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## Abstract

**Background:** Subclinical atherosclerosis (sCVD), measured by coronary artery calcium (CAC) and carotid intima media thickness (CIMT) has been associated with cardiovascular disease (CVD). Genome Wide Association Studies (GWAS) of CVD have focused on Caucasian populations. We hypothesized that these associations would differ in populations from distinct genetic backgrounds. **Methods and Results:** The associations between sCVD and 66 single nucleotide polymorphisms (SNPs) from published GWAS of sCVD and CVD were tested in 8224 Multi-Ethnic Study of Atherosclerosis (MESA) and MESA Family participants (2685 Caucasians (EUA), 777 Chinese (CHN), 2588 African Americans (AFA), and 2174 Hispanic (HIS)) using an additive model adjusting for CVD risk factors, with SNP significance defined by a Bonferroni-corrected  $p < 7.6 \times 10^{-4}$  (0.05/66). **Results:** In EUA there were significant associations with CAC in 9p21 (rs1333049,  $P=2 \times 10^{-9}$ ; rs4977574,  $P=4 \times 10^{-9}$ ), *COL4A1* (rs9515203,  $P=9 \times 10^{-6}$ ), and *PHACTR1* (rs9349379,  $P=4 \times 10^{-4}$ ). In HIS, SNPs were associated with CAC in 9p21 (rs1333049,  $P=8 \times 10^{-5}$ ; rs4977574,  $P=5 \times 10^{-5}$ ), *APOA5* (rs964184,  $P=2 \times 10^{-4}$ ), and *ADAMTS7* (rs7173743,  $P=4 \times 10^{-4}$ ). There were no associations with the 9p21 region in AFA and CHN. Fine mapping of the 9p21 region revealed SNPs with robust associations with CAC in EUA and HIS but no significant associations in AFA and CHN. **Conclusions:** In non-Caucasians in MESA, associations were found with SNPs previously identified in EUA GWAS for CVD and sCVD, but not in all loci, suggesting novel variants and/or pathways in risk of CVD in minority populations.

## Introduction

Cardiovascular disease (CVD) is among the leading causes of death worldwide.<sup>1</sup> Recent technological advances have made possible the identification of CVD before it becomes clinically apparent.<sup>2</sup> Subclinical atherosclerosis is common<sup>3</sup> and can be measured non-invasively through imaging techniques

such as coronary artery calcium (CAC)<sup>4,5</sup> and carotid intima media thickness (CIMT)<sup>6</sup> thereby providing a non-invasive way to risk stratify patients.

Genome-wide association studies (GWAS) have been instrumental in advancing our understanding of the genetic basis of CVD and subclinical CVD by identifying novel loci associated with the development of atherosclerosis (Supplementary Table 1).<sup>7-22</sup> Many of these associations are not explained by conventional CVD risk factors, consistent with the possibility that these loci represent novel atherosclerotic pathways. The most widely replicated of these GWAS results that are associated with CVD and subclinical CVD is the 9p21 locus.<sup>16,17,23</sup> In addition to CVD and subclinical CVD, the 9p21 locus has also been associated with risk of abdominal and intracranial aneurysms<sup>24</sup>, peripheral arterial disease<sup>24</sup>, heart failure<sup>25</sup>, sudden cardiac death<sup>26</sup> and stroke.<sup>27</sup> Nonetheless, association of 9p21 with markers of the early stages of atherosclerosis has not been found, including arterial elasticity and retinal microvascular abnormalities.<sup>28</sup>

The number of GWAS loci identified with complex human phenotypes has increased exponentially since the completion of the human genome project, yet the majority of GWAS have been performed on populations of European descent.<sup>29</sup> Moreover, relatively few GWAS of CVD and/or subclinical CVD have focused on non-European populations.<sup>14,21</sup> Small candidate gene studies have complemented these GWAS but are few in number.<sup>30,31</sup> This apparent disparity has raised questions regarding the relevance of GWAS findings to populations of different genetic backgrounds.<sup>32</sup> This issue is particularly relevant to CVD and subclinical CVD given the varying prevalence of these phenotypes in different ethnicities.<sup>33,34</sup>

In the present study, we use the Multi-ethnic Study of Atherosclerosis (MESA) to investigate whether significant associations found in recent GWAS of CVD and subclinical CVD are also associated with measures of subclinical CVD, CAC and CIMT in a genetically diverse cohort of African, Hispanic and Asian ancestry.

## **Methods**

### **Study Design**

MESA is a longitudinal cohort study designed to investigate the impact of subclinical CVD and CVD risk factors on the development of clinically overt CVD. The first clinic visits occurred during 2000 - 2002 in

6,814 participants recruited from 6 field centers across the United States, and all participants were free of clinical CVD at the baseline examination. Approximately 38% of the recruited participants are Caucasians (EUA), 12% Chinese (CHN), 28% African American (AFA) and 22% Hispanic (HIS). Many ancillary studies have added to the original MESA cohort by focusing on specific phenotypes and/or participant populations.<sup>35</sup> One of these studies, the MESA Family Study, recruited African American and Hispanic family members specifically for genetic analyses..

### **Genotype Data**

The 66 single nucleotide polymorphisms (SNPs) used in this study are described in supplemental Table 1 and were obtained from GWAS data on 8,224 consenting MESA participants (2,685 EUA, 777 CHN, 2,588 AFA, and 2,174 HIS) from the National Heart, Lung, and Blood Institute SNP Health Association Resource (SHARe) project. Study participants were genotyped using the Affymetrix 6.0 GWAS set, which includes MESA and MESA Family participants. SNPs not present on this chip were imputed using the 1000 genomes Phase 1 v3 as a reference. Genotypes were filtered for SNP level call rate <95% and individual level call rate <95%, and monomorphic SNPs as well as SNPs with heterozygosity >53% were removed. Allele frequencies were calculated separately within each racial/ethnic group, and only those SNPs with minor allele frequencies >0.01 were included in genetic association analyses. We further filtered imputed SNPs based on imputation quality >0.5, using the observed versus expected variance quality metric, and filtered genotyped SNPs for Hardy-Weinberg equilibrium P-value  $\geq 10^{-5}$ .

### **Subclinical CVD Measurement**

The Agatston coronary calcium score (CAC) was measured by either electron-beam tomography or multi-detector computed tomography, as described previously.<sup>4</sup> All scans were read at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Measurements of CAC were adjusted between the different field centers and imaging machines by using a standard calcium phantom of known density, which was scanned with each participant. The Agatston calcium score was calculated as described previously<sup>36</sup> and the mean value from two scans used for analysis.

Imaging of the internal carotid and common carotid arteries were performed by B-mode ultrasonography of the right and left, near and far walls, and images were recorded using a Logiq 700 ultrasound device (General Electric Medical Systems, Waukesha, WI). Maximal carotid intima media thickness (CIMT) of the

internal (CIMT-i) and common (CIMT-c) carotid arteries was measured as the mean of the maximum CIMT of the near and far wall of the right and left sides at a central ultrasound reading center (Department of Radiology, New England Medical Center, Boston, MA) as described previously.<sup>37</sup>

## Statistical Analyses

Given skewed distributions, the common (CIMT-c) and internal IMT (CIMT-i) values were log normalized. CAC was analyzed as a continuous variable by obtaining the log of the raw CAC score plus one (CAC-c) or as a dichotomous variable (CAC-d) with CAC > 0. Analyses were first performed stratified within each racial/ethnic group. For analysis involving EUA and CHN, an unrelated subset of individuals was constructed by selecting at most one individual from each pedigree. For analysis of phenotypes with a substantial familial component, among AFA and HIS, the analysis was performed using a linear mixed-effects model (continuous variables) and by generalized estimating equations (dichotomous variables). Associations between each SNP and each individual phenotype was determined using separate multiple linear regressions (continuous variables) or logistic regressions (dichotomous variables) assuming an additive model. Two models were used to analyze the data. Model 1 accounted for age, sex, site of ascertainment, and principal components. Model 2 included Model 1 plus HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglycerides, body mass index (BMI), hypertension status (self-report of physician-diagnosed hypertension along with use of antihypertensive medication or systolic blood pressure of 140 mm Hg or greater and/or diastolic blood pressure of 90 mm Hg or greater), diabetes status (fasting blood glucose was 126 mg/dL or greater or use of diabetes medications), and tobacco use (self-reported tobacco use within the past 30 days). Fixed effect meta-analysis was used to combine results across all four race/ethnic groups, as implemented in METAL.<sup>38</sup>

Significance thresholds were defined by Bonferroni correction, determined by the number of SNPs used in each analysis. In the case of the initial analysis, significance was defined by a Bonferroni-corrected  $p < 7.6 \times 10^{-4}$  given 66 SNPs tested (0.05/66). To assess genetic heterogeneity seen in stratified analyses of the four MESA race/ethnic groups, we used the  $I^2$  heterogeneity metric to quantify the proportion of total variation across studies attributable to heterogeneity rather than chance.<sup>39</sup>

Fine mapping of the 9p21 region (100 kb upstream or downstream from SNPs rs1333049, rs4977574, and rs16905644) was performed for each ethnic group by selecting all SNPs on the chromosome 9

imputation set (NCBI Build 37) between positions 21997022-22225503. A total of 3,282 SNPs were identified (598, 631, 1256 and 797 SNPs in EUA, CHN, AFA and HIS, respectively). This list of SNPs was supplemented by adding novel SNPs identified by deep sequencing efforts in this region.<sup>40,41</sup> Analyses for these SNPs were performed as described above. Given that each ethnicity has its own LD structure, the significance threshold was defined by Bonferroni correction as determined by the number of independent SNPs used in the analysis for each ethnicity.

## Results

### Study Sample Characteristics

Table 1 illustrates the baseline demographic characteristics of the MESA participants included in this study as well as their CVD risk factors and measures of subclinical atherosclerosis. Sample sizes reflect the inclusion of individuals from the original MESA cohort, combined with those from the ancillary MESA Family cohort as described above. The samples sizes are similar in the different ethnicities with the exception of CHN, which had a relatively lower number of study participants (n=691). There is a lower BMI in the CHN (median 23.7), higher prevalences of hypertension (60%) and smoking (19.3%) in the AFA, and higher prevalence of CAC (56.9%) and median CAC score (115.7) in the EUA populations. There were no differences across ethnicities for CIMT.

### Association of SNPs with subclinical CVD

The association of previously identified SNPs associated with CVD and subclinical CVD with CAC-c in the four MESA ethnicities is shown in Figure 1. After Bonferroni correction, multiple SNPs were associated with CAC-c in EUA and HIS, one in AFA, and no SNP significantly associated with CAC-c in CHN. The significant SNPs in EUA, HIS and AFA, and the closest gene in the locus, *P*-values and effect sizes, are shown in Table 2. In EUA there were significant associations with CAC-c in 9p21 (rs1333049,  $P=2 \times 10^{-9}$ ; rs4977574,  $P=4 \times 10^{-9}$ ), *COL4A1* (rs9515203,  $P=9 \times 10^{-6}$ ), and *PHACTR1* (rs9349379,  $P=4 \times 10^{-4}$ ). All of these associations, except for *COL4A1*, have effects in the same direction as shown in the initial discoveries. The difference in the direction of effect of rs9515203 in *COL4A1* is not expected, given that the alleles in this study are the same and the minor allele frequencies are equivalent as those previously published. In HIS, SNPs were associated with CAC-c in 9p21 (rs1333049,  $P=8 \times 10^{-5}$ ; rs4977574,  $P=5 \times 10^{-5}$ ), *APOA5* (rs964184,  $P=2 \times 10^{-4}$ ), and *ADAMTS7* (rs7173743,  $P=4 \times 10^{-4}$ ). There were no associations with the 9p21 region in AFA and CHN. The only significant association in AFA with CAC-c was in the *LPA* locus (rs10455872,  $P=5.66 \times 10^{-4}$ ).

Results from meta-analysis across ethnicities (Figure 2) show the associations between previously identified CVD and subclinical CVD SNPs and the four subclinical CVD phenotypes in MESA. After Bonferroni correction, there were four significant associations with CAC-c and CAC-d (Table 3). There was evidence of significant heterogeneity based upon inconsistent direction of association in different

ethnicities, elevated  $I^2$  heterogeneity, and low heterogeneity p-values. This increased heterogeneity may reflect different directions of association in the AFA population. There was also increased heterogeneity observed with respect to the *COL4A1* and CAC-c association. In contrast, the associations between *PHACTR1* and *ADAMTS7* showed significant p-values as well as low heterogeneity metrics in the context of CAC-d and CAC-c respectively. There was one significant association with CIMT-c and two significant associations with CIMT-i. The *SMG6* locus was significantly associated with CIMT-c but there were increased heterogeneity. The *LPA* and *TRIB1* loci were significantly associated with CIMT-i with low heterogeneity indices, indicating a consistent association across ethnicities.

### **Functional Annotation**

To assess the functional significance of the SNPs with significant associations, we used the publically available ENCODE Project Consortium and HaploRegv2 for functional annotation of selected SNPs.<sup>42</sup> The 9p21 locus SNP rs1333049 is in a heterochromatin protein 1 (HP1 site), a protein known to be important in gene expression regulation. rs4977574, also in the 9p21 locus, is located in an enhancer in various types of cells and is also in a DNase site (in LNCaP cells). rs9515203 in the *COL4A1* locus is located in an enhancer in various types of cells and binds RNA polymerase II (POL2). The *PHACTR1* SNP rs9349379 is in a site for myocyte enhancer factor 2 (Mef2), a family of transcription factors important in cellular differentiation and embryonic development. rs10455872 in the *LPA* locus is a modulator of FOXO, a family of transcription factors involved in cellular metabolism and proliferation. The *APOA5* SNP rs964184 and the *ADAMTS7* SNP rs7173743 are both located in DNase sites in various types of cells. Another SNP in *ADAMTS7*, rs1994016, is in the binding site of the transcription factor activator protein 1 (AP-1) which is important in regulating cellular proliferation, differentiation and apoptosis. The *SMG6* SNP rs216172 is in enhancer and DNase sites in numerous cell types.

### **Fine Mapping of 9p21 Region**

Given the inconsistent association across ethnicities between measures of CAC and the 9p21 region, fine mapping of this region was performed in an attempt to uncover as yet unidentified associations. Figure 3 presents the associations between 9p21 SNPs (598 SNPs in EUA, 631 SNPs in CHN, 1256 SNPs in AFA, and 797 SNPs in HIS) and CAC-c demonstrating robust associations in EUA and HIS but no significant associations in AFA and CHN. Supplementary Table 2 lists the significant SNPs in EUA and HIS along



with their location, effect size and p-values. Figure 4 shows Manhattan plots of meta-analyses across ethnicities illustrating the associations between 9p21 SNPs and the four subclinical CVD phenotypes included in this study. There were no significant associations between 9p21 SNPs and CIMT-c and CIMT-i. There was a similar pattern of associations between these SNPs and CAC-d and CAC-c although there were more significant associations with CAC-c. However, as shown in Supplementary Table 3, although these associations are statistically significant, there was inconsistent direction of associations across ethnicities, particularly with respect to AFA, and increased heterogeneity indices.

## DISCUSSION

The association of SNPs identified in GWAS of CVD and subclinical CVD has been observed in populations of mostly European descent. In this study, we evaluate the association of those SNPs with subclinical CVD (CAC and CIMT) in populations from European, African, Hispanic and Asian ancestry in the MESA cohort. Our findings reveal previously unknown associations between various SNPs and different measures of subclinical CVD. We also explored whether these associations were present in different ethnicities.

The vast majority of SNPs tested in this candidate gene study had been previously associated with coronary artery disease (CAD), while a handful had been associated with subclinical CVD (supplemental table 1). We have confirmed the previously reported associations between CAC and SNPs in the 9p21 and *PHACTR1*,<sup>16</sup> while reporting associations between CAC and the *ADAMTS7* and *COL4A1* (Table 3). The *ADAMTS7* and *COL4A1* loci have been previously associated with CAD, but not with CAC. We also report associations between loci previously associated with CAD (*SMG6*, *LPA* and *TRIB1*) and CIMT. We found no association between the 9p21 locus and CIMT in any of the ethnicities tested, as has been previously reported,<sup>43</sup> suggesting a mechanism for CVD risk mediation independent of CIMT. Although our power to detect associations at a Bonferroni corrected p-value  $p < 7.6 \times 10^{-4}$ , minor allele frequency of 10% and beta coefficient of 0.022 was greater than 90%, not all SNPs tested were found to be associated with subclinical CVD in our study. This lack of association could be due to relatively limited power particularly for SNPs with lesser minor allele frequencies and/or effect sizes. However, the fact that some loci are associated with CAD and CAC or CIMT while others are not raises the intriguing possibility that not all genetic mechanisms for CAD are mediated via subclinical CVD as currently characterized by the imaging techniques in this study.<sup>16</sup> Functional annotation of the SNPs in these loci reveals that they likely mediate the development of CVD and/or subclinical CVD by means of regulating gene expression and cellular proliferation.

There were notable differences in subclinical CVD associations based on ethnicity. The 9p21 locus was only significantly associated with CAC in EUA and HIS ancestry populations. The *COL4A1* and *PHACTR1* SNPs were only associated with CAC in the group of EUA ancestry. The *LPA* SNP was only associated with CAC in AFA ancestry and the *APOA5* and *ADAMTS7* SNPs only achieved significance in

the HIS group. Of note, SNPs previously associated with atherosclerosis in Han Chinese<sup>14,44,45</sup> were not associated with subclinical CVD in CHN in this study. With the exception of CHN, these differences are unlikely to be explained by power as the sample sizes are equivalent across the different ethnicities. However, these findings could be explained by ethnic differences in the heritability of the subclinical CVD trait. For example the heritability of CAC in AFA has been estimated at 30% which is lower than the 50% estimated for EUA, which suggests the possibility of ethnic differences in the impact of genetic variation on subclinical CVD.<sup>46,47</sup> Moreover, these differences in heritability could reflect a greater impact of environmental factors on subclinical CVD or a potential greater role for gene-environment interactions.

Differences in linkage disequilibrium (LD) structure in different ethnicities<sup>48</sup> could potentially explain the different genetic associations with subclinical CVD. It is possible that SNPs used in this study are not adequate markers of the functional SNPs responsible for a potential genetic effect on subclinical CVD. As such, lower LD between the marker SNPs and functional SNPs could decrease the effect size and therefore the power for detection. We attempted to overcome this limitation for one of the loci included in the present study. The 9p21 locus is one of the most consistently associated loci to emerge from modern genetic analysis of CVD susceptibility. There were no associations between the 9p21 SNPs in this study and subclinical CVD in AFA and CHN and our meta-analysis across ethnicities shows increased heterogeneity in this region. Prior studies have also failed to show association with 9p21 and subclinical CVD and CVD in AFA.<sup>21,23,25</sup> In order to uncover a potentially as-yet-unmeasured functional SNP in this locus we performed fine mapping in this region but again found no significant associations in AFA and CHN despite robust associations in EUA and HIS. Although, this fine mapping experiment suggests that there is no association between the 9p21 locus and CAC in AFA and CHN, race-specific maps of this region achieved de novo sequencing would be needed to solidify this observation.

It is possible that different biological pathways in the development of subclinical CVD and CVD are at play in different ethnicities. This observation is supported by the fact that greater European admixture in AFA populations has been previously associated with higher CAC<sup>49</sup>. However, it is important to note that although certainly related, subclinical CVD and CVD represent different phenotypic endpoints with potentially different pathophysiology depending on ethnicity. For example, AFA have been shown to have lower CAC scores but higher CVD as compared to other ethnicities.<sup>34,50</sup>

In conclusion, this is the first study testing the association of previously identified SNPs in GWAS of CVD and subclinical CVD with subclinical CVD across three different ethnicities of non-European descent. Although our study is limited by power to detect associations compared to the larger power in the original GWAS describing the associations, we describe several associations with subclinical CVD. Furthermore, we point out lack of association between 9p21 and CAC in AFA despite a fine mapping effort in this region suggesting a lack of signal in this region in AFA.

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