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Genetics of Subclinical Coronary Atherosclerosis

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Abstract

Purpose of Review.—This review highlights recent findings regarding genetics of coronary artery calcification (CAC), a marker of subclinical atherosclerosis burden, that is a precursor of clinical coronary artery disease.

Recent findings.—CAC quantity is heritable. Genome wide association studies of common single nucleotide polymorphisms have identified genomic regions explaining ~2.4% of CAC heritability. Low frequency and rare variants explain additional variation in CAC. Evidence suggests that there may be different genetic etiologies for variation in CAC progression than for cross-sectional measures of CAC. Studies integrating multiple -omics data are providing new insights into the pathobiology of subclinical coronary atherosclerosis.

Summary.—The future is promising for innovative studies utilizing whole genome sequencing data as well as other -omics such as epigenomic modifications of genes and gene expression. These studies may provide multiple sources of data pointing to the same gene or pathway, thus providing greater confidence in findings.

Keywords

MESH; Atherosclerosis; Coronary Artery Disease; Disease Progression; Genomics; Epigenomics

INTRODUCTION

Coronary artery disease (CAD), which causes 1 in 7 deaths in the US, is a major preventable health burden. Direct and indirect costs of CAD are \$204.8 billion dollars per year [1]. CAD is due primarily to development and progression of subclinical atherosclerosis, which develops over several decades and often remains asymptomatic until the occurrence of an acute, life-threatening event. One-half of sudden CAD deaths occur in persons without previous symptoms [2]. Two subclinical measures of atherosclerosis have been used to predict CAD. One is carotid intima-media thickness (cIMT), a measure of the intimal and

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Compliance with Ethical Standards

Conflict of Interest: Dr. Bielak and Dr. Peyser report grants from NIH/NHLBI during the conduct of study.

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medial layers of the carotid artery walls, and the other is coronary artery calcification (CAC), a marker of subclinical coronary atherosclerosis. cIMT and CAC are moderately correlated within individuals [3,4]. CAC is a better predictor of incident CAD than cIMT presumably because it appears in a more clinically relevant vascular territory [5]. Thus, this review will focus solely on CAC.

Risk factors for CAC include older age, male sex, family history of premature CAD, elevated levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C), reduced high-density lipoprotein cholesterol levels, hypertension, physical inactivity, obesity, type 1 and type 2 diabetes, and cigarette smoking [6]. While subclinical coronary atherosclerosis is a heritable trait, most genetic variants influencing this trait are unknown.

Development and progression of atherosclerosis can be viewed as a complex inflammatory process involving multiple biologic pathways and environmental cues [7]. Cellular processes leading to atherosclerotic plaques are initiated by injury to the endothelial and smooth muscle cells of the arterial wall. This injury is followed by increased adherence and migration of monocytes and T lymphocytes into the subendothelial space. Monocytes develop into macrophages, accumulate lipids, and together with the accompanying lymphocytes, form the fatty streaks that develop into fibrous plaques. The fibrous plaques increase in extent and thickness and undergo calcification and vascularization [7]. This chronic condition is converted to an acute event by plaque rupture and resultant thrombosis [8].

The study of the genetic architecture of subclinical coronary atherosclerosis has public health importance. Identification of susceptibility genes for subclinical coronary atherosclerosis may improve our understanding of its pathogenesis and lead to discovery of improved molecular markers of CAD as well as improved preventive strategies and treatments.

In this review, we first summarize the biological and clinical significance of CAC. We then review our current understanding of the genetic underpinnings of CAC, including 1) its heritability, 2) common and rare variants that have been associated with variation in CAC, and 3) new areas of research including genetic risk scores, progression of CAC, clonal hematopoiesis of indeterminate potential (CHIP), epigenomics, and the integration of other - omics to enhance understanding of the genetic architecture of subclinical coronary atherosclerosis.

Biology of CAC.

CAC is an organized, regulated process similar to bone formation, but it occurs only when other aspects of coronary atherosclerosis are also present [9]. The mineral in CAC is hydroxyapatite, the same mineral in bone, and mechanisms of CAC development are similar to osteogenesis. While CAC has been found in preatheromas, it most often occurs in fibroatheromas found in the third or fourth decade of life [10].

Studies of smooth muscle cells (SMCs) and their derivatives reveal sources of calcification. SMCs release matrix vesicles that undergo calcification in atherosclerosis [11]. Foam cells

derived from SMCs express osteopontin mRNA [12]. The expression of this gene is induced by factors present in the fatty streak in the developing atherosclerotic lesion. Osteocalcin, which is actively involved in transport of calcium out of vessels, binds directly to hydroxyapatite and has been identified within atherosclerotic plaques [13]. Osteonectin has a high affinity for hydroxyapatite and type I collagen. Matrix Gla protein (MGP) and osteopontin, two regulators of calcification in bone, are also highly expressed in plaques [14]. Osteopontin mRNA, expressed by macrophages surrounding the plaque, is related to the degree of atherosclerosis [15]. A calcifying vascular cell (CVC) has been found in large and medium arteries [16]. This cell has many characteristics of pericytes and is induced to calcify by expression of the same set of genes expressed during bone formation. CVCs aggregate into nodules, form hydroxyapatite mineral, and produce bone differentiation factors. CVCs also produce extracellular matrix that influences the rate and amount of calcification produced *in vitro* [17].

The role of calcification in the inflammatory response of atherosclerosis is still not fully understood. It has been hypothesized that oxidized lipids may trigger the immune system to respond as it does to a bacterial infection [18]. The immune system will often respond to a long-standing infection or inflammation in soft tissues with heterotopic bone formation around the site that will serve to wall off the infectious organism. While calcification may represent the body's attempt to strengthen the plaque [19], a soft plaque may have a point of weakness induced by inflammation sitting on an area of calcification [8]. This may predispose the plaque to rupture because the tissue interface of differing physical properties is subjected to the pulsatile changes of the arterial blood pressure [8,13].

These and other findings indicate that calcification is an active process and not simply a passive precipitation of calcium phosphate crystals. The calcific burden within a plaque is modulated by many different factors under genetic control [20–25].

Clinical Significance of CT Measures of CAC.

CAC can be measured non-invasively and without contrast with computed tomography (CT) [9]. The most commonly used measure of quantity of CAC is the score that was first described by Agatston and colleagues in 1990 [26]. The Agatston score is calculated by multiplying the calcific area (mm^2) of each hyperattenuating foci by a weighting coefficient based on the peak CT number (in Hounsfield Units (HU)) in the foci. The coefficient ranges from 1 to 4 where 1 = peak 130 to 199 HU; 2 = peak 200 to 299 HU; 3 = peak 300 to 399 HU; and 4 = peak 400 HU. The total score is summed across the four major epicardial arteries: the left main, left anterior descending, circumflex, and right coronary arteries.

There is a strong, positive relationship between CT measured CAC and both histological [27] and *in vivo* ultrasound measures of combined calcified and noncalcified plaque [28]. After adjustment for verification bias (i.e., the bias introduced through use of symptomatic patients with their CAD status verified by angiography), the sensitivity and specificity to detect 50% stenosis are 97% (95% CI = 95.1%, 98.8%) and 72% (95% CI = 68.6%, 75.4%), respectively [29]. Importantly, sensitivity and specificity do not vary by ethnic group [30].

Race/ethnic group differences in CAC exist and are not explained by race/ethnic group differences in CAD risk factors [31]. CAC is a strong, independent predictor of myocardial infarction (MI), cardiovascular disease (CVD) deaths, and all-cause mortality in men and women across different race/ethnic groups [32,33]. At the same burden of CAC, African Americans (AAs) have increased events compared to those of European Ancestry (EA) [32,33]. Notably, increased CAC independently predicts higher risk of events across all age groups [34] including in those at low lifetime risk of CAD based on their other CAD risk factors [35].

In addition to MI, CVD deaths, and all-cause mortality, CAC also is an independent predictor of stroke [36], heart failure [37], and atrial fibrillation [38]. Recently, CAC has been associated with non-CVD outcomes including cancer, chronic kidney disease, pneumonia, chronic obstructive pulmonary disease, and hip fracture [39]. These associations may represent shared risk factors (both measured and unmeasured) between CVD and non-CVD outcomes as well as vulnerability to development of tissue injury. These associations may also reflect that CAC is a measure of arterial aging [40].

CAC Quantity is Heritable.

Our own study of 698 asymptomatic EA adults from 302 families examined the heritability of quantity of CAC. We found that more than 40% of the inter-individual variation in quantity of CAC not explained by traditional CAD risk factors was attributable to genetic factors [41]. This study suggests there is a substantial genetic component for variation in CAC quantity, even after accounting for the effects of genes that may act through some measured traditional CAD risk factors [41]. In a study of five AA cohorts, heritability of CAC quantity was estimated to be ~ 30% and tended to be lower than what has been observed among EA cohorts [42].

Common Variants Underlie CAC.

Associations between variants in genes regulating vitamin D metabolism and CAC quantity were meta-analyzed in three independent EA cohorts [43]. There was a significant association between a common single nucleotide polymorphism (SNP) (rs2762939) in *CYP24A1* (Cytochrome P450 Family 24 Subfamily A Member 1) and CAC quantity. This gene plays a crucial role in calcium homeostasis and in maintaining vitamin D metabolism which suggests a role of vitamin D metabolism in subclinical coronary atherosclerosis.

A genome-wide association study (GWAS) of CAC carried out in almost 10,000 EA individuals (with replication in an independent sample of more than 6,000 EA individuals) identified common noncoding SNPs in the 6p24 and 9p21 regions, near *PHACTR1* (Phosphatase and Actin Regulator 1) and *CDKN2A/CDKN2B* (Cyclin Dependent Kinase Inhibitor 2A and Cyclin Dependent Kinase Inhibitor 2B), respectively [44,45]. Both of these genomic regions are associated with MI and CAD [46]. *PHACTR1* is an actin and protein phosphatase 1 binding protein that regulates the reorganization of the actin cytoskeleton. It plays a role in tubule formation and in endothelial cell survival. Beaudoin et al., (2015) found that gene expression of *PHACTR1* increases in heart tissue following MI [47]. Recently, the lead SNP in *PHACTR1* (rs9349379) has been shown to be the putative causal

variant that regulates the expression of Endothelin 1 (*END1*) located 600 kb upstream of *PHACTR1* [48]. *EDN1* encodes a preproprotein that is proteolytically processed to generate a secreted peptide that is a potent vasoconstrictor.

While 9p21 is the most replicated region of human atherosclerosis, there are no protein coding genes in the region. In addition, there are no associations with this region and common CAD risk factors suggesting this region exerts effects through other mechanisms. Evidence suggests that 9p21 risk variants alter the expression of the non-coding RNA ANRIL, thereby altering the activity of two nearby cyclin-dependent kinase inhibitors (CDKN2A and CDKN2B) that are involved in regulating the cell cycle and cell proliferation. ANRIL is also involved in cell adhesion. Modulation of ANRIL expression affects epigenetic regulation of target gene expression in cis and in trans [49–51]. More recently, a circular ANRIL RNA (circANRIL) has been identified that is thought to promote protection from atherosclerosis by culling overproliferating cell types in atherosclerotic plaques [52]. Recently, Schmidt et al. (2017) have shown that genetic effects of the common variant in the 9p21 region are strongest in those with the lowest incomes and thus there is interaction with an indicator of socioeconomic status [53].

A CAC GWAS in almost 6,000 AAs, however, did not find evidence for association in either of these two regions; nor did the study identify any new regions [42]. The study was powered to detect effects comparable to effects identified in the EA studies suggesting the genetic architecture of CAC in AAs and EA individuals may differ.

Admixture mapping for CAC can detect genetic associations by identifying genomic regions where an association exists between genetic ancestry and CAC. Admixture mapping peaks are generally not as well defined as GWAS signals. Admixture mapping in AAs with type 2 diabetes identified genomic regions where EA ancestry was associated with CAC [54]. Divers et al. (2013) reported that they replicated the 9p region association and identified interesting regions on chromosomes 1 and 4 [54]. The signal on chromosome 1 is near the Low-density Lipoprotein Receptor Related Protein 8 gene (*LRP8*) which encodes an apolipoprotein E receptor. The signal on chromosome 4 is near Protein Kinase, CGMP-Dependent, Type II (*PRKG2*) in a region that includes genes involved in bone metabolism. Another admixture mapping study in AAs [55] identified Bone Morphogenetic Protein 6 (*BMP6*), on chromosome 6 which is expressed in vascular calcific lesions and was among the top signals in GWAS in AAs [42].

In a study of ~25,000 EA and AA individuals, having at least one copy of the *APOE* ϵ 2 allele was associated with a 22% decrease in CAC versus those with no copies of this allele. This was the only novel finding in those of both ancestries [56]. In further analyses conditioning on LDL-cholesterol levels, having at least one copy of the *APOE* ϵ 2 allele remained statistically significantly associated with lower CAC levels. The *APOE* ϵ 2 allele association with decreased CAC may be due to increased clearance of atherogenic LDL and remnant lipoprotein particles [56]. Furthermore, having at least one copy of the *APOE* ϵ 2 allele was significantly associated with decreased risk of coronary heart disease (Odds Ratio 0.77; 95% CI: 0.71, 0.84) which is consistent with many other studies [57]. While rs7412 is on the HumanExome BeadChip used in this study and defines whether ϵ 2 allele is present or

not, rs429358, which defines whether $\epsilon 4$ is present or not, was not included on the HumanExome BeadChip. Given the absence of $\epsilon 4$ from the HumanExome BeadChip, it was unknown whether $\epsilon 2$'s apparent effect on reduced CAC quantity was due to a referent that includes a previously described risk allele ($\epsilon 3 + \epsilon 4$). Almost 6,000 EA individuals in the study had the major *APOE* genotypes assessed by polymerase chain reaction. Each *APOE* genotype's association with CAC was compared to $\epsilon 3/\epsilon 3$, the referent. $\epsilon 2/\epsilon 3$ was associated with 10.8% reduced CAC (95% CI: -19.6, -0.01%) and $\epsilon 2/\epsilon 2$ with 27.4 % reduced CAC (95% CI: -45.2, -0.04%) [56].

Rare Genetic Variants Underlie CAC.

Mutations associated with extreme levels of lipid traits have been associated with CAC. The Amish population of Lancaster County, PA, for example, is enriched with two such variants due to a founder effect. One mutation, a truncating mutation (R19X) in *APOC3*, is associated with lower postprandial triglyceride levels following an oral fat challenge test [58]. This variant is associated with lower quantity of CAC in carriers compared to non-carriers of this variant. The second mutation, the R3527Q variant in *APOB*, interferes with the folding of apolipoprotein B, which reduces the ability of the LDL-C particle to bind to the LDL receptor. This mutation is a known cause of familial defective apolipoprotein B-100. Due to a founder effect, approximately 12% of all Amish in the Lancaster community carry the *R3500Q* mutation, compared to only 0.1% to 0.4% in EAs. This mutation is strongly associated with CAC, highlighting the role of high LDL-C levels in the development of CAC [59]. In a study of individuals from the Netherlands with Low Density Lipoprotein Receptor (*LDLR*) mutations (Familial Hypercholesterolemia) individuals with the rare mutation show extensive CAC [60].

Genetic Risk Scores.

Risk scores such as the Framingham Risk Score combine information of major CAD risk factors to estimate an individual's risk of having an event over a specified period of time (e.g., next 10 years) [61]. Morrison et al., (2007) extended this concept to aggregate the contribution of multiple SNPs, previously identified in genomic studies of CAD, into a single genetic risk score (GRS) [62]. Salfati et al., (2015) investigated a GRS based on 49 established variants for CAD with the presence of subclinical coronary atherosclerosis among subjects asymptomatic for clinical CAD [63]. Study participants were stratified into 4 age groups (15-34, 35-54, 55-74, and >75 years). The youngest age group was ascertained through the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study and their subclinical coronary atherosclerosis was assessed from histologic samples. The remaining participants were ascertained through five ongoing studies and CT measures of CAC were used as markers of subclinical coronary atherosclerosis. The GRS was significantly associated with advanced subclinical atherosclerosis across all age groups. In sensitivity analyses, the results were similar when the GRS was restricted to SNPs that were not associated with traditional risk factors or when the models were adjusted for traditional risk factors. Importantly, the GRS was significantly associated with subclinical coronary atherosclerosis across the life-course.

Progression of CAC.

In a study of 877 asymptomatic EA individuals, the heritability of CAC progression was 40% after adjusting for baseline CAD risk factors and baseline CAC [64]. Progression of CAC was based on 2 CT measures of CAC obtained an average of 7.3 years apart. The genetic correlation between baseline CAC quantity and CAC progression was estimated to assess pleiotropic effects. There was evidence suggesting that there were unique genes for each trait as well as a shared genetic component [64]. Candidate gene studies have identified specific polymorphisms associated with CAC progression but not with the individuals' baseline CAC quantity [65,66]. A SNP in Matrix gla protein was associated with CAC progression, but not with cross-sectional measures of CAC [67].

Aherrahrou et al., (2017) investigated *PHACTR1*'s expression over time in *in vitro* murine embryonic stem cell (mESC)-derived smooth muscle cells (SMC) [68]. They found evidence that down-regulation of *PHACTR1* in mESC-derived SMCs decreased calcification; additionally gene expression of *PHACTR1* increased in response to a calcification stimulus and was further enhanced as calcification progressed [68].

Consistent with earlier studies [64], the work of Aherrahrou et al., (2017) suggests there may be different genetic etiologies for variation in CAC progression than for variation in cross-sectional measures of CAC [68].

CHIP.

Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related disorder that is common in older individuals. CHIP is the presence of an expanded somatic bloodcell clone in individuals without other hematologic abnormalities. In a study of somatic mutations leading to CHIP, CHIP carriers were identified based on a list of 74 genes previously found to be recurrently mutated in myeloid cancers [69]. The presence of CHIP was significantly associated with the presence of CAD in a study of over 8,000 individuals (4726 with CAD and 3529 controls). In samples obtained from the BioImage Study, among those without incident coronary heart disease, CHIP carriers had a median CAC score that was 3.3 times higher in carriers versus non-carriers [69]. They also found the presence of CHIP was significantly associated with increased atherosclerosis in mice [69]. This study provided important insights into how aging may contribute to increased subclinical coronary atherosclerosis and CAD events.

DNA Methylation.

Epigenetics is the study of heritable alterations in phenotypes and gene expression that occur without changes in the DNA sequence. Epigenetic mechanisms are flexible genomic factors that not only can change genome function under exogenous influence (e.g., diet), but also provide a molecular substrate that allows for stable propagation of gene expression states from one generation of cells to the next. The best understood epigenetic mechanism is DNA methylation (DNAm), a covalent modification that, in mammals, occurs predominantly at cytosines followed by guanines (i.e., CpG dinucleotides) to form 5-methylcytosines. DNAm states may vary over an individual's lifetime, and have been shown to regulate biological

processes underlying coronary atherosclerosis through associations with risk factors such as inflammation, hypertension, diabetes, and smoking [70–73].

An epigenome-wide association study identified DNAm sites associated with MI risk [74]. In a recent study of DNAm at two examinations five years apart, those with stable CAD risk factors had a different DNAm profile compared to those with worsening risk factors over time [75]. Among DNAm sites associated with risk factor trajectories, one-fourth were stable over time, while the rest were dynamic. One-third of DNAm sites associated with trajectories of risk factors were also associated with progression of CAC from 0 (i.e., not detectable) to CAC >0 five years later [75].

Integrating Across Omics.

Rather than using a single variant or single gene approach, Sen et al., (2014) used an integrated multi-omic and functional approach to identify genes associated with CAC [76]. RNA sequencing was used in a sample of individuals with extreme quantities of CAC (the cases) and matched controls with no detectable CAC to identify dysregulated transcripts in peripheral blood transcriptome data. They found that *TREML4* (Triggering Receptor Expressed On Myeloid Cells Like 4) was upregulated in the CAC cases. At rs2803496, a *TREML4* cis-eQTL, having the C allele was associated with a 6.5-fold increased relative risk of having a CAC score >1,200. Additionally, they found that *TREML4* localized in macrophages surrounding the necrotic core of atherosclerotic plaques with calcification. This study demonstrated a fruitful approach for clinical gene discovery.

Future Areas of Research.

A critical next step is further investigations of low frequency and rare variants in coding and non-coding regions using whole genome sequencing (WGS) data. Additionally, further integrating WGS data with other -omics such as RNA expression profiles and metabolomics will help uncover factors associated with subclinical coronary atherosclerosis. To this end, the National Institutes of Health's National Heart, Lung and Blood Institute (NHLBI) Trans-Omics for Precision Medicine (TOPMed) effort is generating scientific resources to increase our understanding of processes that underlie multiple disorders including subclinical coronary atherosclerosis and related traits and risk factors. One of the many benefits of TOPMed is that studies with genotypic data but without WGS will be able to impute genotypes to a TOPMed reference panel with improved accuracy for low-frequency and rare variants.

Mendelian randomization is one method to evaluate possible causal relationships suggested by association analyses by using genetic information, intermediate biomarkers, and outcomes [77]. It has the ability to control for confounding and reverse causation [78]. However, interpreting Mendelian randomization studies is not straightforward and is based on important assumptions including that measured and unmeasured factors do not confound the exposure-outcome relationship [79]. In addition to providing evidence of causality, Mendelian randomization studies may provide evidence to refute causal evidence.

The majority of genetic studies have been conducted in samples of EA individuals. However it is important to conduct further studies across ancestrally diverse groups.

The future is promising for the investigation of context dependent genome effects (gene-by-environment interactions) [80]. Recently, a large multi-ethnic GWAS study identified novel genes for blood pressure, a major risk factor for CAD, when including SNP-by-smoking interactions in the models [81].

There are sex differences in the biology and manifestations of both subclinical and clinical CAD; therefore, using the same disease definition for men and women may not be appropriate. Few genetic studies, however, have considered the sexual dimorphism in atherosclerosis with appropriate study designs [82]. A better understanding of differences in the etiology of subclinical coronary atherosclerosis between women and men would enhance future prevention efforts.

CONCLUSIONS

Genetic studies published to date have identified a few common variants associated with CAC quantity explaining only a small fraction (~2.4%) of the genetic contribution to CAC quantity. Known common variants for CAD explain an additional 4%. The ability to analyze the entire genome through WGS will provide detailed data on every rare and low frequency variant, as well as common variants not well imputed, to identify potentially causative variants involved in CAC variation. Additionally, the ability to examine epigenomic modifications of genes regulating adaptive cellular pathways and the integration of other - omics can provide further understanding of mechanisms for variation in CAC and subclinical coronary atherosclerosis.

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