Genetic Regulation of Atherosclerosis-Relevant Phenotypes in Human Vascular Smooth Muscle Cells

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ABSTRACT

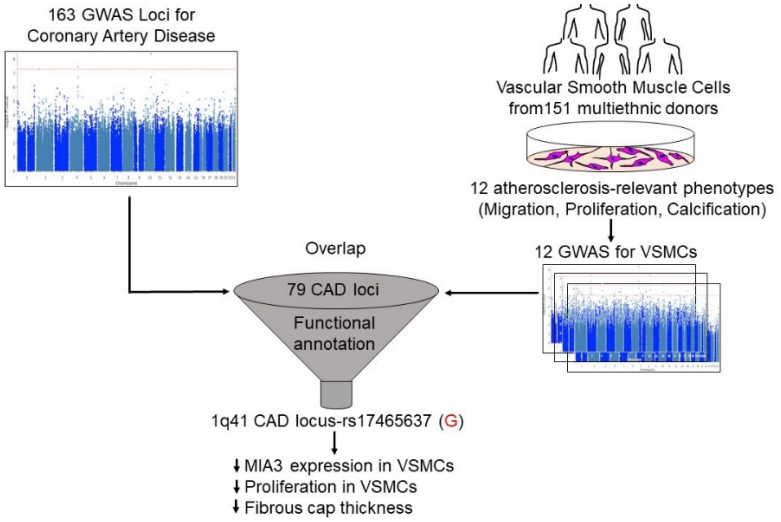
***Rationale*:** Coronary artery disease (CAD) is a major cause of morbidity and mortality worldwide. Recent genome-wide association studies (GWAS) revealed 163 loci associated with CAD. However, the precise molecular mechanisms by which the majority of these loci increase CAD risk are not known. Vascular smooth muscle cells (VSMCs) are critical in the development of CAD. They can play either beneficial or detrimental roles in lesion pathogenesis, depending on the nature of their phenotypic changes.

***Objective*:** To identify genetic variants associated with atherosclerosis-relevant phenotypes in VSMCs

***Methods and Results*:** We quantified twelve atherosclerosis-relevant phenotypes related to calcification, proliferation, and migration in VSMCs isolated from 151 multi-ethnic heart transplant donors. After genotyping and imputation, we performed association mapping using 6.3 million genetic variants. We demonstrated significant variations in calcification, proliferation, and migration. These phenotypes were not correlated with each other. We performed GWAS for twelve atherosclerosis-relevant phenotypes and identified four genome-wide significant loci associated with at least one VSMC phenotype. We overlapped the previously identified CAD GWAS loci with our dataset and found nominally significant associations at 79 loci. One of them was the chromosome 1q41 locus, which harbors *MIA3*. The G allele of the lead risk SNP rs67180937 was associated with lower VSMC *MIA3* expression and lower proliferation. Lentivirus-mediated silencing of MIA3 in VSMCs resulted in lower proliferation, consistent with human genetics findings. Further, we observed a significant reduction of MIA3 protein in VSMCs in thin fibrous caps of late-stage atherosclerotic plaques compared to early fibroatheroma with thick and protective fibrous caps in mice and humans.

***Conclusions*:** Our data demonstrate that genetic variants have significant influences on VSMC function relevant to the development of atherosclerosis. Further, high MIA3 expression may promote atheroprotective VSMC phenotypic transitions, including increased proliferation, which is essential in the formation or maintenance of a protective fibrous cap.

Keywords:
VSMCs, calcification, migration, proliferation, genetic variants, smooth muscle cell.



Nonstandard Abbreviations and Acronyms:

BrDU = Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
CAD = Coronary artery disease
eQTL = expression quantitative trait locus
GTEx = Genotype-Tissue Expression
IL-1β = Interleukin-1 β
GWAS = Genome-wide association studies
MIA3 = Melanoma inhibitory activity protein 3
PDGF-BB = Platelet-derived growth factor BB
TGF-β1 = Transforming growth factor beta 1

INTRODUCTION

Vascular smooth muscle cells (VSMCs) are the major cell type in blood vessels. They are crucial for healthy arteries providing contractile function and structural support. Healthy VSMCs maintain quiescence and express genes essential for contraction to regulate blood pressure, yet they also retain remarkable plasticity. In pathological conditions, such as the development of atherosclerotic lesions, contractile VSMCs are thought to switch to a dedifferentiated phenotype characterized by increased proliferation, migration, and extracellular matrix synthesis[1,2]. Mouse lineage tracing studies showed that a large percentage of cells in atherosclerotic plaques, including a subset of the foam cells, were VSMC-derived[3]. Despite decades of intense research, environmental and genetic factors controlling VSMC phenotypes within lesions are poorly understood.

Advanced atherosclerosis is the underlying cause of coronary artery disease (CAD)[4]. Heritability estimates for CAD vary from 40% to 70%, suggesting strong genetic contributions to disease pathology[5]. Previous studies of the genetic architecture of CAD using linkage analysis in families with a history of myocardial infarction identified rare genetic variants with strong effects[5]. However, common forms of CAD are complex and involve a large number of variants with modest effect sizes. The most recent meta-analysis of genome-wide association studies (GWAS) performed in more than half a million individuals identified 163 loci significantly associated with CAD[6,7]. Most of the underlying genes and the related mechanisms of these loci remain unknown.

Approximately one-third of the CAD loci are associated with traditional risk factors such as blood lipids, blood pressure, body mass index, diabetes, and smoking behavior[8]. The remaining loci harbor genes implicated in novel mechanisms of disease risk related to the vessel wall[9]. For example, 9p21.3, the most significantly associated GWAS locus for CAD, was recently shown to affect VSMC adhesion, contraction, and proliferation[10]. Similarly, some preliminary work on GWAS loci that harbor the *ADAMTS7*, *TCF21*, *PHACTR1*, *GUCY1A3*, and *PLPP3* genes began to provide insight into the molecular mechanisms of atherosclerosis susceptibility and pointed to processes that involve VSMC dedifferentiation[11–13] and migration[14] as well as endothelial nitric oxide signaling[15] and mechanotransduction[16]. Identifying the genetic regulatory mechanisms associated with vessel wall processes may identify new therapeutic approaches for treating CAD.

Previous studies have investigated the candidate mechanisms of CAD GWAS loci by measuring the impact of the CAD-associated variants on gene expression[17–20]. However, in this study, we took a different approach by studying the impact of CAD-associated variants on atherosclerosis-relevant VSMC phenotypes: calcification, proliferation, and migration. We characterized a unique source of VSMCs isolated from the ascending aortas of 151 healthy and ethnically-diverse donors. We conducted GWAS of these cellular traits and identified four loci associated with calcification (3) and proliferation (1). We showed that 48% of CAD associated loci are also associated with VSMC cellular traits. Finally, we validated the predicted role of CAD associated gene *MIA3* on VSMC function.

METHODS

A detailed description of the methods and the experimental procedures are provided in the Online Data Supplement. Please see the Major Resources Table in the Supplemental Materials. The data and GWAS summary statistics that support the findings of this study will be available in the database of Genotypes and Phenotypes (dbGap).

RESULTS

Characterization of genotypic and phenotypic variation among VSMC donors.

Our approach and main results are summarized in **Figure 1**. We characterized VSMCs from 151 ethnically-diverse healthy heart transplant donors (118 male and 33 female) for atherosclerosis-relevant phenotypes under distinct environmental conditions. We had genotype information for 6.3 million variants with at least 5% minor allele frequency in our population. Clustering of the donor genotypes with 1000 Genomes reference population samples identified 6, 12, 64, and 69 of the individuals with East Asian, African, Admixed American, and European ancestry, respectively (**Figure 2A**). Next, we quantified the phenotypic variation in VSMC calcification, proliferation, and migration among all the donors (**Online Figures I-IV**).

First, we quantified the amount of calcification in media containing high inorganic phosphate or osteogenic stimuli. These two media formulations have been shown to recapitulate different aspects of arterial calcification that occurs in advanced stages of atherosclerosis[22]. We observed 787-fold difference among the cells from donors with the highest and lowest calcification potential in high phosphate media and 577-fold differences in osteogenic conditions (**Online Figure V-A and Online Table III**).

Second, we quantified DNA synthesis as a proxy for proliferation in control media and media containing PDGF-BB, IL-1β, or TGF-β1 (**Online Figure III**). All three cytokines have been shown to play a role in VSMC proliferation and the development of atherosclerosis[1,41–44]. We observed 228-, 315-, 235-, and 160-fold difference between the slowest and fastest proliferating cells in control media or media containing PDGF-BB, IL-1β, or TGF-β1, respectively (**Online Figure V-B1 and Online Table III**). To determine if there was a donor-dependent response to cytokine stimulation, we calculated the ratio of proliferation in cytokine-stimulation conditions compared to the control condition. Cells isolated from each individual responded differently to cytokine stimulation. For example, in IL-1β-containing media, cells from 18% of the donors proliferated less, whereas cells from 82% of the donors proliferated more compared to control media (**Online Figure V-B2**). These results suggest gene-by-environment interactions in the proliferative response of VSMCs under atherogenic cytokine stimulation conditions.

Third, we quantified the amount of migration to a PDGF-BB gradient over 24 hours using a modified Boyden chamber assay (**Online Figure IV**). We observed the expected increase in cells migrating to the PDGF-BB-containing lower chamber, consistent with PDGF-BB as a pro-migratory stimulus for VSMCs in atherosclerosis[45]. Using the continuous migration monitoring data, we calculated three distinct phenotypes: difference in (i) area-under-the-curve, (ii) the rate of initial migration, and (iii) the time to reach the maximum amount of migration between PDGF-BB and control media. These phenotypes represent VSMCs' total capacity for migration, the rate with which they can migrate, and how fast they can respond to PDGF-BB stimulus, respectively. We observed 66-, 130-, and 25-fold differences in these three traits among the donors (**Online Figure V-C and Online Table III**).

Overall, we assessed 12 quantitative phenotypes related to calcification, proliferation, and migration (**Figure 1**). We tested for repeatability of cellular phenotyping to rule out the contribution of technical artifacts in the quantification of VSMC phenotypes. We performed the calcification, proliferation, and migration experiments using cells from four donors at two different passages (P3 and P5). We repeated each experiment three times on different days. The hierarchical clustering of the phenotype data showed grouping based on the donor, suggesting that donor-to-donor variation was larger than within-donor variation (**Online Figure VI**). Finally, we calculated the Spearman correlation of all phenotypes with each other. 12 pairs of phenotypes had a significant correlation ($R > |0.18|$, FDR < 5%) (**Figure 2B**). The majority of these pairs were within a phenotypic group. For example, VSMCs that highly proliferate when stimulated with one of the cytokines were more likely to highly proliferate when stimulated with the other cytokines. Only one pair had a negative correlation, showing that the cells that had slow proliferation in control media responded highly to the PDGF-BB stimulation.

Collectively, these data suggest that genetic variation among individuals have a significant impact on atherosclerosis-relevant VSMC phenotypes.

Genetic variants associated with VSMC phenotypes.

To identify genetic loci associated with VSMC phenotypes, we performed association mapping of the 6.3 million variants and 12 traits using a linear mixed model to account for multi-ethnic population composition. Since the phenotypes were not correlated with each other, we studied the results at the genome-wide statistical significance threshold of P-value < $5x10^{-8}$, which is typically used in GWAS. Four loci were associated with two distinct VSMC phenotypes (**Table 1, Figure 3& Online Figure VII**): three were associated with calcification (rs56062640 with P-value = $4.8x10^{-8}$, rs12777350 with P-value = $4.0x10^{-9}$ and rs112162751 with P-value = $3.3x10^{-8}$) and one with proliferation (rs982228 with P-value = $3.4x10^{-8}$). To account for the impact of ancestral diversity in our population on the significance of associations, we determined that the first 17 principal components (PCs) calculated from the genotypes explained 90% of the genetic variation. Genotype PCs and the 12 traits were not significantly correlated at the 1% FDR cut-off; however, 15 of the 204 phenotype-PC pairs showed nominally significant correlations (P-value < 0.05) (**Online Table III**). We, therefore, performed association mapping either with only the genetic relatedness matrix or with the genetic relatedness matrix and the 17 genotype PCs. When the genotype PCs were added to the linear mixed model as fixed effects, one locus became more significant and three loci became less significant (**Online Table IV**). Since there was no significant correlation between the genotype PCs and the traits, we focused on the association results derived from the linear mixed model without the PCs as fixed effects.

All the significant SNPs were in non-coding or intronic regions of the genome, suggesting that potential effects might occur through changes in gene expression. Conditioning on the lead SNPs revealed a single signal at each locus (**Online Figure VIII**). The locus associated with VSMC calcification in response to the osteogenic stimulus overlapped *SYNPO2*, a typical SMC marker, which encodes Myopodin (**Figure 3**). The G allele of the most significantly associated SNP in the locus, rs112162751, is associated with increased calcification. Recent studies showed that *SYNPO2* is upregulated in high-calcified plaques compared to low-calcified plaques and calcified plaques from symptomatic patients[46]. The most significant association was between the rs12777350 genotype and calcification in response to the osteogenic stimulus (**Figure 3**). The T allele of the rs12777350, located in the eighth intron of *LIPJ*, was associated with decreased calcification (P-value=$4.0x10^{-9}$, $\beta = -0.57$). *LIPJ* encodes Lipase Family Member J, but its role in VSMC calcification is not known.

Functional annotation of the VSMC loci.

To identify cardiometabolic phenotypes that colocalize with the VSMC loci, we performed a phenome-wide association study (PheWAS) using results from the GWAS catalog[47], UK Biobank[48], and Athero-Express[49], which is a study of 2,450 patients comprising carotid endarterectomy samples in which atherosclerotic plaque characteristics were assessed and genetic data were available (n = 1,443). We looked up the association of index SNPs and their proxies (LD r^2 >0.8) in the VSMC loci with various traits. We found suggestive associations with

plaque characteristics for three of the VSMC loci at a nominal association significance (P-value < 0.05) (**Online Table V**). For example, the G allele of rs11216251 is associated with increased VSMC calcification and calcified area in atherosclerotic plaques (P-value=$1.5x10^{-2}$, β=3.24). We also found suggestive associations with CAD and other cardiovascular traits driven by vascular SMC-dysfunction (**Online Table VI**).

Since the variants in the significant VSMC loci are in non-coding regions, to identify the effector transcript(s), we determined the colocalization of expression quantitative trait loci (eQTL) using gene expression data from the mammary artery of ~600 individuals in The Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task (STARNET) cohort[17], from the aortic artery of 387 donors in The Genotype-Tissue Expression (GTEx) v8 cohort[20], or the coronary artery SMCs (CoASMCs) isolated from 52 donors[18]. No artery tissue eQTLs colocalized with the VSMC loci (**Online Table VI**). This suggests that the VSMC loci may regulate gene expression only in aortic VSMCs since the tissues contain many cell types.

Table 1: Genomic loci associated with VSMC cellular phenotypes. Minor allele frequency of the SNP in our population is shown in the MAF column.

VSMC Phenotype	Lead SNP	Ref/Alt Allele	SNP location	MAF	Nearest Genes	*P*-value	β (SE)	MAF in 1000 Genomes reference populations AFR/AMR/ASN/EUR
Calcification (Osteogenic media)	rs56062640	G/A	chr2:40303792	0.09	*SLC8A1-AS1*	$4.8x10^{-8}$	-0.53 (0.09)	0.12/0.05/0.00/0.09
Calcification (Osteogenic media)	rs112162751	G/A	chr4:119801647	0.08	*SYNPO2*	$3.3X10^{-8}$	-0.54 (0.09)	0.06/0.08/0.16/0.01
Calcification (Osteogenic media)	rs12777350	C/T	chr10:90361836	0.06	*LIPJ*	$4.0X10^{-9}$	-0.57 (0.09)	0.00/0.05/0.11/0.06
Proliferation (IL-1β)	rs982228	G/A	chr5:162738838	0.13	*CCNG1*	$3.4X10^{-8}$	-0.37 (0.06)	0.01/0.15/0.36/0.15

Association of VSMC phenotypes with genetic susceptibility to CAD.

We calculated the genetic risk score (GRS) of the 151 donors in our population using the 163 lead CAD SNPs identified in GWAS[7]. We had genotype information on 155 of the 163 SNPs and used proxy SNPs for another three loci in our population. Overall, 158 CAD loci could be tracked in our dataset. There was a 1.5-fold difference in the CAD GRS between the donors with the highest and lowest risk scores. There was no significant association between the CAD GRS and VSMC phenotypes at the Bonferroni-corrected *P*-value threshold of $4.1x10^{-3}$ (0.05/12 phenotypes); however, VSMC migration was negatively correlated with CAD GRS at nominal significance (**Online Figure IX**).

In a second approach, to identify which of the 158 CAD GWAS loci might be impacting susceptibility to CAD via a VSMC-selective role, we investigated whether there was an association with any of the VSMC phenotypes. None of the CAD loci showed association at the Bonferroni-corrected *P*-value of $2.6x10^{-5}$ (0.05/(12 phenotypes*158 variants)). However, 79 CAD loci showed a nominal association with at least one VSMC phenotype (*P*-value < 0.05) (**Figure 4**). Only 13 of the 79 CAD loci have an association with serum lipid levels suggesting that our results were enriched for loci functioning in the vessel wall. The majority of the CAD loci were found to be associated with proliferation (33 loci). 11 and 20 were found to be associated with migration and calcification, respectively. Furthermore, 10 of them overlapped with more than one VSMC phenotype. The different risk alleles showed distinct effects on VSMC phenotypes. For example, while the risk allele of rs67180937 in the *MIA3* locus was associated with lower proliferation response to TGF-β1 (*P*-value= 0.04, β =-2.1), the risk allele of rs3825807 in the *ADAMTS7* locus was associated with higher proliferation response to TGF-β1 (*P*-value=0.04, β =2.4) (**Figure 4**). We also found that in independent risk SNPs (LD $r^2 = 0$) in three loci (*TGFB1*, *LPL*, and *COL4A2*) were associated with distinct VSMC phenotypes (**Online Figure X**). For example, the risk allele of the independent SNPs rs12980942 and rs8108632 in the *TGFB1* locus were associated with higher calcification (*P*-value=0.05, β =2.0) and lower proliferation (*P*-value=0.04, β =-2.1), respectively. These results demonstrate the complexity of the functional impact of the CAD GWAS loci and are consistent with the detrimental or beneficial roles VSMCs play in atherosclerosis.

We functionally annotated the 79 loci using the PheWAS approach based on the GWAS catalog[47], UK Biobank[48], and eQTL colocalization based on GTEx v8 gene expression datasets[20] (**Online Figure XI**). 32 of the 79 loci had an eQTL effect in an artery tissue. The SNP rs67180937 had the highest significant association with CAD (*P*-value=$1.6x10^{-13}$, OR=1.08)[6] among the 32 eQTLs and was not associated with blood lipid levels (*P*-value=0.61-0.86)[50]. The risk allele (G) of SNP rs67180937 is associated with lower proliferation (**Figure 5A**) in our dataset (*P*-value= 0.04, β =-2.1). We identified SNP rs67180937 in the 1q41 locus as a *cis*-eQTL for *MIA3* expression in arterial tissue (*P*-value=$8.6x10^{-7}$, β =-0.17)[20]. The SNP was not associated with MIA3 expression in monocytes/macrophages [51] or aortic endothelial cells[52]. The risk allele (G) was significantly associated with lower MIA3 expression in aortic (*P*-value=$8.6x10^{-7}$, β =-0.17) and tibial arteries (*P*-value=$3.6x10^{-5}$, β =-0.09) from 387 and 584 GTEx donors, respectively (**Figure 5B**). While these tissues contain many cell types, the absence of a significant association between *MIA3* expression and rs67180937 in monocytes/macrophages and aortic endothelial cells suggests that the regulatory impact of the variants may be in VSMCs. In support of this, we found that seven SNPs in high linkage disequilibrium (LD $r^2 > 0.7$) with rs67180937, resided in an enhancer identified by H3K27ac epigenetic marker in human coronary artery SMCs[53] (**Online Figure XII**). Therefore, we measured *MIA3* expression in 143 homozygous and heterozygous carriers of the risk or non-risk alleles of rs67180937 in VSMCs from our human donors and also found that lower *MIA3* was associated with the risk allele (*P*-value=$6.4x10^{-5}$, β =-0.39) (**Figure 5C**). To validate our human genetics-predictions, we transduced VSMCs from two heterozygous carriers of the risk allele with three constructs encoding an shRNA against the *MIA3* transcript separately. *MIA3* expression was downregulated 72-80 % compared to controls transduced with scrambled shRNA (**Figure 5D**). The silencing of *MIA3* resulted in a significant decrease in proliferation (10%-69% decrease) compared to control with two of the shRNAs (**Figure 5E**). There was also a significant positive correlation between *MIA3* expression and SMC proliferation in control condition or in response to TGF-β1 stimulation, consistent with our genetic predictions (r=0.37, *P*-value=$1.1x10^{-5}$ shown in **Figure 5F** and r=0.37, *P*-value=$8.9x10^{-6}$ shown in **Online Figure XIII**).

Collectively, these studies suggest that the risk variant in the 1q41 CAD locus affects atherosclerosis by reducing *MIA3* expression, which, in turn, negatively regulates the proliferation of VSMCs, which is important for the formation of a protective fibrous cap **(Figure 1)**.

Based on our human genetics and *in vitro* results, we postulated that MIA3 might play a role in fibrous cap stability in atherosclerotic lesions. Hypercholesterolemic *ApoE*-null mice deficient in Oct4 expression in their VSMCs have been shown to have lower indices of plaque stability in their atherosclerotic lesions compared to wild-type controls, including marked reductions in the number of SMCs within lesions and the fibrous cap[40]. Therefore, we quantified the number of MIA3 expressing ACTA2-positive VSMCs in vulnerable lesions of hypercholesterolemic $VSMC^{Oct4\text{-}\Delta/\Delta}$ $ApoE^{-/-}$ mice and compared the results to $VSMC^{Oct4\text{-}WT/WT}$ $ApoE^{-/-}$ control mice. These mice were fed a Western diet for 18 weeks and had advanced vulnerable atherosclerotic lesions as described previously[40]. Oct4 deletion in VSMC did not affect *MIA3* expression using either RNAseq or ChIP-seq analysis[40]. Immunofluorescence staining for MIA3 protein demonstrated its presence in the VSMCs of the aorta and fibrous cap (**Figure 5G & Online Figures XIV & XV**). Fewer ACTA2-positive VSMCs were also MIA3-positive in vulnerable plaques of the $VSMC^{Oct4\text{-}\Delta/\Delta}$ $ApoE^{-/-}$ mice compared to $VSMC^{Oct4\text{-}WT/WT}$ $ApoE^{-/-}$ control mice (**Figure 5H**). We also observed a significant reduction of MIA3 expression in ACTA2-positive VSMCs in the thin-cap region of late-stage fibroatheroma compared to the VSMCs in the protective thick fibrous cap in human coronary artery lesions (**Figure 5I & Online Figures XVI-XVII**). In contrast, no significant changes in MIA3 protein abundance were seen in CD68-positive macrophages. This suggests that lower MIA3 in VSMCs, resulting in lower proliferation, may render the atherosclerotic plaques vulnerable to rupture as a result of the weakened fibrous cap and thereby may increase CAD risk.

DISCUSSION

VSMCs are the major cell types capable of synthesizing components of the fibrous cap in atherosclerotic plaques whose rupture or erosion may trigger myocardial infarction[54]. The number of VSMCs in fibrous caps is directly correlated with plaque stability[55]. Migration and proliferation of VSMCs to the fibrous cap and their subsequent calcification are important determinants of plaque stability. In this study, we sought to identify the genetic determinants of the processes by which VSMCs may contribute to the stability of the fibrous cap (**Figure 1**). Since it is not possible to study these processes in detail in the arteries of living humans, we used cultured VSMCs isolated from an ethnically-diverse population of 151 heart transplant donors to understand the genetic regulation of naturally occurring variation in VSMC calcification, proliferation, and migration. However, since the number of donors with different ancestries were not equal, we were not able to assess the impact of transethnic genetic differences on VSMC function.

Quantification of VSMC phenotypes in cell culture relevant to atherosclerosis, which takes decades to develop in arteries, presents several challenges. For example, the culture conditions lack the key interactions with other cell types and environmental conditions in the vessel wall and the atherosclerotic plaque. Further, environmental insults accumulated over a long period cannot be adequately replicated *in vitro*. However, CAD-associated variants are predicted to affect vascular wall function[9]. Previous studies[18] employed cultured human coronary artery SMCs (HCASMCs) to investigate the genetic basis of gene expression and identified quantitative trait loci and performed fine-mapping of CAD loci. This same group[56] also demonstrated co-clustering of chromatin accessibility regions between cultured HCASMC and *in vivo* coronary artery segments, further supporting the relevance of these cells to native SMCs in the vessel wall and disease biology. Since vascular wall phenotypes are difficult or impossible to measure in cellular detail in the arteries of humans, our approach provides a reasonable proxy to *in vivo* characteristics of VSMCs.

We used DNA synthesis as measured by BrDU incorporation as a proxy for proliferation. However, it is important to note that DNA synthesis can also occur as cells increase their size and ploidy. This mechanism often

occurs *in vivo* and increases with age and hypertension and in conditions of vascular injury[57]. Hixon *et al* found that angiotensin II, but not PDGF-BB or TGF-β1, promoted VSMC polyploidization in capacitance arteries of rat models of hypertension[58]. PDGF-BB is a well-established stimulant of VSMC proliferation[57]; however, TGF-β1 was shown to induce both hyperplasia and hypertrophy in rat aortic SMCs[59]. In our study, BrDU incorporation was significantly correlated in conditions of PDGF-BB and TGF-β1 stimulation ($r=0.97$ P-value=3.0×10^{-6} shown in **Figure 2B**); therefore, we measured DNA synthesis as a proxy for quantifying VSMC proliferation. Further, BrDU incorporation has been shown to have a significant correlation with *in vitro* VSMC proliferation[23–25].

Pro-inflammatory cytokines have a significant presence in atherosclerotic plaques[60]. The recent success of canakinumab, a neutralizing antibody against IL-1β, which significantly reduced cardiovascular events in clinical trials, points to an important role of pro-inflammatory cytokines in atherosclerosis[61]. Therefore, we prioritized studying the impact of PDGF-BB, TGF-β1, and IL-1β on VSMC phenotypes, all of which have been shown to play significant roles in VSMC-mediated processes in atherosclerosis and are present in the atherosclerotic vessel wall[1,41–44].

Our results showed a large phenotypic variation among the donors. It is possible that some of the variations in VSMC phenotypes are due to lifestyle-induced epigenetic changes. However, a large scale study of epigenetic marks in freshly isolated tissues and cells as well as their cultured counterparts showed little correlation between the epigenomic landscape of freshly isolated and cultured cells[62]. Another source of variation could be the heterogeneity in the anatomical location of cells isolated from the arterial tissue. While we were careful to isolate the cells using the same protocol, it took more than a decade to collect the large number of donor tissues for this study; therefore, slight variations in VSMC isolation and cultivation methods cannot be ruled out. It is also possible that more proliferative SMC clones in the isolated cell population have gained increased representation. For these reasons, we chose to perform our experiments *in vitro* under identical conditions to be able to eliminate as much of the environmental effects as possible. Further, we showed that phenotypic variations due to the technical aspects of the experiments were much smaller than the variation among the donors.

Given the size of our study population, we were able to calculate the correlations among the phenotypes, which showed that calcification, proliferation, and migration are not correlated. Our results suggest that these phenotypes are independent of each other and are regulated by distinct genetic loci. This is in contrast to studies demonstrating that changes in the local environment, such as the presence of pro-inflammatory stimuli, especially PDGF-BB, changes in extracellular matrix composition, and distinct shear stress patterns induce coordinated changes in VSMC migration and proliferation[63]. This could be due to the subtle impact of genetic variants on VSMC phenotypes, which can be overpowered by environmental stimuli. It is also interesting to note that we observed gene-by-environment interactions in the proliferative response of VSMCs to cytokine stimulation. While 99% of the donors showed an increase in proliferation and migration in response to PDGF-BB stimulation consistent with previous studies[64,65], the proliferative response of VSMCs to IL-1β- and TGF-β1 stimulation was dependent on the genotype of the donors. This could explain some of the contradictory results reported in previous studies where cells from a single donor were studied. For example, whereas a study showed that TGF-β1 stimulation increased human VSMC proliferation[66], another one showed inhibition[67].

The VSMCs used in this study were isolated from healthy ascending aortas, whereas the GWAS for CAD was performed using data from individuals where lesions in coronary arteries have been assessed. It is possible that CAD risk variants may have distinct functions in coronary or arterial SMCs. One example is the transcription factor *TCF21* in the 6q23 CAD GWAS locus. While *TCF21* is expressed in human coronary artery SMCs, it is not expressed in human aortic SMCs[68] suggesting a coronary SMC-specific role in CAD risk. On the other hand, the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study clearly demonstrated the co-occurrence of atherosclerotic lesions throughout the arterial system[69]; therefore, we expect a great deal of overlap in the function of CAD GWAS variants in coronary and arterial SMCs.

Having established donor-dependent phenotypic differences, we performed GWAS on the VSMC phenotypes and identified four loci associated with at least one of the traits at genome-wide significance. To the best of our knowledge, only one other study performed GWAS for disease-relevant cellular phenotypes to identify quantitative trait loci[70]. Multi-dimensional phenotyping of disease-relevant cells to perform GWAS may uncover the impact of the genetic variants on biological mechanisms that underlie the disease. We were able to identify genome-wide significant loci by studying only 151 donors, a small number by GWAS standards, most likely because it is easier to observe the impact of genetic variants on detailed cellular phenotypes similar to expression quantitative trait locus studies measuring the impact of genetic variants on gene expression[71]. Larger numbers of donors should lead to the identification of more loci associated with VSMC phenotypes.

Variants in the CAD loci harboring *CDKN2BAS*[72] and *ADAMTS7*[73] were shown to be associated with the proliferation and migration of VSMCs. For example, the G allele of rs3825807, which is a coding variant in *ADAMTS7*, was associated with the reduced migratory ability of VSMCs compared to the A allele. The same allele was associated with increased proliferation under TGF-β1 stimulation but not with migration in our dataset. We did not find significant associations between the variants in the *CDKN2BAS* locus with VSMC proliferation. The differences in our results with published studies may be due to the cell source or culture conditions. These previous studies used either genome-edited iPSC-derived VSMCs or umbilical VMSCs, as well as different cell culture media and stimuli that may have collectively contributed to the disagreement with our results.

Some of the CAD loci with unknown mechanisms are predicted to act in the vessel wall where the disease process occurs[74]. We hypothesized that a subset of the CAD-variants affects VSMC phenotype and function at the cellular and molecular level. We identified an overlap between 79 loci and at least one VSMC phenotype. We provided evidence for the regulatory impact of 1q41 CAD locus on *MIA3* expression and proliferation of VSMCs. While this locus had been shown to be associated with increased CAD risk, the causal gene in the locus, and its effect on VSMC function were not known. MIA3 protein is localized to the endoplasmic reticulum (ER) exit site, where it loads cargo molecules, such as collagen VII, into COPII carriers to promote their secretion out of the ER[75,76]. A recent mouse study showed that MIA3 is involved in the secretion of collagens I to IV and IX from VSMCs[77]. We found that the risk allele was associated with lower *MIA3* expression and lower proliferation compared to the non-risk allele. We corroborated these results *in vitro*. While there is evidence that *MIA3* expression is associated with cell proliferation, migration, and extracellular collagen secretion in endothelial, mural, and monocytes cells[77–79], the role of MIA3 in regulating the transition of VSMC phenotype from quiescent to pathological state is unknown. The 1q41 CAD locus is associated with MIA3 down-regulation, which led to lower VSMC proliferation that may be related to the formation of a thin fibrous cap, which in turn increases the risk for plaque rupture. Indeed, our results of MIA3 immunostaining in human coronary atherosclerotic lesion confirmed this correlation. However, the expression of MIA3 in macrophages was not significantly changed in different stages of plaque progression. Our integrative analyses identified MIA3 as a CAD-associated gene that might offer clues into potentially targetable VSMC-mediated disease mechanisms. However, more work is needed to explore the molecular mechanisms by which MIA3 affect VSMC proliferation and fibrous cap stability in mouse models of atherosclerosis.

AUTHOR CONTRIBUTIONS

RA and MC conceived the study; LG, AA, JH, LC, GFA, EF, DF, RAW, and NA performed wet-lab experiments; VPN, JYS, JS, DL, AB, SWV, AWM, LM, MUK, JLMB, SOG, GP, CLM, GKO, AF, MN, AMF, and JAB participated in data analysis and lookup. RA and MC drafted the manuscript. MC directed the study. All authors contributed to the final manuscript.

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DISCLOSURES

Drs Navab and Fogelman are principals in Bruin Pharma, and A.M.F is an officer in Bruin Pharma.

SUPPLEMENTAL MATERIALS

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FIGURE LEGENDS

Figure 1: Schematic representation of the overall study design and major results. Illustration of phenotypic and genotypic characterization of VSMC. The risk variant in the 1q41 CAD locus affects atherosclerosis by reducing MIA3 expression, which, in turn, negatively regulates the proliferation of VSMCs, which is important for the formation of a protective fibrous cap.

Figure 2: Genotypic and phenotypic characterization of VSCM donors. A) Principal component analysis of the genotypes of the 151 donors in our population and 1000 Genomes populations. The colors indicate different 1000 Genomes reference samples. Donors in our study are represented with "+" and black color. **B)** Heatmap of pairwise correlations between 12 VSMC cellular phenotypes. The size of the circles and different colors represent the correlation range (r) from –1 to +1. Orange indicates a perfect negative correlation, whereas blue indicates a perfect positive correlation. The color key of the correlations is shown at the bottom. "light color" indicates a low correlation, and "dark color" indicates a high correlation. Statistically significant (FDR<5%) correlations are shown with colored circles.

Figure 3: Regional association and genotype-phenotype plots of the four genome-wide significant loci associated with VSMC phenotypes. Associations with genetic variants around the lead SNP for each significant locus are shown using LocusZoom for **(A-C)** calcification under the osteogenic stimulus and **(D)** relative proliferation in IL-1β-containing media. The left y-axis represents the –log10 (*P*-value) of the SNP associations. The right y-axis shows the recombination rate. Linkage disequilibrium (r^2) of each SNP with the lead SNP is color-coded. **(E-H)** Box and whisker plots of the associated VSMC phenotypes in relation to the genotype of the four lead variants. *P*-values were determined using the linear mixed-model regression in performing GWAS.

Figure 4: Heatmap of effect sizes for significant associations between CAD variants and VSMC phenotypes. 79 of the 158 CAD GWAS loci genotyped in our population showed a nominal association (*P*-value<0.05) with at least one VSMC phenotype. Rows show 12 VSMC phenotypes, and columns show the index variants in the CAD loci. The color key of the correlations is shown on the left. The colors refer to the SNP weight (beta) direction and magnitude, ranging from -3 (blue) to 3.5 (red). Only statistically significant associations (*P*-value<0.05) are indicated with a colored box. Negative effect sizes (blue) indicate that risk allele was associated with lower VSMC phenotype, whereas positive effect sizes (red) indicate that risk allele was associated with a higher VSMC phenotype.

Figure 5: Association of MIA3 with VSMC proliferation. The risk allele (G) of SNP rs67180937 is associated with **A)** lower proliferation, **B)** lower MIA3 expression in human arteries aorta, and **C)** VSMCs. *P*-values in **A** and **C** were determined using the linear mixed-model regression, whereas the *P*-value in **B** was obtained from the GTEx database. VSMCs from two heterozygous carriers of the risk allele were transduced with three distinct shRNAs against MIA3 showed significant **D)** MIA3 downregulation and **E)** lower proliferation**. F)** Correlation of MIA3 expression with proliferation. **G)** Representative immunofluorescence images of brachiocephalic artery (BCA) lesions from $VSMC^{Oct4\text{-}WT/WT}$ $ApoE^{-/-}$ and $VSMC^{Oct4\text{-}\Delta/\Delta}$ $ApoE^{-/-}$ mice, which were fed 18 weeks of hypercholesterolemic Western diet, were stained for MIA3, ACTA2, and eYFP. **H)** Quantification of the frequency of eYFP+ MIA3+ ACTA+ cells as a percent of total eYFP+ VSMC in advanced BCA lesions from $VSMC^{Oct4\text{-}WT/WT}$ $ApoE^{-/-}$ and $VSMC^{Oct4\text{-}\Delta/\Delta}$ $ApoE^{-/-}$ mice. Colocalization of MIA3 with ACTA2 and eYFP is shown in Supplementary Figure 14. Single-channel images with isotype controls are shown in Online Figure XV. **I)** Results of MIA3, ACTA2, CD68, Pico-Sirius Red (Bright Field), and Movat staining in human coronary artery fibrous cap atheroma. IgG control images are shown in Online Figures XVI and XVII. The representative images in **G** and **I** were chosen since they captured the critical lesion features, including the three anatomical layers, cell composition, and evident staining. *P*-values presented in panels **E** and **F** are from one-way ANOVA with Tukey's multiple comparison test, while in **H** are from comparisons using the T-test.

NOVELTY AND SIGNIFICANCE

What Is Known?

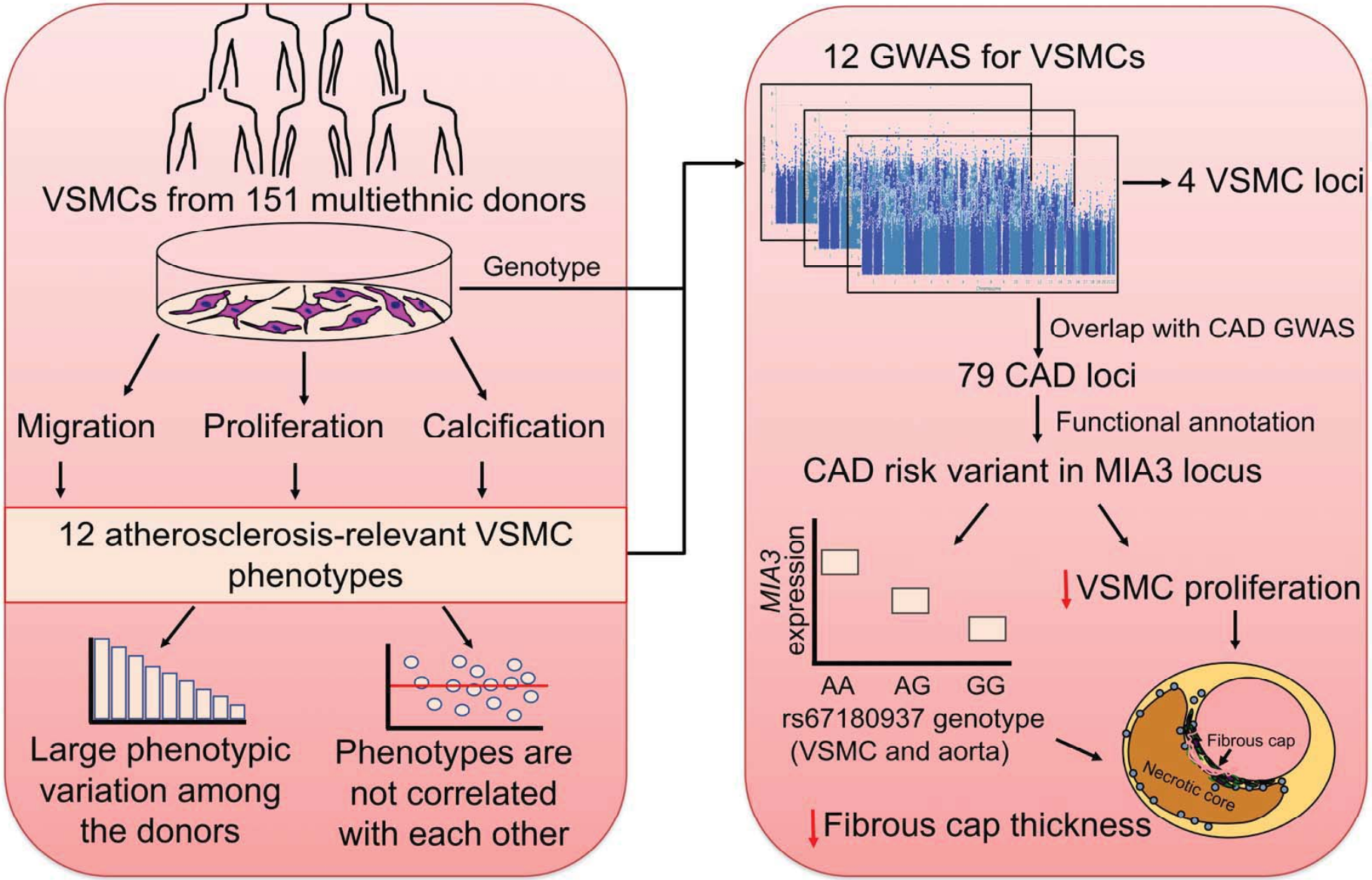
- Atherosclerosis is the underlying cause of coronary artery disease (CAD), which has a significant but poorly defined genetic component.
- Previous genome-wide association analyses (GWAS) identified multiple loci associated with increased risk for CAD.
- The majority of these loci are predicted to act in the vessel wall where the disease occurs but their cellular and molecular mechanisms are unknown.

What New Information Does This Article Contribute?

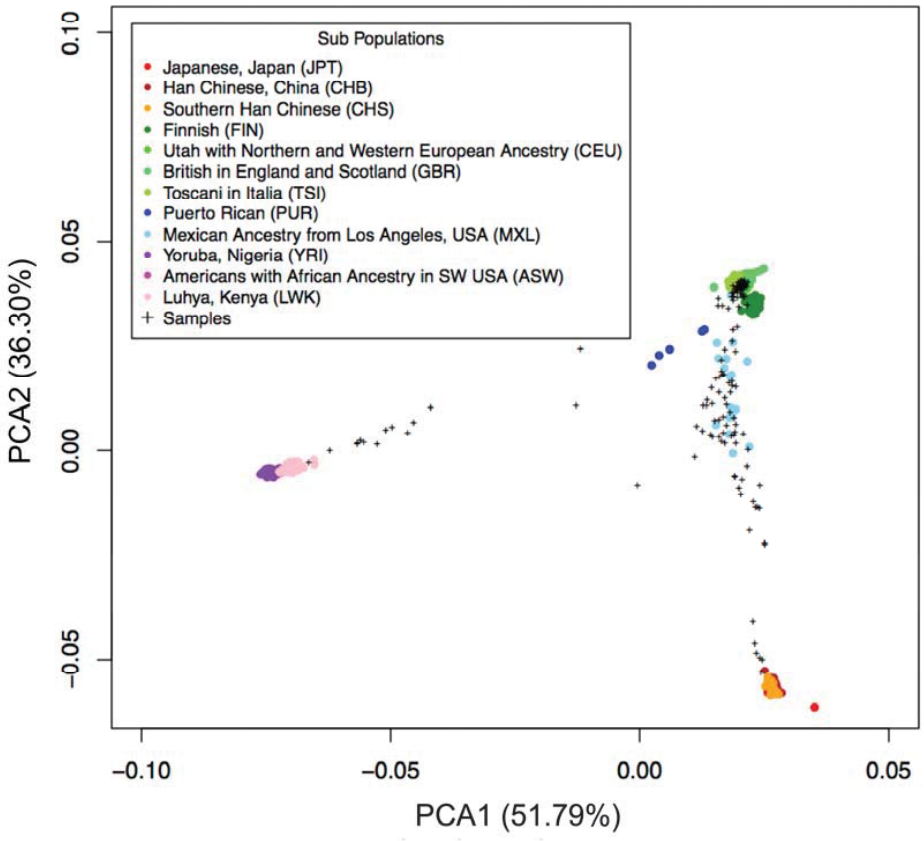
- Characterization of atherosclerosis-relevant traits, migration, proliferation, and calcification, in vascular smooth muscle cells (VSMCs) from 151 multi-ethnic heart transplant donors identified significant phenotypic variation.
- Genome-wide association studies identified four loci associated with atherosclerosis-relevant cellular phenotypes in VSMCs.
- 79 of the 163 coronary artery disease-associated loci were associated with one of the VSMC phenotypes.
- MIA3 protein, which plays a role in collagen secretion, is the likely causal gene in the 1q41 coronary artery disease locus and affects VSMC proliferation and fibrous cap thickness.

CAD is a major cause of morbidity and mortality worldwide. The majority of the 163 GWAS loci associated with CAD have unknown casual mechanism but are predicted to act within the vessel wall, pointing to novel biology. We hypothesize that a subset of the CAD loci affects VSMC functions, which play significant roles in the development of the disease. We demonstrated a large variation in three atherosclerosis-relevant phenotypes among 151 multi-ethnic heart transplant donors. We showed that more than half of the CAD GWAS loci were associated with one of the VSMC phenotypes. We predicted MIA3 as the likely causal gene in the 1q41 locus. The allele, which increases CAD risk, is associated with lower gene expression and proliferation. Staining of MIA3 in late-stage atherosclerotic lesions showed that MIA3 abundance was lower in lesions that have a thin cap, which is associated with plaque instability, compared to lesions that have a thick cap. Our study provided evidence for the complex role VSMCs play in CAD-associated GWAS loci and the development of atherosclerotic coronary disease.

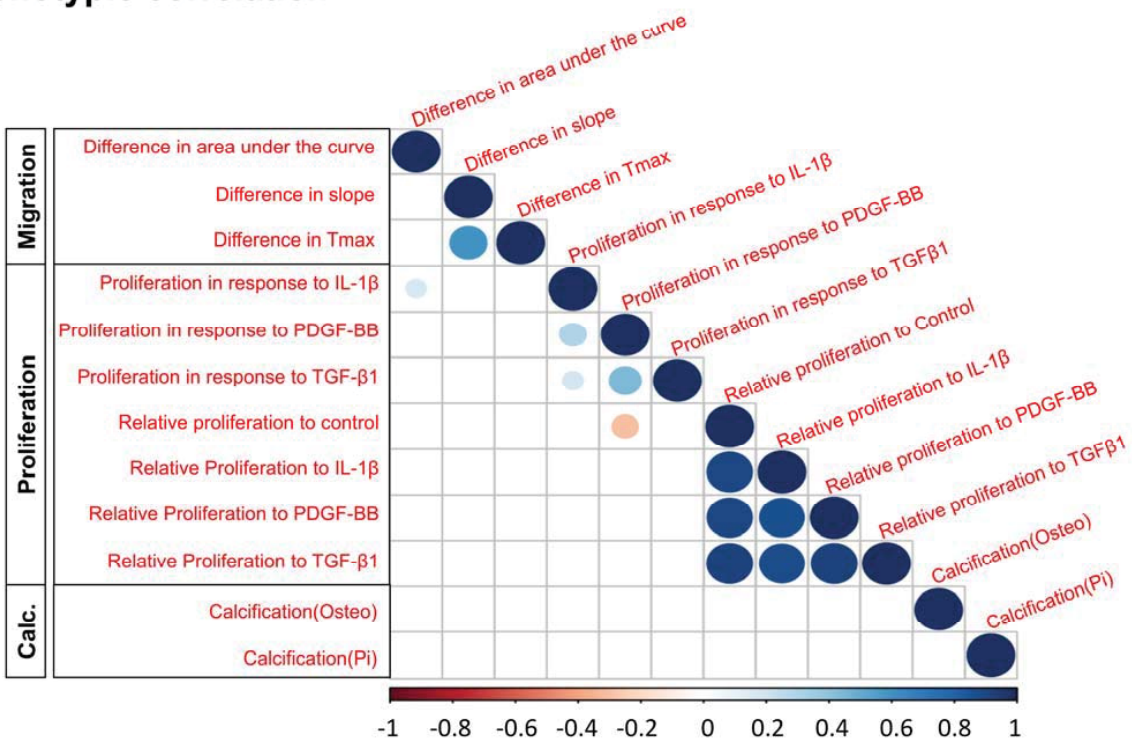
FIGURE 1



A) Population structure of the VSMC donors



B) Phenotypic correlation



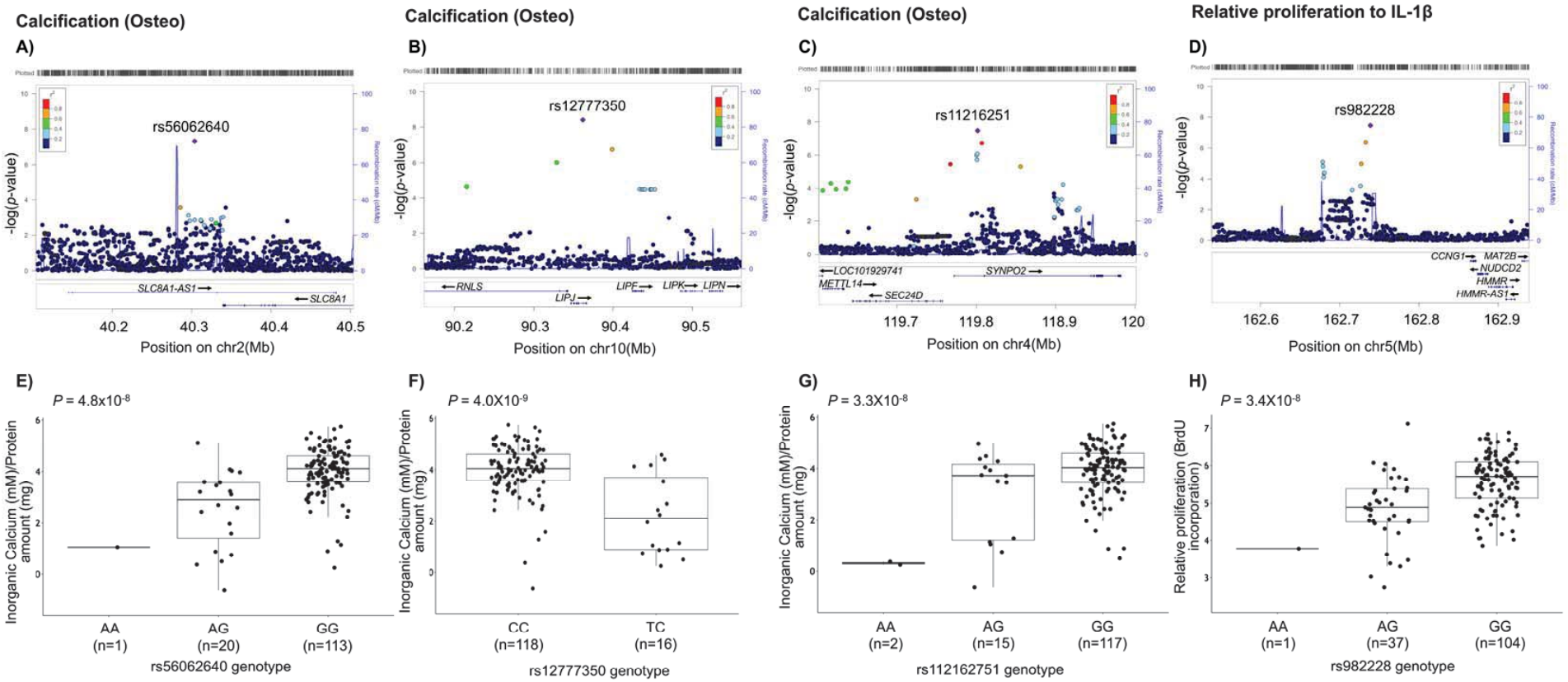


FIGURE 4