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Association of Single Nucleotide Polymorphisms on Chromosome 9p21.3 With Platelet Reactivity

A Potential Mechanism for Increased Vascular Disease

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Background—Genome-wide association studies have identified a locus on chromosome 9p21.3 to be strongly associated with myocardial infarction/coronary artery disease and ischemic stroke. To gain insights into the mechanisms underlying these associations, we hypothesized that single nucleotide polymorphisms (SNPs) in this region would be associated with platelet reactivity across multiple populations.

Methods and Results—Subjects in the initial population included 1402 asymptomatic Amish adults in whom we measured platelet reactivity (n=788) and coronary artery calcification (CAC) (n=939). Platelet reactivity on agonist stimulation was measured by impedance aggregometry, and CAC was measured by electron beam CT. Twenty-nine SNPs at the 9p21.3 locus were genotyped using the Affymetrix 500K array. Twelve correlated SNPs in the locus were significantly associated with platelet reactivity (all $P \leq 0.001$). The SNP most strongly associated with platelet reactivity, rs10965219 ($P=0.0002$), also was associated with CAC ($P=0.002$) along with 9 other SNPs (all $P < 0.004$). Association of rs10965219 with platelet reactivity persisted after adjustment for CAC, a measure of underlying atherosclerotic burden known to affect platelet reactivity. We then tested rs10965219 for association with platelet function in 2364 subjects from the Framingham Heart Study and 1169 subjects from the Genetic Study of Aspirin Responsiveness. The rs10965219 G allele (frequency $\approx 51\%$ across all 3 populations) was significantly associated with higher platelet reactivity in the Framingham Heart Study ($P=0.001$) and trended toward higher reactivity in the Genetic Study of Aspirin Responsiveness ($P=0.087$); the combined P value for metaanalysis was 0.0002.

Conclusions—These results suggest that risk alleles at 9p21.3 locus may have pleiotropic effects on myocardial infarction/coronary artery disease and stroke risk, possibly through their influence on platelet reactivity. (*Circ Cardiovasc Genet.* 2010;3:445-453.)

Key Words: genetics ■ cardiovascular diseases ■ coronary disease ■ stroke ■ blood platelets

Myocardial infarction (MI) is one of the leading causes of death and infirmity worldwide, with >800 000 individuals suffering an MI annually in the United States alone.¹ Susceptibility to MI is at least partly inherited, and recent studies have identified several single nucleotide polymorphisms (SNPs) that are associated with MI.² Among these

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SNPs is a block of common SNPs located on chromosome 9p21.3 that was associated with MI, coronary artery disease (CAD), or both in 3 different genome-wide association

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studies (GWAS).³⁻⁵ Association with these SNPs has now been consistently and robustly replicated in multiple populations,³⁻¹⁶ and the locus is estimated to account for 10% to 15% of the population-attributable risk for CAD in 1 study³ and up to 21% in a second.⁴ Some of these same MI/CAD-associated SNPs have been associated with other vascular phenotypes, including coronary artery calcification (CAC)^{3,7,17}; aortic and intracranial aneurysms⁹; and more recently, ischemic stroke.^{11,18}

SNPs at the chromosome 9p locus are not known to be associated with traditional cardiovascular disease (CVD) risk factors, such as blood pressure or lipids, and robust associations have not been reported with subclinical atherosclerosis as measured by carotid arterial wall thickness.^{19,20} It is possible, however, that the chromosome 9p locus may influence mechanisms common to both MI/CAD and stroke. Platelets, for example, play a critical role in vascular repair, and when activated under certain pathological conditions, they can lead to thrombus formation and vessel occlusion.²¹ Moreover, therapeutic interventions that decrease platelet reactivity (ie, aspirin, clopidogrel) decrease risk of the vascular occlusive events that underlie MI and stroke.^{22,23} We therefore hypothesized that MI/CAD-associated SNPs at chromosome 9p21.3 would be associated with increased platelet reactivity as assessed by *ex vivo* platelet aggregation measures. To address this hypothesis, we examined the associations of MI-associated SNPs on chromosome 9p with platelet reactivity in an Amish population and then sought to replicate observed associations in 2 independent populations. Additionally, we sought to confirm the associations of chromosome 9p SNPs with CAC in the Amish to replicate previously reported associations^{3,7,17} and to determine whether CAC predisposing risk alleles would be associated with increased platelet aggregation and if so, whether the effect of 9p SNPs on platelet reactivity would be independent of underlying atherosclerotic burden.

Methods

The initial study population included 788 Amish individuals from the Heredity and Phenotype Intervention (HAPI) Heart Study²⁴ in whom platelet reactivity had been measured as well as a partially overlapping sample of 939 subjects in whom CAC had been measured as part of the Amish Family Calcification Study.²⁵ Recruitment of subjects for these 2 studies was carried out between 2002 and 2006. The participant pool was largely healthy and asymptomatic from a CVD perspective. HAPI Heart Study participants ranged in age from 20 to 80 years; exclusions from participation in the study included severe hypertension (systolic blood pressure >180 mm Hg, diastolic blood pressure >105 mm Hg), kidney disease, liver disease, untreated thyroid disease, and malignancy of any type. Participants of the Amish Family Calcification Study aged ≥ 30 years were recruited without regard to any CVD phenotype. Informed consent was obtained from all participants. The study protocols were approved by the Institutional Review Boards at the University of Maryland School of Medicine and other participating institutions.

Examination procedures for both studies included a medical and family history and focused physical examination at the Amish Research Center (Strasburg, Pa). A morning blood sample was collected following an overnight fast for clinical chemistries and DNA analysis.

Platelet reactivity studies were carried out in 788 HAPI Heart Study subjects.²⁴ Before enrollment into the platelet reactivity arm of the HAPI Heart Study, subjects were instructed not to take vitamins,

herbal supplements, or medications 2 weeks before and during the study period. At an initial clinic visit, a fasting blood sample was obtained for measurement of complete blood cell count with differential. Subjects with platelet counts between 100 000/ μ L and 500 000/ μ L and white blood cell counts <20 000/ μ L were eligible for platelet studies. In these subjects, a second blood sample was collected in a syringe with sodium citrate anticoagulant at a final concentration of 0.0105 mol/L for the baseline platelet activity measures. Reactivity studies included whole-blood platelet impedance aggregometry with a Chrono-Log 4-channel aggregometer (Havertown, Pa) within 3 hours after the blood draw, with incubation wells set to 37°C and stirring speed to 1000 rpm. Prewarmed cuvettes were each filled with equal amounts of citrate anticoagulated whole blood and Hank's Balanced Salt Solution (Sigma-Aldrich; St Louis, Mo). After a 5-minute incubation period, a prewarmed probe was inserted into each cuvette, the aggregation baseline was set to 0, and the impedance circuit was calibrated to 50%. Collagen (Chrono-Log) was added at final concentrations of 0.5, 1.0, 2.0, and 5.0 μ g/mL, and peak aggregation at 5 minutes was measured.

CAC was measured in 939 Amish subjects by electron beam CT on an Imatron C-150 scanner (GE; South San Francisco, Calif) using a standard protocol that included 30 to 40 3-mm contiguous transverse slices between the aortic root and the apex of the heart, gated to 80% of the R-R interval obtained during a single breath hold.²⁵ CAC was quantified using the Agatston score method, incorporating both density and area. We defined presence of calcification as a CAC score ≥ 1 (density >130 Hounsfield units in >3 contiguous pixels [>1 mm²]). The sum of the scores in the left main, left anterior descending, circumflex, and right coronary arteries was considered the CAC score. Interscan reproducibility for quantification of CAC with this software was previously reported to range from 89% to 94%. The interreader and intrareader reproducibilities were each $\approx 99\%$.

Genotyping of Amish subjects was performed using the Affymetrix GeneChip Human Mapping 500K Array set. We selected 42 SNPs in the 175-kb region of interest falling between positions 21 948 524 and 22 124 094 bp on chromosome 9p21.3 (National Center for Biotechnology Information Map Viewer Build 36.3). The GTYPE-generated chip files were analyzed using the Bayesian Robust Linear Model with Mahalanobis Distance Classification genotype calling algorithm. Of the 42 SNPs genotyped in the 175-kb region of interest, we excluded from analysis 3 SNPs with call rates <0.93 and 10 SNPs with minor allele frequencies <0.02. This exclusion left a total of 29 analyzable SNPs. The mean genotyping call rate of these SNPs was 98.6%.

Replication Cohorts

Framingham Heart Study

Platelet aggregation was measured from platelet-rich plasma in 2364 subjects who also underwent a GWAS using the Affymetrix 500K platform and an additional 50K Affymetrix gene-focused Molecular Inversion Probe array. Subjects with prevalent CVD and those using aspirin or anticoagulant medications were excluded from analysis. The extent of platelet aggregation was determined through a 4-channel aggregometer (BioData Corp, Horsham, Pa) as the minimum threshold concentration required to induce a biphasic response within 5 minutes postepinephrine addition (doses, 0.01, 0.03, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0 μ M); thus, a higher threshold aggregation reflected more agonist required to initiate aggregation and less baseline aggregation. Mean age \pm SD of the study subjects was 54.8 \pm 9.8 years, and 43.6% were men. Details of the assay and trait definition have been previously described.²⁶

Genetic Study of Aspirin Responsiveness

Whole-blood impedance aggregometry was carried out in 1169 European-American subjects from 327 families who had undergone GWAS analysis using the Illumina 1M platform at deCODE Genetics (Reykjavik, Iceland). Subjects with prevalent CVD were excluded from platelet aggregation studies as were those with a history

of bleeding disorders, hemorrhagic events, or aspirin intolerance; those with serious comorbidities; and those who were taking aspirin or anticoagulant medications who could not safely discontinue use before these studies. Mean age of the study subjects was 45 ± 13 years, and 44.9% were men. Platelet aggregation studies were conducted under a similar protocol as in the Amish HAPI Heart Study, with the exception that the Genetic Study of Aspirin Responsiveness (GeneSTAR) protocol did not include a $0.5\text{-}\mu\text{g/mL}$ concentration of collagen agonist; therefore, for this replication study, platelet reactivity was assessed following stimulation with a $1.0\text{-}\mu\text{g/mL}$ concentration of collagen. Details of the assay and trait definition have been previously described.^{26,27}

To ensure that allele calls were consistent between the Amish study and Framingham Heart Study (FHS), both of which were genotyped on the Affymetrix platform, and GeneSTAR, which was genotyped on the Illumina platform, we genotyped rs10965219 in a subset of samples from the Amish study and GeneSTAR with TaqMan (Applied Biosystems Inc, Carlsbad, Calif) and aligned the TaqMan genotype calls with our respective GWAS genotype calls. Because no TaqMan assay was available for rs10965219, we genotyped in its place rs1360590, an SNP in high linkage disequilibrium (LD) ($r^2=0.93$ in HapMap CEU with rs10965219). Genotyping was carried out in 96 samples from each study. Genotype concordance rates were 98.7% and 94.5% in the Amish study and GeneSTAR, respectively.

Statistical Analysis

The observed distribution of genotypes was tested for deviation from Hardy-Weinberg equilibrium using Pearson χ^2 test. Pairwise LD correlation statistics (r^2) were computed using the Haploview software program (www.broad.mit.edu). Association analyses of platelet reactivity and CAC quantity in Amish subjects were performed under the measured genotype variance component mixed model that assesses the additive effect of genotype on the quantitative trait while simultaneously estimating the effects of age, age², sex, and a polygenic component to account for phenotypic correlation due to relatedness.²⁸ In the additive model, genotype is coded as the number of copies of the reference allele (0, 1, or 2), and the significance of the genotype effect is assessed using a 1-*df* test. The polygenic component was modeled using the relationship matrix derived from the pedigree structure constructed from the entire Lancaster Amish settlement dating back 14 generations because all subjects are related. We have implemented this mixed-model analysis in an in-house software program, called Mixed Model Analysis for Pedigrees, that is computationally efficient for large pedigrees such as the Amish.²⁹ Because the distribution of CAC scores was positively skewed and not all subjects had detectable CAC, the scores were natural log-transformed after adding 1. Age- and sex-residualized CAC scores were approximately normally distributed, as were the distributions of the platelet aggregation measures in response to each dose of collagen agonist. We estimated that our study would have 80% power to detect SNPs, accounting for $\geq 2\%$ of the variation in each of platelet aggregation ($n=788$) and CAC score ($n=939$) at an alpha level of 0.005 (see following discussion).

Association analyses of the FHS and GeneSTAR replication cohorts were performed within each cohort and then combined for metaanalysis using a weighted *z* score-based fixed effects metaanalysis approach. Both FHS and GeneSTAR evaluated age- and sex-adjusted models for aggregation phenotypes. FHS analyzed the \log_{10} transformation of epinephrine concentrations that produced a half-maximal response. FHS and GeneSTAR included the principal components from EIGENSTRAT 2.0³⁰ ($n=8$ and $n=2$, respectively) as covariates to account for potential population admixture. Linear mixed effects models were used in the respective studies to test the association under an additive model between an SNP and specific phenotype adjusted for age, sex, and principal components. Additional adjustments were made for diabetes, hypertension, current smoking, body mass index, low-density lipoprotein (LDL) cholesterol, and fibrinogen in GeneSTAR. SNP genotypes were included as fixed effects using an additive model (0 for 1 major allele, 1 for the heterozygote, and 2 for the minor allele homozygote genotype) for

Table 1. Characteristics of 788 Amish Subjects Undergoing Platelet Reactivity Studies

Trait	Men (n=428)	Women (n=360)
Age, y	41.5 \pm 13.1	44.5 \pm 13.8
Body mass index, kg/m ²	25.5 \pm 3.2	27.6 \pm 5.3
Systolic blood pressure, mm Hg	120.9 \pm 11.9	120.7 \pm 16.0
Hypertension, %*	8.6	12.8
Diabetes, %	0.7	0.6
Total cholesterol, mg/dL	203.3 \pm 44.9	213.9 \pm 47.4
HDL-cholesterol, mg/dL	53.1 \pm 12.8	59.4 \pm 15.4
LDL-cholesterol, mg/dL	137.7 \pm 41.5	140.2 \pm 44.7
Triglycerides, mg/dL	62.4 \pm 34.7	71.4 \pm 44.1
Current smoking (pipes only), %	19.9	0.0
Presence of CAC, %†	66.6	49.5
ln(CAC+1)†	3.36 \pm 2.85	2.09 \pm 2.49
Platelet aggregation in response to collagen 0.5 $\mu\text{g/mL}$ (mean \pm SE), Ω	10.4 \pm 2.8	11.8 \pm 2.7

Data are presented as mean \pm SD unless otherwise indicated. HDL indicates high-density lipoprotein.

*Defined as systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg or reported current use of antihypertensive medications.

†Presence of CAC defined as an Agatston score >1 ; values based on 939 subjects in whom CAC was measured.

the original genotypes and dosage (probabilistic estimations) for the imputed genotypes. We tested whether the SNP additive effects differed from 0. FHS used the R kinship and genome-wide association analyses with family packages,³¹ accounting for familial relatedness, whereas GeneSTAR used PROC MIXED in SAS version 9.1.3 for Linux OS with the option for EMPIRICAL variance and including the family identification number in the random effects to account for relatedness.

Our metaanalysis approach does not compute summary effect sizes across studies (which would be inappropriate given that somewhat different measures of platelet aggregation were used in the Amish study, FHS, and GeneSTAR). Using the METAL software program (<http://www.sph.umich.edu/csg/abecasis/Metal/index.html>), we defined a reference allele and generated a *z*-statistic summarizing the magnitude of the *P* value for association (under the additive model) and direction of effect for each study. An overall *z*-statistic was then computed as a weighted average of the individual statistics, and a corresponding *P* value for that statistic was computed. The weights were proportional to the square root of the number of individuals in each study and scaled such that the squared weights summed to 1. In constructing the overall *z*-statistic, we multiplied the β coefficient reflecting the SNP association with platelet aggregation in FHS by -1 to make the directions of effect similar among the 3 studies. In the Amish study and GeneSTAR, the measurements reflect platelet aggregation, whereas in FHS, values reflect the dose of agonist required to initiate aggregation, and higher doses reflect lower aggregation.

Results

Characteristics of the 788 Amish subjects in whom platelet aggregation studies were performed are shown in Table 1. This sample included 428 men and 360 women, and the mean age was 42.8 ± 13.5 years. Participating subjects were relatively healthy as evidenced by the low prevalence of hypertension (8% to 13%) and diabetes ($<1\%$). Less than 2% of the study subjects reported current use of antihypertensive or cholesterol-lowering medications, and 1.4% of subjects reported a prior cardiovascular event (ie, history of MI, stroke,

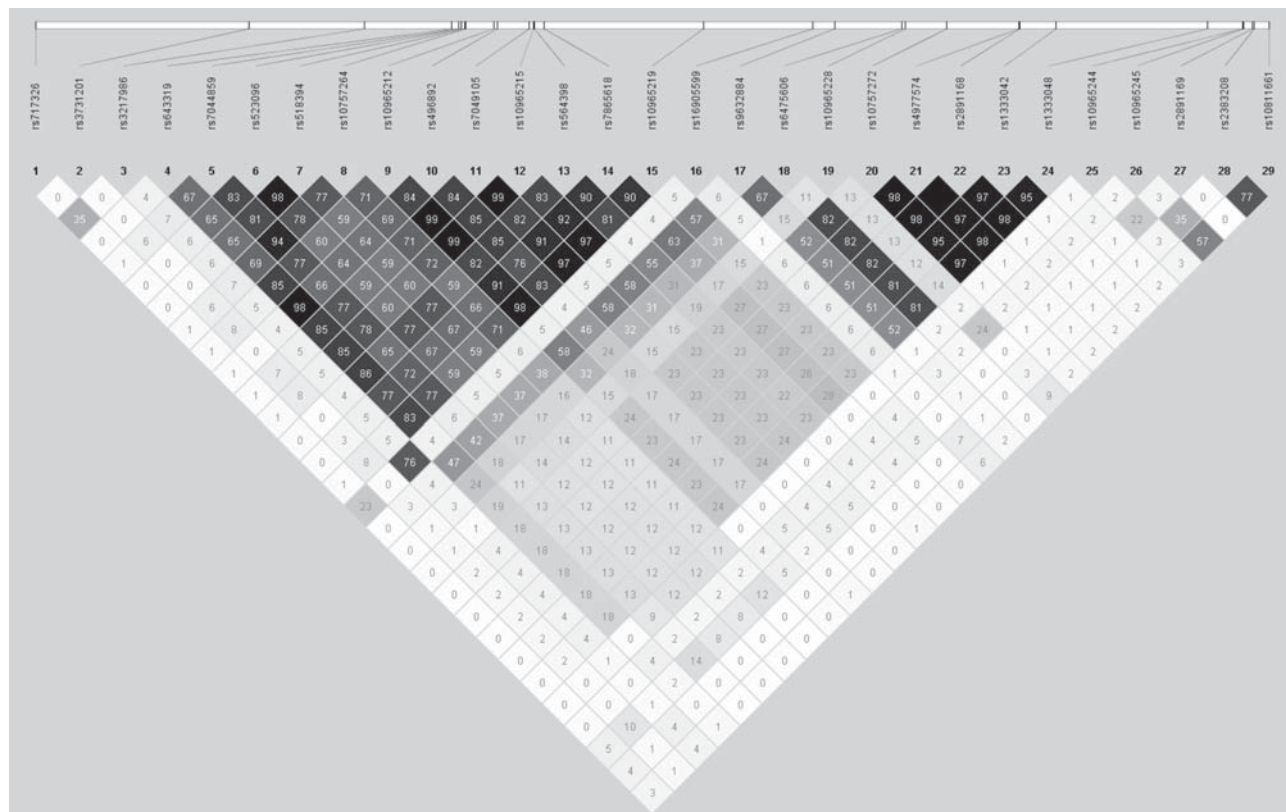


Figure. LD structure shown as r^2 in the chromosome 9p21.3 region in Amish subjects. All genotyped SNPs passing quality control with minor allele frequencies >0.02 are shown. Genomic locations are given according to National Center for Biotechnology Information Map Viewer Build 36.3. Figure created with Haploview software.

a coronary angiogram with a detectable blockage). Mean platelet reactivity was slightly higher in women than in men (11.8 versus 10.4 Ω with collagen 0.5 $\mu\text{g}/\text{mL}$). CAC was measured in 939 Amish subjects (430 men and 509 women) of whom 325 were included in the platelet aggregation studies. Mean age of the subjects in whom CAC was measured was 50.3 years in men and 53.4 years in women, and 8.2% of subjects reported a prior cardiovascular event. The proportion of subjects with detectable CAC was 57.9% (66.7% men and 50.4% women).

As shown in the Figure, the 29 SNPs in the region of interest formed essentially 2 large haplotype blocks in Amish subjects, the first extending approximately 49 kb from rs643319 to rs10965219 (SNPs 4 to 15 in Figure) and the second extending approximately 37 kb from rs10757272 to rs1333048 (SNPs 20 to 24 in Figure). The genotype distributions of all SNPs conformed with Hardy-Weinberg expectations (all $P>0.001$). SNPs associated with MI/CAD in previously published studies fall in both blocks.

SNPs and Platelet Aggregation Associations in the Amish

Because the 29 tested SNPs were largely clustered in 2 blocks, we did not regard each SNP as being independently tested and so did not apply the Bonferroni correction for the total number of SNPs. Instead, we corrected for 12 independent tests, providing a statistical significance threshold of 0.05/12, or $P<0.004$. The 12 independent tests were based on subtracting from the 29 SNPs any SNP that was highly

correlated in the Amish subjects (ie, $r^2>0.65$) with an already-selected SNP. Thus, we selected SNPs 1 to 3, 1 SNP from block 1 (SNPs 4 to 15), SNP 16, 1 SNP from SNP17/SNP18, SNP 19, 1 SNP from block 2 (SNPs 20 to 24), SNPs 25 to 27, and 1 SNP from SNP 28/SNP 29 (see Figure). After adjusting for age, age², and sex, all 12 SNPs in block 1 were associated with variation in platelet reactivity in response to 0.5 $\mu\text{g}/\text{mL}$ collagen ($P\leq 0.001$). The strongest associations observed for platelet aggregation were with SNPs rs10965212, rs7049105, rs10965215, and rs10965219 ($P=0.0001$ to 0.0002), all of which were in very high LD (all pairwise $r^2\geq 0.97$) (Table 2).

All 12 SNPs in block 1 were only weakly associated with platelet aggregation in response to collagen agonist at the 1- $\mu\text{g}/\text{mL}$ concentration (all $P=0.01$ to 0.07). No SNPs were associated with platelet aggregation in response to collagen agonist at the 2.0- or 5.0- $\mu\text{g}/\text{mL}$ concentrations (data not shown).

SNPs and CAC Associations in the Amish

After adjusting for age² and sex, CAC scores were significantly associated with SNPs in both haplotype blocks (Table 2). In block 1, associations with CAC quantity were observed with 5 SNPs in the 36-kb region bounded by rs643319 (22 007 846 bp) and rs10965219 (22 043 687 bp), with P values of the associated SNPs ranging from 0.001 to 0.004. All 5 SNPs in block 2 also were associated with transformed CAC scores in the 37-kb region bounded by rs10757272

Table 2. Additive Model *P* Values Representing the Associations of 29 SNPs on Chromosome 9p21.3 With ln(CAC+1) and Platelet Aggregation, Adjusted for Age, Age², and Sex in the Amish

SNP rs No.	Position (bp)	Minor Allele Frequency	<i>P</i> for Association with ln(CAC+1) (n=939)	<i>P</i> for Association With Platelet Aggregation (0.5 μg/mL Collagen) (n=788)
rs717326	21 948 524	0.070	7.43E-01	9.44E-01
rs3731201	21 978 896	0.082	8.20E-02	7.00E-02
rs3217986	21 995 330	0.074	9.76E-01	3.43E-01
Block 1				
rs643319	22 007 836	0.434	1.13E-03*	2.10E-04*
rs7044859	22 008 781	0.476	2.55E-02	1.02E-03*
rs523096	22 009 129	0.496	2.10E-02	1.05E-03*
rs518394	22 009 673	0.489	2.70E-02	6.24E-04*
rs10757264	22 009 732	0.477	4.45E-02	6.08E-04*
rs10965212	22 013 795	0.473	4.34E-03	1.37E-04*
rs496892	22 014 351	0.435	8.32E-03	3.10E-04*
rs7049105	22 018 801	0.471	2.64E-03*	1.25E-03*
rs10965215	22 019 445	0.468	3.35E-03*	1.07E-04*
rs564398	22 019 547	0.430	2.34E-03*	4.40E-04*
rs7865618	22 021 005	0.452	8.36E-03	5.18E-04*
rs10965219	22 043 687	0.480	2.00E-03*	1.91E-04*
rs16905599	22 059 144	0.056	8.74E-01	1.22E-01
rs9632884	22 062 301	0.473	4.61E-02	2.90E-02
rs6475606	22 071 850	0.461	8.97E-03	1.64E-02
rs10965228	22 072 380	0.154	2.72E-01	4.70E-03
Block 2				
rs10757272	22 078 260	0.497	2.97E-04*	6.79E-02
rs4977574	22 088 574	0.497	2.13E-04*	8.13E-02
rs2891168	22 088 619	0.497	2.13E-04*	8.13E-02
rs1333042	22 093 813	0.482	9.42E-04*	8.04E-02
rs1333048	22 115 347	0.490	3.18E-03*	1.37E-01
rs10965244	22 120 389	0.188	3.11E-01	1.47E-01
rs10965245	22 120 515	0.088	2.88E-01	9.01E-01
rs2891169	22 121 825	0.456	3.36E-01	1.23E-02
rs2383208	22 122 076	0.127	8.27E-01	1.27E-01
rs10811661	22 124 094	0.143	6.01E-01	2.96E-01

SNPs in the region bounded by rs643319 (22 007 836 bp) and rs1333049 (22 115 500 bp) have been associated with MI or CAD in McPherson et al,³ Helgadottir et al,⁴ Samani et al,⁵ and Zeggini et al,³² among others. Haplotypes in the region bounded by rs7044859 (22 008 781 bp) and rs1333048 (22 115 347 bp) have been associated with ischemic stroke.^{11,18}

**P*<0.004 (see text).

(22 078 260 bp) and rs1333048 (22 115 347 bp) (*P*=0.0002 to 0.003).

Because there is some, albeit modest, LD among SNPs in blocks 1 and 2 (eg, $0.10 < r^2 < 0.28$ in Amish subjects), we performed a further analysis that included SNPs in both regions as independent variables to determine whether the associations observed with the block 1 and block 2 clusters were independent. The SNP rs564398 was selected as the representative CAC-associated SNP in block 1 and rs4977574 as the representative CAC-associated SNP in block 2. In this analysis, the effect sizes of both SNPs were markedly reduced, and rs564398 no longer remained associated with CAC at the *P*<0.01 significance threshold.

SNP rs10965219 and CAC and Platelet Aggregation Associations

For each SNP associated with both CAC and platelet aggregation, the same allele that was associated with increased

CAC score also was associated with increased platelet aggregation. We selected for more detailed analysis SNP rs10965219 as representative of the rs10965212-rs7049105-rs10965215-rs10965219 SNP cluster that was in near perfect LD and strongly associated with platelet aggregation. Table 3 shows the proportion of subjects with any CAC and the mean CAC scores and platelet aggregation values by rs10965219 genotype. The rs10965219 G allele (frequency in Amish, 0.520) was associated with higher presence of any CAC (*P*=0.01), higher CAC score (*P*=0.0002), and increased platelet aggregation (*P*=0.0002). After adjusting for age and sex, this SNP accounted for 1.7% of the variation in preaspirin platelet aggregation and 1.1% of the variation in transformed CAC score.

There was no association between transformed CAC scores and platelet aggregation in the 325 subjects in whom both measures were obtained (age- and sex-adjusted $r^2=0.019$;

Table 3. Distribution of Vascular and Platelet Phenotypes in 924 Amish Subjects According to rs10965219 Genotype

Trait	GG Genotype	GA Genotype	AA Genotype	Age- and Sex-Adjusted <i>P</i>
CAC, n	219*	508	197	
Presence of CAC, %†	63.9	56.6	54.8	0.01
ln(CAC+1) (mean±SE)	3.24±0.19	2.53±1.63	2.56±1.87	0.002
Platelet aggregation measures, n	193	415	162	
Platelet aggregation in response to collagen 0.5 μg/mL (mean±SD), Ω	11.4±2.6	11.2±2.7	10.1±3.4	0.0002

*A total of 924 subjects were phenotyped for CAC, and 767 were phenotyped for platelet aggregation at this locus.

†Presence of CAC defined as an Agatston score >1.

$P=0.73$). Furthermore, the statistically significant association observed between the rs10965219 genotype and platelet aggregation was not diminished by adjusting for CAC scores or for any of the conventional CVD risk factors besides age and sex. Genotype for rs10965219 was not associated with body mass index; systolic blood pressure; total, high-density lipoprotein, or LDL cholesterol; or triglycerides in this sample (data not shown). Thus, the genotype-platelet aggregation association does not appear to be mediated by traditional CVD risk factors or by atherosclerotic burden as measured by CAC.

Replication Studies

We assessed the association of rs10965219 genotype with platelet reactivity in the FHS and GeneSTAR. This SNP was genotyped directly in FHS but imputed in GeneSTAR using the MACH imputation program. The imputation quality score (defined as the average posterior probability for the most likely genotype) for this SNP was 0.80, and the r^2 (defined as the squared correlation between imputed and true genotypes) was 0.56. The frequencies of the rs10965219 G allele were 0.507 and 0.506 in FHS and GeneSTAR compared with 0.520 in the Amish study. In each study, the G allele was associated with higher platelet reactivity, with the association achieving statistical significance in the FHS ($P=0.001$) but not in GeneSTAR ($P=0.087$) (see Table 4). The combined evidence for association across the 2 replication cohorts through metaanalysis was 0.0002.

Discussion

The chromosome 9p21.3 locus has recently attracted much attention because of the consistent associations observed

between SNPs at this locus and CVD-related traits. As in the FHS¹⁷ and other studies,^{3,7} we found common SNPs at this locus to be associated with CAC, a well-validated marker of atherosclerosis and predictor of MI. The novel contribution of the present study is our demonstration that some of these MI/CAD-associated alleles also are associated with increased platelet aggregation, an important risk factor for both MI and stroke. We found the chromosome 9p SNPs to be associated across 3 different populations to 2 different platelet aggregation phenotypes, 2 reflecting whole-blood platelet aggregation in response to collagen and 1 reflecting threshold aggregation concentration in response to epinephrine agonist in platelet-rich plasma. On the one hand, the differences among the platelet aggregation phenotypes among the 3 studies argue for the robustness of the genotype association with platelet aggregation; on the other hand, one also could say that no single phenotype association has been replicated. Although differences in the platelet aggregation measures and their scales preclude direct comparison of effect sizes among the 3 studies, there is a suggestion that the platelet aggregation association may be stronger in the Amish study (with collagen dose of 0.5 μg/mL) than in GeneStar (with collagen dose of 1.0 μg/mL). A stronger association in Amish may reflect increased sensitivity to differences in aggregation at a lower dose of collagen agonist. This speculation is supported by the fact that higher doses of collagen cause a more robust platelet activation response than lower doses, including secretion of platelet granules and thromboxane release, which are absent or incomplete at lower doses. It is thus possible that higher doses of collagen may overwhelm gene-association signals of subtle difference in platelet function

Table 4. Association of Platelet Reactivity With rs10965219 Genotype in the Amish, FHS, and GeneSTAR Populations

	Amish (n=788)	FHS (n=2364)	GeneSTAR (n=1169)
Age (mean±SD), y	42.8±13.5	54.4±9.8	44.6±13.2
Male sex, %	54.3	43.6	44.9
Platelet reactivity measure	Whole-blood platelet aggregation in response to collagen 0.5 μg/mL in Ω	Threshold aggregation concentration in response to epinephrine	Whole-blood platelet aggregation in response to collagen 1.0 μg/mL in Ω
rs10965219 genotype			
β (SE)*	-0.541 (0.154)	0.058 (0.017)*	-0.574 (0.335)
Age- and sex-adjusted <i>P</i>	0.0007	0.001	0.087†

*β corresponds to the effect of each G allele copy on platelet aggregation. In FHS, higher values of threshold aggregation concentration indicate that aggregation occurs with higher concentrations of agonist and, hence, lesser aggregability. Thus, a positive β coefficient in FHS has the same interpretation as a negative coefficient in the Amish study and GeneSTAR. Effect estimates cannot be compared across studies because of differences in the phenotypes.

†Additional adjustment was made for smoking, hypertension, diabetes, LDL cholesterol, body mass index, and fibrinogen in GeneSTAR.

that are pathophysiologically relevant and are discoverable using lower doses.

The association with platelet aggregation is intriguing, suggesting that increased platelet reactivity leading to thrombosis may be a mechanism whereby this locus contributes to MI/CAD. It seems unlikely that the SNP-platelet reactivity association can be explained by associations of these SNPs with CAC because there was virtually no correlation between CAC and platelet aggregation in the Amish. Association with platelet reactivity adds to the growing number of traits that SNP variation at this locus has been shown to influence—a list that now includes in addition to MI/CAD and stroke, abdominal aortic and intracranial aneurysms,⁹ periodontal disease,³³ and familial melanoma.³⁴ Moreover, an SNP in a different LD block approximately 10-kb centromeric to this MI/CAD-associated region has been consistently associated with type 2 diabetes mellitus.^{32,35,36} The diabetes-associated locus appears to be entirely distinct from the MI/CAD-associated locus.

Because SNPs in the MI/CAD-associated region are in relatively high LD, it has been difficult to pinpoint the specific causal SNPs. In our Amish population, associations with CAC quantity extended over an 86-kb region that includes 2 large LD blocks, similar to the pattern seen in other white populations.¹⁹ However, the most highly associated SNPs in each block were not independently associated with CAC, suggesting that the association could be driven by a single (yet to be identified) variant marked by both SNPs somewhere in this region.

Our data suggest that variants affecting CAC are associated with SNPs in LD with both blocks 1 and 2, whereas variants associated with platelet reactivity are associated most strongly with SNPs in block 1. There are no annotated protein-coding genes that map to the CAC- and platelet aggregation-associated SNPs in block 1, although this region does map to a recently identified noncoding RNA called *ANRIL*.³⁷ Noncoding RNAs can alter expression of associated protein-coding genes through a number of mechanisms.^{38,39} Such mechanisms include the knockdown of mRNAs and the alteration of gene transcription through the recruitment of chromatin-modifying enzymes or epigenetic silencing. *ANRIL* has been shown to be expressed in vascular endothelial cells, monocyte-derived macrophages, coronary smooth muscle cells, and other cell types known to be affected by atherosclerosis, making it a strong candidate gene for the chromosome 9p disease associations reported.⁴⁰ Recently, Jarinova and colleagues⁴¹ reported that a conserved sequence within this locus has enhancer activity and that the associated haplotype alters the regulatory sequence of *ANRIL* and changes expression levels of this gene. Through additional experiments, these authors then showed that *ANRIL* expression changes correlated with changes in expression of other genes, particularly those in pathways associated with cell proliferation. Genotype associations with differential *ANRIL* gene expression also have been independently reported by other groups.^{42,43} Thus, risk alleles in the 9p21.3 region may act by altering *ANRIL* expression levels and, in turn, potentially influence a wide variety of vascular responses. Ultimately, a molecular profiling of the appropriate locus se-

quences is mandatory to identify the causal variant, which reliably associates with CAD-related phenotypes. Understanding more fully the molecular basis underlying the 9p21.3 association may have important implications for understanding, preventing, and treating heart disease.

In summary, we confirm that common SNPs on chromosome 9p21.3 are associated with CAC, a well-recognized subclinical marker of CAD and a predictor of MI. We find that 1 mechanism underlying the well-replicated association of this locus with MI/CAC is likely to involve increased platelet reactivity, also a risk factor for CVD events. This observation is consistent with the observation that this locus is a risk factor for thromboembolic disease more generally, including stroke. Additional studies, including in subjects from other ethnic groups and with varying degrees of CVD severity, to further define the importance of platelet reactivity in those who carry the at-risk genotype may provide mechanistic insights toward personalized medicine through genotype-specific interventions that target platelet reactivity.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Common genetic variants on chromosome 9p21.3 are associated with myocardial infarction, coronary artery disease, coronary artery calcification (CAC), and ischemic stroke. To gain insights into mechanisms underlying these associations, we examined variations in this region and platelet reactivity across multiple populations. One variant, rs10965219, showed consistent effects on platelet reactivity in 4321 subjects across 3 studies. The variant also was associated with CAC. Further, the association between the variant and platelet reactivity persisted after adjusting for variation in CAC. These results suggest that risk alleles at 9p21.3 may have pleiotropic effects on myocardial infarction, coronary artery disease, CAC, and stroke risk, possibly through their influence on platelet reactivity. Additional studies to further define the importance of platelet reactivity in those who carry the at-risk genotype may provide mechanistic insights toward personalized medicine through genotype-specific interventions that target platelet reactivity.