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ORIGINAL ARTICLE

Variation in 24 hemostatic genes and associations with non-fatal myocardial infarction and ischemic stroke

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Summary. *Background:* Arterial thrombosis involves platelet aggregation and clot formation, yet little is known about the contribution of genetic variation in fibrin-based hemostatic factors to arterial clotting risk. We hypothesized that common variation in 24 coagulation–fibrinolysis genes would contribute to risk of incident myocardial infarction (MI) or ischemic stroke (IS). *Methods:* We conducted a population-based, case–control study. Subjects were hypertensive adults and postmenopausal women 30–79 years of age, who sustained a first MI ($n = 856$) or IS ($n = 368$) between 1995 and 2002, and controls matched on age, hypertension status, and calendar year ($n = 2689$). We investigated the risk of MI and IS associated with (i) global variation within each gene as measured by common haplotypes and (ii) individual haplotypes and single nucleotide polymorphisms (SNPs). Significance was assessed using a 0.2 threshold of the false discovery rate q-value, which accounts for multiple testing. *Results:* After accounting for multiple testing, global genetic variation in factor (F) VIII was associated with IS risk. Two haplotypes in FVIII and one in FXIIIa1 were significantly associated with increased IS risk (all q-values < 0.2). A plasminogen gene SNP was associated with MI risk. All are new discoveries not previously reported. Another 24 tests had P-values < 0.05 and q-values > 0.2 in MI and IS analyses, 23 of which are new and hypothesis generating. *Conclusions:* Apart from the association of FVIII variation with IS, we found little evidence that common variation in the 24 candidate fibrin-based hemostasis genes strongly influences arterial thrombotic risk, but our results cannot rule out small effects.

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Introduction

Fibrin clot formation and platelet aggregation create arterial thrombi that impede circulation and can precipitate adverse cardiovascular events such as myocardial infarction (MI) and ischemic stroke (IS). Acquired risk factors for MI and IS have been extensively characterized, yet little is known about the contribution of genetic variation in hemostatic factors to arterial clotting risk. Genetic investigations of coagulation, anticoagulation, fibrinolytic, and antifibrinolytic factors published to date have primarily investigated single nucleotide polymorphisms (SNPs) or haplotypes defined by a few SNPs. Although individual study findings have often been inconsistent, meta-analyses of the data on factor (F) II G20210A, FV R509Q (Leiden), and plasminogen activator inhibitor type 1 (PAI-1) 4G/5G indicate that coronary disease and IS risks are increased by 4–47% [1–3]. Meta-analyses of data on several other variants in the clotting pathway (FVII G10976A, FXIIIa1 V34L, β-fibrinogen G-455A and C-148T) have suggested no association with either MI or IS. Findings for individual variants across other genes are limited and have not been included in meta-analyses [1–4].

The aim of these analyses was to describe the association of common genetic variation in 24 fibrin-based clotting candidate genes with first-time MI and IS in hypertensive adults and postmenopausal women. Our approach involved global testing of gene-wide variation for each of the 24 genes with MI risk and IS risk separately and exploration of haplotype and SNP contributions to risk.

Methods

Setting and design

The setting for this observational study was Group Health (GH), a large integrated healthcare system in western Washington State. These analyses were part of a series of ongoing, population-based, case–control studies of MI, ischemic and hemorrhagic stroke, venous thrombosis, and atrial fibrillation [5–8]. For these analyses, we combined data from the study of MI and IS risk in adults with treated hypertension and the study of MI and IS risk in peri- and postmenopausal women. This study was approved by the GH Human Subjects Review Committee and all study participants provided written informed consent.

Study population

Subjects were pharmaceutically treated hypertensive men and women and peri- and postmenopausal women 30–79 years of age who were GH members. Case subjects were GH members who suffered an incident MI or IS between 1 January 1995 and 31 December 2002, and who were alive at the time of study recruitment. The date of the event served as an index date. Control subjects were a random sample of GH members that comprised a pool of control subjects shared by several case–control studies conducted at GH [5–8]. Control subjects were also GH members and were frequency matched by age (within decade), sex, treated hypertension status, and calendar-year of identification to MI cases, the largest case group. Control subjects included in this analysis had no prior history of MI or IS and their index date was a randomly chosen date within the calendar year from which they were selected as a control.

Case subjects were screened using ICD-9 codes from GH and non-GH hospitalizations. Trained medical record abstractors reviewed the medical records of all potential cases to verify the event. For MI, verification was based on symptoms, electrocardiogram findings, enzyme levels, and physician diagnosis and treatment. For IS, verification required the rapid onset of a neurologic deficit that persisted for at least 24 h or evidence of infarction on brain imaging studies or at surgery [5,9]. We excluded case subjects whose MI or stroke was a complication of a procedure or surgery.

Measures

Genetic variation The 24 candidate genes were selected *a priori* based on their role in regulating hemostasis. Specifically, the genes code for proteins affecting coagulation (FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, FXIIIa1, and FXIIIb; α-, β-, and γ-fibrinogen; and tissue factor), anticoagulation (antithrombin, proteins C and S, endothelial protein C receptor, thrombomodulin, and tissue factor pathway inhibitor), fibrinolysis [plasminogen, and tissue-type plasminogen activator (tPA)], and antifibrinolysis [PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI)]. All 24 genes were sequenced by the Seattle Program for Genomic Applications (PGA) using DNA obtained from a panel of 24 African-Americans and 23 European-Americans. Genes were sequenced starting 2.5 kb upstream of the start of transcription and extending 1.5 kb beyond the end of transcription. For large genes, sequencing was limited to exons and promoter regions. Details can be found at http://pga.gs.washington.edu/.

Among variants with a minor allele frequency of 5% or greater in the PGA panel, ldSelect (ldSelect version 1.0, University of Washington, Seattle, WA, USA) was used to identify maximally informative sets of SNPs to describe genetic variation using linkage disequilibrium [10]. This process was carried out separately for European- and African-American sequence data. From within ancestry-stratified SNP sets, a single variant was hierarchically chosen based on the following priorities: non-synonymous, untranslated regions (5′ or 3′), promoter, intron, and synonymous. In addition, we identified non-synonymous SNPs that were not already selected and that had a minor allele frequency of 4% or greater in either racial group.

Blood collection and genotyping A blood sample was collected from all consenting subjects and DNA was extracted from white blood cells using standard procedures. Genotyping was performed by Illumina® (Illumina Inc., San Diego, CA, USA) with a GoldenGate custom panel using BeadArray® technology. Illumina® personnel were blinded to case–control status. The final list of SNPs in the 24 genes numbered 306, of which 280 were successfully genotyped on the Illumina® platform. Among the 280 successful SNPs, 99.94% of nucleotide pairs were successfully called. Agreement between Illumina® results and results from a substudy of 1472 subjects that used restriction fragment length polymorphism technology to genotype two variants (FV Leiden and FII G20210A) was excellent (kappa = 0.99 and 1.00, respectively).

Haplotype construction and SNP selection Phased haplotypes were estimated for each gene using PHASE software (PHASE version 2.0, University of Washington, Seattle, WA, USA), which computes probabilities for each haplotype pair consistent with the observed data [11]. When ambiguous haplotypes were encountered, multiple, probability-weighted haplotype pairs were created. We combined haplotypes with a frequency of less than 2% in control and case populations into a single 'other' haplotype. We split the FV gene into two sections below a point of high recombination (PGA site 36090) to minimize the number of haplotypes below the 2% threshold. Halves were analyzed separately.

From among the 280 SNPs that were genotyped in the study population, 109 SNPs were found to be either extremely rare or non-varying, or tightly linked ($r^2 \geq 0.8$) with another SNP. Many of these low-informative SNPs were selected from African-American bins. These variants were excluded from SNP analyses leaving 171 SNPs characterizing genetic variation across the 24 candidate genes. RS numbers, PGA identifiers,

and Hardy–Weinberg equilibrium *P*-values for all SNPs are listed in Table S1 of the Supplementary material.

Demographic and clinical information Demographic and health-status information was obtained by review of the entire GH ambulatory medical record up to the index date. Information was collected on treated hypertension and diabetes, menopausal and smoking status, and history of coronary heart disease (angina or coronary revascularization), transient ischemic attack, and chronic heart failure. Clinical measures were also collected and included most recent weight and height, blood pressure, and lipid measures. Information on race, ancestry, and tobacco use was collected from the participant by telephone interview.

Statistical Analyses

Haplotype and SNP models For each gene, weighted logistic regression was used to estimate haplotype associations. To express haplotype uncertainty, we used probability weights produced by PHASE and robust 'sandwich' errors, clustered by subject [12]. The number of copies of each haplotype (0, 1, or 2) was used as the predictor, and the most common haplotype in the gene was used as the reference level and was not included in the model. The estimates were adjusted for race, and matching variables sex, age, index year and hypertension. For each haplotype, this model provides an estimate of the risk associated with each additional copy of the specified haplotype compared with an additional copy of the reference haplotype. This assumes an additive model of inheritance where each additional allele modifies risk similarly. These are presented as odds ratios (ORs) with 95% CIs. As a global test of variation within each gene, we compared the haplotype model that characterized all common variation within the gene with a model that included no haplotype information using a Wald test. Formally, this tests the null hypothesis that all gene-wide variants have an OR equal to 1. In addition, for each SNP, unweighted logistic regression models were used to estimate the association with MI and stroke risk while adjusting for race and matching variables. A single predictor coded 0, 1, or 2 (representing the number of copies of the minor allele) was created for each SNP, giving estimates of the OR associated with each additional copy of the minor allele. For the two X-linked genes, FVIII and FIX, males were coded 0 or 1 for haplotype and SNPs and females were coded 0, 0.5, and 1 to express the uncertainty of which allele was influential while maintaining a single-allele risk estimate. Models were not adjusted for other risk factors besides age, sex, and race, because acquired characteristics do not generally confound genetic associations.

Multiple testing and false discovery rate analysis Twenty-five global gene association tests were performed on the 24 candidate genes (FV gene was split into two parts). In the absence of a true association for any gene, we would expect a *P*-value of less than 0.05 for at least one of the 25 gene-wide association tests. A total of 180 haplotypes and 171 SNPs were tested for individual associations across the 24 genes. By chance alone, we would expect *P*-values of less than 0.05 in nine of the haplotype-specific tests and in at least nine of the SNP-specific tests.

To assist in the interpretation of *P*-values given the number of statistical tests performed, false discovery rate (FDR) *q*-values were calculated separately for gene-wide, haplotype and SNP analyses [13]. Rather than expressing the probability of one false positive result among all tests, the *q*-value estimates the proportion of results declared interesting that are actually false. A *q*-value threshold of 0.2 was selected to separate false discoveries from true discoveries in this candidate-gene investigation. A lower threshold is often used in genome-wide association studies, which does not use prior information to select candidate genes [14].

Interesting haplotype or SNP variants We examined 24 genes, and so can practically report measures of global association for each gene. However, with 180 haplotypes and 171 SNPs to assess, we summarized the more interesting findings. After computing *P*-values and *q*-values for all haplotypes and SNPs separately for each outcome, we identified and listed haplotypes and SNPs with *P*-values less than 0.05 in the models described above. In genes where the listed haplotype effect was primarily attributable to a single SNP, we listed the SNP and de-listed the haplotype to avoid redundancy of information. We have used a similar approach to report findings on these 24 hemostatic genes and venous thrombosis in postmenopausal women [15].

Results

Included in the analyses were 856 GH members who suffered an incident MI (365 in hypertensive men, 242 in hypertensive women, and 249 in non-hypertensive women) and 368 who suffered an incident IS (114 in hypertensive men, 150 in hypertensive women, and 104 in non-hypertensive women) between 1 January 1995 and 31 December 2002, and who provided a blood sample. Controls were without a history of MI or IS and numbered 2689 (1128 hypertensive men, 847 hypertensive women, and 714 non-hypertensive women). Characteristics of the two case groups and controls are listed in Table 1. The study population was largely of European ancestry and the average age ranged from 65 to 68 years for case and control groups. Cardiovascular risk factors, such as smoking, diabetes, elevated systolic blood pressure, and treated hyperlipidemia, were more common in cases than in controls.

Gene associations

Table 2 provides a summary of the 24 candidate clotting-related genes investigated and their global association with MI and IS risk. The proportion of all haplotypes with an incidence of 2% or less was small with 90% of alleles assigned a unique haplotype in 20 genes and nearly 80% in the remaining four

Table 1 Characteristics of study participants

Characteristic	Myocardial infarction $n = 856$	Ischemic stroke $n = 368$	Control $n = 2689$
Mean age, years (SD)	65.3 (9.9)	68.0 (9.1)	64.8 (9.8)
Female, *n* (%)	491 (57)	254 (69)	1561 (58)
White, *n* (%)	780 (91)	335 (91)	2451 (91)
Current smoker, *n* (%)	157 (18)	46 (13)	270 (10)
Treated hypertension, *n* (%)	614 (72)	264 (72)	1975 (73)
Treated diabetes, *n* (%)	207 (24)	90 (24)	306 (11)
Treated hyperlipidemia, *n* (%)	142 (17)	45 (12)	345 (13)
Coronary heart disease, *n* (%)	149 (17)	39 (11)	237 (9)
Chronic heart failure, *n* (%)	40 (5)	16 (4)	68 (3)
History of transient ischemic attacks, *n* (%)	33 (4)	39 (11)	68 (3)
Mean body mass index, kg m^{-2} (SD)	30.1 (6.1)	30.0 (6.9)	29.4 (6.3)
Systolic blood pressure, mmHg (SD)	142 (20.2)	146 (22.1)	138 (18.5)
Diastolic blood pressure, mmHg (SD)	80 (11.4)	82 (11.6)	80 (10.6)

SD, standard deviation.

genes. For global tests of genetic relation to MI, there was no gene with a *q*-value less than 0.2 and only one gene with a *P*-value less than 0.05 (plasminogen). For global tests in relation to IS, only the FVIII gene had a *q*-value less than 0.2. No other gene had a *P*-value less than 0.05.

Haplotype and SNP associations

Tables S2–S26 in the Supplementary material present the basic analyses that were conducted for MI and IS outcomes for each gene. We present Table 3, the FV table, as an example. Table 3 provides a matrix of nucleotides that summarize the association between SNP variation and haplotypes within the upper portion of the FV gene. Shaded cells identify the minor allele. Haplotype- and SNP-specific ORs, 95% CIs, and *P*-values are presented. For MI, we found that one of the 10 haplotypes was associated with an increased risk (haplotype 9: OR 1.8; 95% CI 1.2–2.7) compared with the reference haplotype. The MI high-risk haplotype was marked by SNP PGA-46058 (non-synonymous substitution of T to S at amino acid 915, rs9332695),

Table 2 Candidate gene studied and global tests of association with risk of incident myocardial infarction (MI) or ischemic stroke (IS)

Gene descriptives					Global risk association			
					MI		IS	
Gene name (HUGO abbreviation)	Proportion of gene sequenced, %	Number of SNPs investigated	Number of common haplotypes observed*	Haplotype coverage, %	*P*-value	FDR *q*-value	*P*-value	FDR *q*-value
Coagulation								
Factor (F) II or prothrombin (F2)	92.2	8	8	95.8	0.6688	0.9010	0.3103	0.7052
FV lower half of gene (F5)	71.3[†]	5	8	91.8	0.5541	0.9010	0.1325	0.5521
FV upper half of gene (F5)	71.3[†]	9	10	94.9	0.1139	0.8325	0.4475	0.7219
FVII (F7)	61.6[‡]	6	6	95.5	0.7186	0.9010	0.8336	0.9061
FVIII (F8)	29.9[§]	7	7	96.7	0.1025	0.8325	0.0023	0.0575
FIX (F9)	100.0	11	10	85.1	0.1667	0.8335	0.9217	0.9217
FX (F10)	86.2	10	12	79.9	0.3077	0.9010	0.4300	0.7219
FXI (F11)	98.4	9	14	93.8	0.3709	0.9010	0.0509	0.5295
FXII (F12)	100.0	2	3	100.0	0.4686	0.9010	0.7188	0.8985
FXIIIa1 (F13A1)	16.5[§]	9	14	91.7	0.7400	0.9010	0.1059	0.5295
FXIIIb (F13B)	95.8	5	5	97.1	0.3134	0.9010	0.4620	0.7219
Fibrinogen α (FGA)	100.0	5	6	99.0	0.7568	0.9010	0.3790	0.7219
Fibrinogen β (FGB)	97.1	4	6	100.0	0.8497	0.9272	0.0996	0.5295
Fibrinogen gamma (FGG)	100.0	4	6	99.9	0.4784	0.9010	0.0745	0.5295
Tissue factor (F3)	71.3	5	8	99.4	0.1332	0.8325	0.9201	0.9217
Anticoagulation								
Antithrombin (SERPINC1)	93.1	5	6	96.3	0.9415	0.9415	0.8199	0.9061
Protein C (PROC)	92.8[‡]	8	7	95.7	0.4225	0.9010	0.2957	0.7052
Protein C receptor (PROCR)	96.8	4	6	98.5	0.4703	0.9010	0.2393	0.6878
Protein S (PROS1)	58.6[†]	6	7	97.9	0.8926	0.9298	0.7791	0.9061
Thrombomodulin (THBD)	85.0[¶]	4	5	97.7	0.6041	0.9010	0.5885	0.7743
Tissue factor pathway inhibitor (TFPI)	98.1	5	8	99.7	0.4697	0.9010	0.4029	0.7219
Fibrinolysis								
Plasminogen (PLG)	44.3[§]	10	13	87.0	0.0226	0.5650	0.5840	0.7743
Tissue-type plasminogen activator (PLAT)	93.8	12	12	90.3	0.7528	0.9010	0.2381	0.6878
Antifibrinolysis								
Plasminogen activator inhibitor 1 (SERPINE1)	90.8	5	9	98.9	0.8530	0.9272	0.2476	0.6878
Thrombin activatable fibrinolysis inhibitor (CPB2)	95.6	13	9	85.5	0.5806	0.9010	0.5628	0.7743

HUGO, Human Genomics Organization; SNP, single nucleotide polymorphism. *Haplotypes with a minor allele frequency less than 2% were grouped into an 'other' haplotype. [†]Missing many introns. [‡]Missing 2 exons. [§]Missing all introns. [¶]Missing a third of single exon.

Table 3 Association between factor V genetic variation and risk of myocardial infarction and ischemic stroke

	FV upper SNPs	38592 (R534Q: FVL)		42713	45765 (N817T)	45888 (K858R: HR2)	46058 (T915S)	66464	66872	68717	72877 (M2148T)								
	Control MAF	2.3	39.7	5.7	27.9	1.3	6.6	20.5	47.1	4.7	Control (n = 2689) MAF	MI (n = 856) MAF	MI OR (95% CI)	*P*-value	Stroke (n = 368) MAF	Stroke OR (95% CI)	*P*-value	Stroke OR (95%CI)	*P*-value	MI OR (95%CI)	*P*-value
Haplotype 1		G	C	A	A	A	G	C	A	T	31.8	31.6	1.00 (reference)		33.0	1.00 (reference)		**1.53 (1.00–2.34)**	**0.0484**	1.03 (0.73–1.47)	0.8491
Haplotype 2		G	T	A	G	A	G	C	G	T	25.0	23.3	0.94 (0.81–1.09)	0.4086	24.5	0.94 (0.77–1.15)	0.5636	1.02 (0.86–1.20)	0.8308	1.10 (0.98–1.23)	0.1077
Haplotype 3		G	T	A	A	A	G	T	A	T	13.5	12.1	0.90 (0.75–1.08)	0.2550	11.7	0.82 (0.63–1.06)	0.1300	0.98 (0.71–1.37)	0.9171	1.00 (0.79–1.26)	0.9931
Haplotype 4		G	T	A	A	A	T	T	G	T	6.4	6.7	1.03 (0.81–1.30)	0.8126	6.0	0.91 (0.64–1.29)	0.6060	0.96 (0.80–1.14)	0.6363	0.91 (0.81–1.03)	0.1489
Haplotype 5		G	T	C	A	A	G	C	G	T	5.4	5.4	1.01 (0.78–1.30)	0.9634	5.0	0.85 (0.59–1.24)	0.4082	1.07 (0.54–2.12)	0.8366	**1.74 (1.16–2.62)**	**0.0072**
Other		-	-	-	-	-	-	-	-	-	5.1	5.7	1.11 (0.87–1.42)	0.3994	6.0	1.10 (0.79–1.55)	0.5706	0.94 (0.68–1.30)	0.7242	1.06 (0.85–1.32)	0.6011
Haplotype 6		G	C	A	A	A	G	C	A	C	4.6	5.5	1.19 (0.92–1.54)	0.1798	3.7	0.77 (0.51–1.16)	0.2125	0.90 (0.73–1.10)	0.2935	0.94 (0.82–1.08)	0.3660
Haplotype 7		G	T	A	A	A	G	C	G	T	4.6	5.0	1.07 (0.83–1.39)	0.6013	5.0	1.03 (0.71–1.50)	0.8701	1.02 (0.87–1.19)	0.8111	0.97 (0.87–1.09)	0.6158
Haplotype 8		A	T	A	A	A	G	C	G	T	2.3	2.5	1.04 (0.72–1.49)	0.8424	3.8	1.45 (0.93–2.28)	0.1022	0.82 (0.54–1.23)	0.3395	1.20 (0.94–1.53)	0.1471
Haplotype 9		G	C	A	A	T	G	C	A	T	1.3	2.2	**1.77 (1.17–2.67)**	**0.0071**	1.2	0.93 (0.46–1.89)	0.8375				
												Global *P*-value = 0.1139			Global *P*-value = 0.4475						

CI = Confidence interval
MAF = Minor allele frequency
MI = Myocardial infarction
OR = Odds ratio
SNP = Single nucleotide polymorphism

which was also associated with MI risk (OR 1.7; 95% CI 1.2–2.6). The minor allele frequency of the haplotype and SNP were identical in controls (1.3%). For IS, the FV Leiden variant was associated with risk (OR 1.5; 95% CI 1.0–2.3) yet the haplotype marked by this variant (haplotype 8) was not in part because reference groups were not the same for the two comparisons. Minor allele frequency of the variant and haplotype were identical in controls (2.3%).

A total of 180 haplotypes were tested across the 24 candidate genes. For MI, seven (3.9%) of the 180 tests had *P*-values less than 0.05, which represents two fewer than the nine that would have been expected by chance alone at the α-level of 0.05. For IS, eight (4.4%) of the 180 tests had *P*-values less than 0.05. The seven MI and the eight IS risk haplotypes were not the same. A total of 171 SNPs were tested across 24 candidate genes. For MI, five (2.9%) of the 171 tests had *P*-values less than 0.05. For IS, nine (5.3%) of the 171 tests had *P*-values less than 0.05. None of the MI and IS risk SNPs was the same. Among all haplotype and SNP associations tested, only four had FDR *q*-values less than 0.2. Three were associated with IS risk: FVIII haplotype 2 (OR 2.0; 95% CI 1.3–2.9; *q*-value 0.0630); FVIII 'other' haplotype (OR 2.8; 95% CI 1.4–5.6; *q*-value 0.1860); and FXIIIa haplotype 11 (OR 1.8; 95% CI 1.3–2.6; *q*-value 0.0630). One was associated with MI risk: plasminogen PGA-18114 (rs3823055; OR 1.2; 95% CI 1.1–1.4; *q*-value 0.1710). Table 4 lists the summary of these findings. When analyses were conducted separately among subjects with hypertension and among women, respectively, risk estimates were similar and not statistically different: FVIII haplotype 2 for IS, 1.7 vs. 2.2; FVIII 'other' haplotype for IS, 2.4 vs. 2.1; FXIIIa1 haplotype 11 for IS, 1.9 vs. 1.6; plasminogen PGA-18114 for MI, 1.3 vs. 1.2.

Comment

This investigation assessed the risk of arterial clotting associated with common genetic variation in 24 candidate coagulation, anticoagulation, fibrinolysis, and antifibrinolysis genes. Factor VIII was globally associated with IS risk and no gene was globally associated with MI risk. Three haplotypes, two in FVIII and one in FXIIIa1, were significantly associated with a 96%, 181%, and 81% increase in IS risk, respectively (all FDR

Table 4 Summary of interesting associations between SNPs and haplotypes and risk of myocardial infarction (MI) or ischemic stroke (IS)

				MI				IS			
Gene	Variant identifier (location)*	Susbstitution	MAF (%)	OR (95%CI)†	*P*-value	FDR *q*-value	Status	OR (95%CI)†	*P*-value	FDR *q*-value	Status
FV	46058	T915S	1	1.74 (1.16–2.62)	0.0072	0.4104	New	1.07 (0.54–2.12)	0.8366	–	–
FV	Factor V Leiden	R534Q	2	1.03 (0.73–1.47)	0.8491	–	–	1.53 (1.00–2.34)	0.0484	0.8162	Rep
FVIII	25167	G > A	2	0.75 (0.39–1.43)	0.3780	–	–	2.10 (1.01–4.37)	0.0460	0.8162	New
FVIII	55941	C > G	27	1.04 (0.84–1.27)	0.7361	–	–	1.56 (1.16–2.08)	0.0029	0.2480	New
FVIII	95826	D1260E	18	0.94 (0.74–1.20)	0.6192	–	–	1.70 (1.22–2.35)	0.0015	0.2480	New
FVIII	Hap 2	–	13	0.86 (0.65–1.15)	0.3242	–	–	1.96 (1.34–2.87)	0.0006	0.0630	New
FVIII	Hap other	–	3	0.65 (0.37–1.14)	0.1337	–	–	2.81 (1.42–5.58)	0.0031	0.1860	New
FIX	12806	T > A	5	1.54 (1.06–2.24)	0.0229	0.9714	New	0.99 (0.53–1.85)	0.9787	–	–
FIX	Hap 2	–	18	1.37 (1.04–1.82)	0.0262	0.7860	New	1.29 (0.85–1.95)	0.2312	–	–
FIX	Hap 5	–	5	1.81 (1.19–2.75)	0.0052	0.4920	New	1.20 (0.61–2.36)	0.6034	–	–
FX	9501	G > T	14	0.99 (0.84–1.16)	0.8626	–	–	0.76 (0.59–0.97)	0.0303	0.8162	New
FX	Hap 7	–	6	0.90 (0.71–1.15)	0.4183	–	–	0.61 (0.41–0.90)	0.0135	0.4037	New
FXI	3450	T > C	25	1.00 (0.88–1.14)	0.9891	–	–	0.81 (0.67–0.99)	0.0351	0.8162	New
FXI	10942	A > T	12	1.06 (0.90–1.25)	0.4890	–	–	1.29 (1.04–1.62)	0.0231	0.8162	New
FXI	Hap 13	–	1	1.56 (0.95–2.54)	0.0769	–	–	2.16 (1.21–3.85)	0.0092	0.3312	New
FXI	Hap 8	–	4	1.38 (1.04–1.82)	0.0244	0.7860	New	0.56 (0.30–1.04)	0.0680	–	–
FXIIIa1	Hap 11	–	2	1.01 (0.76–1.34)	0.9430	–	–	1.81 (1.28–2.56)	0.0007	0.0630	New
FGG	Hap other	–	< 1	1.56 (0.39–6.31)	0.5311	–	–	5.41 (1.54–19.0)	0.0086	0.3312	New
TF	Hap 2	–	20	1.17 (1.01–1.35)	0.0396	0.9445	New	1.12 (0.90–1.38)	0.3094	–	–
TF	Hap 6	–	4	1.39 (1.07–1.79)	0.0125	0.5625	New	1.11 (0.75–1.63)	0.6113	–	–
PROCR	837	C > G	10	1.00 (0.83–1.21)	0.9599	–	–	0.74 (0.55–0.99)	0.0441	0.8162	New
PROCR	3600	T > C	5	0.74 (0.56–0.97)	0.0293	0.9714	New	0.99 (0.69–1.41)	0.9488	–	–
PLG	18114	G > A	29	1.21 (1.08–1.36)	0.0010	0.1710	New	1.01 (0.85–1.20)	0.9173	–	–
PLG	31439	D472N	28	0.83 (0.73–0.94)	0.0035	0.2993	New	0.85 (0.71–1.02)	0.0802	–	–
PLG	34158	C > T	4	1.20 (0.91–1.58)	0.2026	–	–	1.58 (1.10–2.26)	0.0131	0.7467	New
PLG	Hap 7	–	8	1.34 (1.08–1.66)	0.0082	0.4920	New	0.97 (0.70–1.34)	0.8493	–	–
TPA	Hap 11	–	1	1.13 (0.72–1.77)	0.5991	–	–	1.78 (1.05–3.02)	0.0326	0.7335	New
PAI-1	Hap 8	–	3	1.11 (0.84–1.46)	0.4766	–	–	1.54 (1.08–2.18)	0.0157	0.4037	New

FDR, false discovery rate; FGG, γ-fibrinogen; FV, factor V; FVIII, factor VIII; FIX, factor IX; FX, factor X; FXI, factor FXI; FXIIIa1, factor XIIIa1; Hap, haplotype; MAF, minor allele frequency; PROCR, protein C receptor; PLG, plasminogen; plasminogen activator inhibitor type 1, PAI-1; SNP, single nucleotide polymorphism; TF, tissue factor; TPA, tissue plasminogen activator. *For SNPs, the Seattle PGA identifier is included. †For SNP models, the comparison group is those homozygous for the wild type; for haplotype models, the reference group is the most common haplotype in controls.

q-values < 0.2). One SNP, in plasminogen, was associated with a 21% increase in MI risk. Another 24 SNPs and haplotypes had *P*-values less than 0.05 yet had *q*-values greater than 0.2 in MI and IS analyses.

Ischemic stroke

Haplotype 2 and the 'other' haplotype of FVIII were among three FVIII SNPs that were associated with an increased risk of IS. None of these associations has been previously reported. As described in Table S7, FVIII haplotype 2 is marked (but not exclusively) by the minor allele of two of the three SNPs also associated with IS risk: PGA-95826 (rs1800291), a D to E amino-acid substitution at position 1260; and PGA-55941 (rs1936645), an intronic nucleotide substitution in tight LD with D1260E ($r^2 = 0.73$). The combined 'other' haplotype was composed of 185 rare haplotypes and included all minor alleles for the M2257V substitution (PGA-165293, rs1800297) that was private to African-Americans and that was associated with a non-significant elevated risk in our data (OR 2.6; 95% CI 0.9–7.1). Haplotype 11 of FXIIIa1 was associated with an increased risk of IS and carries minor alleles for V35L and P565L. Neither amino acid substitution was associated with a significant increase in IS in our data, similar to null reports from a meta-analysis [2]. The FV Leiden variant, which was associated with a 53% increase in risk in our data, replicates findings from a recent meta-analysis that found a 33% increase in IS [2]. PAI-1 haplotype 8, associated with a 54% increase in IS risk, is marked by the minor alleles of PGA-664 (rs2227631) and PGA-12219 (rs11178) and this finding appears to be new. The PGA-664 variant is in strong LD ($r^2 = 0.92$) with the G insertion (PGA-837 4G/5G), which has been associated with a 47% increase in IS risk in a recessive risk model that combined data from five studies [2]. The tag SNP for 4G/5G was not associated with risk in our additive model and a *post hoc* exploration of the recessive model did not yield significant findings (OR 1.2; 95% CI 0.9–1.6). The IS associations with FX, FXI, γ-fibrinogen, protein C receptor, plasminogen, and tPA have not been reported in the literature and serve as hypothesis-generating findings.

Myocardial infarction

The PGA-18114 SNP in plasminogen was associated with a 21% increase in risk and this finding has not been reported previously. The 10 other associations with MI listed in Table 4 had a *q*-value less than 0.2. Of those with *P*-values less than 0.05, variants in FV, FIX, FX, FXI, tissue factor, protein C receptor, and plasminogen genes have not been previously reported to be associated with MI risk. Two of these variants were non-synonymous substitutions: FV T915S and plasminogen D472N. Two haplotypes in tissue factor, 2 and 6, were associated with increased MI risk. Haplotype 6 includes the minor allele by PGA-599 (rs958587), which, along with another promoter variant in tight LD, has been associated with increased cardiovascular morbidity and mortality [16,17]. The PGA-599 was not associated with MI risk in our data. Associations with both haplotypes appear to be new findings.

Factor V Leiden was not associated with MI risk in our data (OR 1.0; 95% CI 0.7–1.5), yet our CI overlaps the risk estimate from a recent meta-analysis [1]. Similarly, our CIs for FII 20210A (OR 1.4; 95% CI 0.9–2.1) or the PAI-1 4G/5G tag SNP (OR 1.0; 95% CI 0.9–1.1) overlapped estimates of 25% and 4% increases in MI risk, respectively, reported in the meta-analysis [1]. We did not find FXIIIa1 V35L (also reported as V34L) to be associated with decreased risk in additive (OR 0.9; 95% CI 0.8–1.1) or *post hoc* recessive models (OR 0.9; 95% CI 0.7–1.3) and reports for this variant have been inconsistent [2]. We did not find significant associations between FVII, FVIII, FXII, and thrombomodulin variants and MI risk, unlike previous reports, some of which have been inconsistent [1,18–27]. The A147T variant in TAFI has been inconsistently associated with coronary disease risk and this variant was not identified by the PGA, so it was not genotyped in our data [28,29].

Interpretation of the FDR statistic in candidate gene studies

We used a 0.2 threshold for the *q*-value to guide the interpretation of gene-, haplotype-, and SNP-level findings in this candidate gene study. This threshold identified one significant global gene association among 50 tests performed (two outcomes $\times$ 25 genes), three significant haplotype associations among the 360 performed (2×180), and one significant SNP among the 342 tests performed (2×171). By design, 20% of these discoveries are likely to be false. Without the application of the false discovery rate, two of the global gene tests and 28 of the haplotype and SNP tests would have been considered significant based on a *P*-value of less than 0.05. We did not exclude from analyses three variants (FV Leiden, FII 20210A, and PAI-1 4G/5G) that were associated with MI and IS risk in recent meta-analyses [1,2]. Exclusion of these SNPs and the haplotypes that they mark from analyses would have minor effects on FDR *q*-values.

Limitations

Our comprehensive approach to risk evaluation among candidate genes at whole-gene, haplotype, and SNP levels for two arterial thrombotic outcomes inherently involved multiple tests. To better characterize the risk of reporting false positive associations, we provided FDR *q*-values and a suggested *q*-value threshold. We limited our investigation to the main effects of genes and did not explore gene–gene interactions or gene–environment interactions. Population association studies such as ours are prone to the potential confounding effects of population substructure. We have adjusted for self-reported race in all analyses but the population of African-Americans in our study was too small to meaningfully address risk differences by race. As our study had only candidate genes, methods of population substructure estimation such as STRUCTURE, or 'genomic control' of population stratification could not be applied [30,31]. For large genes, such as FVIII, only portions of the gene had been sequenced for variation, which limits the amount of variation we were able to capture with our approach. Most non-sequenced gene sections were introns, however. With the exception of FX, our approach used the most common haplotype as the reference in haplotype analyses, and other approaches are possible that would lead to different risk estimates and *P*-values. Although we had adequate statistical power to detect MI risks as small as 1.4 for a variant with a incidence of 10%, our power was more limited for IS, where we could detect a risk as small as 1.9 for a variant with the same incidence. The sample size of our study limited our ability to detect risks in the 1.2–1.5 range, especially for IS outcomes. Although we adjusted for multiple testing, these analyses did not involve replication in another population, so any new findings we have reported need this additional level of interrogation before true associations are established. All subjects in these analyses were survivors of their incident event and findings cannot be generalized to fatal events, which may be more thrombogenic and associated more strongly with genetic risk. Results also cannot be generalized to non-hypertensive men and those who suffered an event before the age of 30 or after 79.

This study investigated the risk of arterial thrombosis associated with common variation in 24 key genes in fibrin-based clotting pathways. After accounting for false discoveries, only FVIII was associated with gene-wide global risk and this was for IS exclusively. Three haplotypes, two in FVIII and one in FXIIIa1, were significantly associated with IS risk. A SNP in the plasminogen gene was associated with MI risk. All were new discoveries that need replication in other populations and as well as follow-up functional investigations to determine their impact on factor plasma levels and activity. Another 24 associations had *P*-values less than 0.05 and an FDR *q*-value greater than 0.2. One of these associations has been previously reported and our study serves as a replication; the remaining 24 are new and hypothesis generating and need replication in other study populations. Apart from the association of FVIII

variation with IS, we found little evidence that genetic variation in fibrin-based hemostatic factors strongly influences arterial thrombotic risk, but our results cannot rule out the presence of small effects.

Addendum

N. L. Smith obtained funding for the study and was responsible for the overall integrity of the manuscript including the study design, data analysis, and written manuscript. J. C. Bis assisted with analyses, data interpretation, and critical revisions of the manuscript. S. Biagiotti conducted literature searches, reviewed and interpreted the literature, and composed the literature review. K. Rice, T. Lumley, and C. Kooperberg provided input on study design, expert statistical review, and provided critical revisions to the manuscript. K. L. Wiggins provided data management and critical revisions of the methods section of the manuscript. S. R. Heckbert and B. M. Psaty obtained funding for the study, contributed to the design of the study, assisted with data interpretation, and provided critical revisions to the manuscript.

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Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

Supplementary Material

The following supplementary material is available for this article:

Table S1. Gene, Seattle Program for Genomic Applications identifier and RS number for single nucleotide polymorphisms presented in appendix tables A2 to A26.
Table S2. Association between factor II variants and myocardial infarction and ischemic stroke.
Table S3. Association between factor V (lower) variants and myocardial infarction and ischemic stroke risk.
Table S4. Association between factor V (upper) variants and myocardial infarction and ischemic stroke risk.
Table S5. Association between factor VII variants and myocardial infarction and ischemic stroke risk.
Table S6. Association between factor VIII variants and myocardial infarction and ischemic stroke risk.
Table S7. Association between factor IX variants and myocardial infarction and ischemic stroke risk.
Table S8. Association between factor X variants and myocardial infarction and ischemic stroke risk.
Table S9. Association between factor XI variants and myocardial infarction and ischemic stroke risk.
Table S10. Association between factor XII variants and myocardial infarction and ischemic stroke risk.
Table S11. Association between factor XIIIa1 variants and myocardial infarction and ischemic stroke risk.
Table S12. Association between factor XIIIb variants and myocardial infarction and ischemic stroke risk.
Table S13. Association between α fibrinogen variants and myocardial infarction and ischemic stroke risk.
Table S14. Association between β fibrinogen variants and myocardial infarction and ischemic stroke risk.
Table S15. Association between γ fibrinogen variants and myocardial infarction and ischemic stroke risk.
Table S16. Association between tissue factor variants and myocardial infarction and ischemic stroke risk.
Table S17. Association between antithrombin variants and myocardial infarction and ischemic stroke risk.
Table S18. Association between protein C variants and myocardial infarction and ischemic stroke risk.
Table S19. Association between protein C receptor variants and myocardial infarction and ischemic stroke risk.
Table S20. Association between protein S variants and myocardial infarction and ischemic stroke risk.
Table S21. Association between thrombomodulin variants and myocardial infarction and ischemic stroke risk.
Table S22. Association between tissue factor pathway inhibitor variants and myocardial infarction and ischemic stroke risk.
Table S23. Association between plasminogen variants and myocardial infarction and ischemic stroke risk.
Table S24. Association between tissue-type plasminogen activator variants and myocardial infarction and ischemic stroke risk.
Table S25. Association between type 1 plasminogen activator inhibitor variants and myocardial infarction and ischemic stroke risk.
Table S26. Association between thrombin activatable fibrinolysis inhibitor variants and myocardial infarction and ischemic stroke risk.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2007.02795.x (This link will take you to the article abstract).

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