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Genetic Determinants of Variability in Warfarin Response after the Dose-Titration Phase

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Abstract

Objectives—Genetic factors contribute substantially to variability in warfarin dose requirements and are important in the dose-titration phase; their effects on the stability of anticoagulation later in therapy are not known.

Methods—Using de-identified electronic medical records linked to a DNA-biobank, we studied 140 African-Americans and 943 European-Americans after the warfarin dose-titration phase. We genotyped 12 SNPs in genes (*CYP2C9*, *VKORC1*, *CYP4F2*, *GGCX*, *EPHX1*, *CALU*) associated with altered warfarin dose-requirements and tested their associations with INR variability (INR_{VAR}) and percent time in therapeutic range (TTR) in European-Americans and African-Americans.

Results—One allele copy of rs2108622 in *CYP4F2* was associated with a 15 percent (95% CI, 1, 26, $p = 0.03$) decrease in median INR_{VAR} in European-Americans. In African-Americans, *GGCX* variants rs11676382 and rs699664 were associated with 4.16 (95% CI, 1.45, 11.97, $p = 0.009$) and 1.50 (95% CI, 1.07, 2.08, $p = 0.02$) fold changes in median INR_{VAR} per variant allele copy respectively; rs11676382 was also significantly associated with a 23.19 (95% CI, 5.89, 40.48, $p =$

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Conflicts of Interest:

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0.01) percent decrease in TTR. The total variation in INR_{VAR} explained by both clinical factors and rs2108622 was 5.2% for European-Americans. In African-Americans, the inclusion of *GGCX* variants rs11676382 and rs699664, and *CYP2C9*8* variant rs7900194 explained approximately 29% of the variation in INR_{VAR} .

Conclusions—The stability of anticoagulation after the warfarin dose-titration phase is differentially affected by variants in *CYP4F2* in European-Americans and *GGCX* loci in African-Americans.

Introduction

Warfarin, a vitamin K antagonist, is a cornerstone of oral anticoagulation therapy; however, it has a narrow therapeutic index and patients on warfarin who are under- or over-anticoagulated have increased risk of thrombosis and bleeding, respectively. Consequently, anticoagulation is monitored by measurement of the international normalized ratio (INR), and warfarin dose is adjusted to maintain the INR within a target range, between 2 and 3 for most patients.

There is, however, substantial interindividual variability in the INR response to the same dose of warfarin, and several clinical and genetic factors affect dose requirements [1–4]. Moreover, once the dose of warfarin that an individual patient requires to attain the target INR has been established (the dose-titration phase), the INR can fluctuate despite an unchanged warfarin dose. Thus, the INR is monitored throughout therapy and dosing is adjusted to maintain the INR within the target range.

Approximately 50% of the variability in warfarin dose requirement among individuals is attributable to genetic variations notably those that occur in *CYP2C9* that metabolizes S-warfarin, and in *VKORC1*, the target of warfarin [5]. Consequently, dosing algorithms that incorporate a patient's genotype have evolved [6], although the added contribution of genotype to improvement in quality of anticoagulation is controversial [7, 8]. We and others have shown that the contribution of genotype to predicting an individual's stable warfarin dose is lost within 2 weeks because the stable dose is identified rapidly by empiric titration of dose against the INR [9–11]. However, several lines of evidence [12–15] suggest that the effects of genotype on clinical outcomes persist after an individual's warfarin dose requirement has been determined.

For example, the risk of bleeding or thrombosis was increased long after the initial 2 week dose-titration phase in patients with genotypes associated with altered warfarin sensitivity [12, 13]. The mechanisms whereby genotype might affect complications of warfarin therapy beyond the dose-titration phase are not known. We hypothesized that long-term increased risk associated with genotype is due to greater instability of warfarin response (and thus of INR) in patients with variant genotypes, throughout therapy.

The lack of stability of the INR response despite a stable dose of warfarin in individual patients is a major clinical problem. As a consequence, patients require regular INR monitoring and time above or below the therapeutic range increases the risk of bleeding and thrombosis, respectively. Stability of the INR response during warfarin therapy is most often

assessed using two measurements: percent of time spent in therapeutic INR range (TTR), [16] and INR variability [17, 18]. TTR is a standard measure of the quality of anticoagulation; lower values are associated with increased risk of adverse outcomes in patients on warfarin [19]. However, because TTR does not capture the full amplitude of INR fluctuations within and outside the target INR range, INR variability is also measured. As is the case with lower TTR, higher INR variability is also associated with adverse outcomes during warfarin therapy [18, 20].

Little is known about the effect of genetic variants that alter warfarin dose-requirements on the stability of the INR response after the dose-titration phase. Therefore, we tested the hypotheses that variants in genes (*CYP2C9*, *VKORC1*, *CYP4F2*, *GGCX*, *EPHX1*, *CALU*) [4, 21–25] associated with altered warfarin dose are associated with INR variability and TTR after the warfarin dose-titration phase.

Methods

Study Population

The study was performed using BioVU, the clinical practice-based biobank at Vanderbilt University Medical Center (VUMC) that stores DNA samples linked to a de-identified version of the electronic medical record (EMR). The enrollment and biobanking approaches have been described [26, 27]. Notably, since January 2015, accrual into BioVU has been switched from an opt-out to an opt-in approach, to comply with evolving federal regulations on use of DNA samples in biomedical research. The Institutional Review Board of VUMC approved this study.

We utilized a cohort of 1170 patients who received warfarin for at least three months, achieved a stable dose of warfarin during therapy and who had been successfully genotyped for the variants of interest [22]. Patients were required to have at least 6 INR values after the study start date. Of the 1170 potential subjects, 1083 (140 African-Americans and 943 European-Americans) qualified.

Phenotyping

For each patient in the cohort we identified a study start date. We designated the study start date as occurring 30 days after the first INR ≥ 1.8 (in order to exclude the warfarin dose-titration phase). Subsequently, INRs for the next 360 days were included in the analysis unless the patient discontinued warfarin earlier. Temporary warfarin discontinuation of up to two weeks was allowed, for example, as occurs before surgery. We identified periods of temporary warfarin discontinuation by examining all INR values for each patient during the 360 day study period, identifying values ≤ 1.2 , and reviewing the EMR to ascertain the cause for the low INR. If review of the EMR determined that the low INR was due to temporary discontinuation of warfarin, the time between the last INR while receiving warfarin and the study resumption start date was excluded from the study time. The study resumption start date after a temporary discontinuation of warfarin was set to 10 days after the next INR of ≥ 1.8 .

The primary outcome was INR variability (INR_{VAR}) calculated for each patient using the variance of the INRs according to the modified Fihn Variance Growth Rate (VGR) method [18]. INR_{VAR} measures the time-weighted variance of the INR and reflects the degree to which each INR value deviates from the previous one, thus reflecting INR variability, irrespective of the intensity of anticoagulation (as measured by TTR) [17, 18].

The secondary outcome was TTR calculated by Rosendaal's linear interpolation method [16]. TTR reflects the sum of a patient's time within the therapeutic range divided by the total time of observation and is expressed as percent.

SNP Selection and Genotyping

We studied 12 preselected candidate single nucleotide polymorphisms (SNPs) in six genes *CYP2C9*, *VKORC1*, *EPHX1*, *GGCX*, *CALU*, and *CYP4F2* known to influence warfarin dose that had been previously genotyped in this cohort. Details of genotyping procedures and quality control measures have been described [22]. Briefly, SNPs were genotyped using either the Sequenom (Sequenom, Inc., CA, USA) or Taqman (Applied Biosystems, Inc., CA, USA) genotyping technologies and subjected to standard genotyping quality control procedures. All SNPs passed tests for Hardy Weinberg Equilibrium at a significance level of $p < 0.05$.

Statistical Analyses

Patient demographics and baseline clinical characteristics data were summarized with 10th, 50th and 90th percentiles for continuous variables and percentages for categorical variables. To examine the association of SNPs with INR_{VAR} and TTR, unadjusted and adjusted analyses were performed separately in European-Americans and African-Americans due to differences in SNP minor allele frequencies. In unadjusted analyses, simple linear models were constructed with each target SNP in the model without adjusting for clinical covariates. In adjusted analyses, multiple linear regression models were constructed using all 12 prespecified SNPs while adjusting for pre-specified clinical risk factors for warfarin response: age, sex, BMI, smoking, amiodarone use, concurrent use of enzyme inducers and indication for warfarin use including atrial fibrillation, pulmonary embolism or venous thrombosis (PE/DVT), and stroke. We utilized an additive genetic model and, if indicated by the putative modes of inheritance for the alleles of interest, also examined recessive or dominant models. Prior to model fitting, INR_{VAR} was log-transformed due to skewness, and the reported exponentiated SNP associations with INR_{VAR} should be interpreted as the fold-change in the geometric mean or the median INR_{VAR} associated with a one minor allele increase. We also analyzed the relationship between both INR_{VAR} and TTR and categories of warfarin response (normal, sensitive or highly sensitive) using combinations of *VKORC1* and *CYP2C9* SNPs (and genotypes) to categorize individuals [28].

All analyses were performed using R version 3.1.2; a two-sided significance level of 0.05 was used for statistical inferences.

Results

Study Cohort Characteristics

Baseline cohort characteristics are shown in Table 1. Of the 1083 subjects, 140 (12.9%) were African-American and 483 (44.6%) were female. The median (10th, 90th percentile) age and BMI was 67 (43, 83) years and 28.4 (22.2, 38.3) kg/m², respectively. The most frequent indication for warfarin therapy in European-Americans was atrial fibrillation (56.5%), followed by pulmonary embolism or deep vein thrombosis (PE/DVT, 22.4%). In African-Americans, the most frequent indication was PE/DVT (49.3%) followed by atrial fibrillation (31.4%).

Outcomes and Association with Genotype

TTR was statistically significantly higher in European-Americans than in African-Americans 62.9 (29.9, 88.3) vs 56.1 (23.1, 83.0), with $p = 0.01$ using the Wilcoxon rank sum test, though there was no significant difference in INR_{VAR} between the two ethnicities. ($p = 0.12$).

In European-Americans subjects, the only variant associated with INR_{VAR} was rs2108622 (V433M) in *CYP4F2*. Each copy of the minor T allele was associated with a 15 percent decrease (95% CI, 1, 26, $p = 0.03$) in median INR_{VAR} (Table 2).

In African-Americans, two SNPs in the *GGCX* locus were significantly associated with INR_{VAR}, rs11676382 and rs699664 (Figure 1). For rs11676382, there was a 4.16-fold change (95% CI, 1.45, 11.97, $p = 0.009$) in median INR_{VAR} per copy of the minor G allele. For rs699664, there was a 1.50 fold change (95% CI, 1.07, 2.08, $p = 0.02$) in median INR_{VAR} per copy of the minor C allele. With this SNP, testing with the recessive model also showed a highly significant association ($p < 0.001$) in African-Americans.

No SNPs were significantly associated with TTR in European-Americans (Table 3). However, in African-Americans, a *GGCX* SNP (rs11676382) was significantly associated with TTR. We estimated a 23.19 (95% CI, 5.89, 40.48, $p = 0.01$) percentage point decrease in mean TTR per copy of the minor G allele.

Adjusted Analysis - Full Regression with Genotype and Clinical Factors

In models that included all 12 SNPs and prespecified clinical risk factors, in European-Americans, INR_{VAR} was significantly associated with female gender, concurrent use of amiodarone and enzyme inducers while TTR was associated with older age. In African-Americans, age and BMI were strongly associated with both INR_{VAR} and TTR (Supplementary Table 2). In these models, the effects of the *GGCX* variants on INR_{VAR} in African-Americans were abrogated but a SNP in the *CYP2C9* locus, rs7900194 (*CYP2C9**8) became borderline significant (2.09-fold increase in the geometric mean (or median) of INR_{VAR} per copy of the minor allele, $p = 0.05$). For TTR, the effects of rs11676382 remained significant in African-Americans but the effect of rs2108622 (V433M) in European-Americans did not.

In European Americans, female age, amiodarone use, and use of enzyme inducers explained 4.7% and 2.4% of the variation in INR_{VAR} and TTR, respectively and inclusion of rs2108622 (V433M) into the model only served to explain 5.2% of the total variation in INR_{VAR} .

In African-Americans, age and BMI accounted for 15% of the variation in both INR_{VAR} and TTR. With the inclusion of the significantly associated *GGCX* variants rs11676382 and rs699664, and the *CYP2C9**8 variant rs7900194 (borderline significance in the regression model) the model explained approximately 29% of the variation in INR_{VAR} . For TTR, adding rs11676382 into the model with prespecified clinical factors increased the percent of variation explained from 14.7 % to 19.6%.

Comparison by Warfarin Sensitivity

Subjects were categorized into 3 genotype functional classes (normal, sensitive and highly sensitive) that corresponded to the FDA warfarin response categories based on combinations of *VKORC1* and *CYP2C9* genotypes. There were 690 normal responders, 280 sensitive and 32 highly sensitive responders. There was no significant differences in either TTR ($p = 0.86$) or INR_{VAR} ($p = 0.93$) among the three groups.

Discussion

The major finding is that some genetic variants in the vitamin K recycling pathway contribute to interindividual variations in stability of INR after the dose titration phase of warfarin therapy. Specifically, rs2108622 (V433M) in *CYP4F2* contributed to variation in European-Americans, and rs11676382 and rs699664 in *GGCX* contributed significantly to INR variation in African-Americans.

The *CYP4F2* locus encodes the CYP4F2 protein, a key regulator of vitamin K levels through NADPH-dependent oxidation and inactivation that ensures effective negative feedback regulation. The rs2108622 (V433M) variant in this gene, a C>T missense SNP with global MAF 0.24, designated as the *3 allele (*CYP* allele nomenclature website at <http://www.cypalleles.ki.se/>) has been associated with warfarin dose. Several studies have reported an association between the T allele and increased warfarin dose requirements [29–32]. A recent meta-analysis estimated that subjects with the rs2108622 T allele required an 8.3% higher warfarin dose than those with the CC genotype [29].

We found an association between rs2108622 (V433M) in *CYP4F2* and INR_{VAR} in European-Americans; subjects bearing one or more copies of the T allele had significantly lower INR variability compared with those with the C allele. This association was not significant in African-Americans, likely due to a lack of statistical power (only 1 TT homozygous subject). The finding of decreased INR variability associated with the T allele of this *CYP4F2* variant is concordant with findings in recent studies of genetic risk factors for major bleeding in patients receiving warfarin that suggested a lower risk of bleeding in subjects bearing the minor T allele [13, 15].

The *GGCX* locus encodes gamma-glutamyl carboxylase which catalyzes the gamma-carboxylation of glutamic acid residues on vitamin K-dependent clotting factors. Because of this role in vitamin K recycling, many studies have examined the effects of *GGCX* variants on warfarin dose. Variants in *GGCX* have been associated with warfarin dose requirements in cohorts of varying ethnicities [23, 33]. Rs11676382 C>G in *GGCX* (intronic variant, global MAF = 0.0256) accounted for a 2–6% reduction in warfarin dose per copy of the minor G allele [33]. In the present study, we report an association between increased INR variability and the G allele of rs11676382 in African-Americans. There were no African-American subjects homozygous for the G allele but heterozygotes (n= 7) had a 4 fold change in median INR $_{VAR}$ and almost two fold numerical decrease in TTR than homozygous CC subjects. This difference appears clinically meaningful in terms of risk associated with both decreased intensity and stability of anticoagulation on warfarin [17].

The second *GGCX* variant, rs699664 C>T (missense SNP, global MAF 0.38), has also been associated with warfarin dosing, largely in Asian populations [34, 35] but not in European-Americans [23]. The differences in outcomes from various studies could be due to the marked differences in minor allele frequency among various ethnicities. The minor (C) allele in African-Americans (MAF 0.39) is the major allele in populations of European ancestry (MAF 0.67). We found significant differences in INR $_{VAR}$ in African-American subjects bearing two copies of the minor allele compared with those bearing one and zero copies. Subjects with the CC genotypes had significantly greater INR variability but did not have a lower TTR compared with CT and TT genotypes. Of interest, the *GCCX* variants associated with INR $_{VAR}$ or TTR in African-Americans were not associated with either outcome in European-Americans.

It is interesting that the significant genetic associations with stability of anticoagulation were with variants involved in vitamin K recycling rather than warfarin metabolism, suggesting that variants related to vitamin K pathways are more likely to contribute to variability in anticoagulation, perhaps because of variability in dietary vitamin K intake. Associations between *VKORC1* and *CYP2C9* variants and measurements of warfarin anticoagulation have typically been found to be most relevant early in therapy, particularly in the dose-titration phase. A recent study reported significant differences in TTR and bleeding during warfarin therapy among subjects categorized as normal, sensitive or highly sensitive according to genotype, but differences did not persist beyond 90 days [28]. We found no associations between the same warfarin sensitivity categories and INR $_{VAR}$ or TTR, perhaps because many patients were exposed to warfarin for longer than 90 days. We did however find a borderline significant association between a *CYP2C9**8 variant (rs7900194) and INR $_{VAR}$. The minor A allele has been associated with decreased warfarin clearance and therefore higher dose requirements in African-Americans [36, 37]

Clinical factors also affected the stability of anticoagulation. Concurrent use of amiodarone and enzyme inducing drugs, and female gender were associated with greater INR $_{VAR}$ (in European-Americans) while age and BMI were linked to lower INR $_{VAR}$ (in African-Americans). Use of amiodarone, a potent *CYP2C9* inhibitor is known to be associated with lower warfarin dose requirements, while concurrent use of enzyme inducers is associated with higher dose requirements. Older age has been associated with lower warfarin dose

requirements and was also associated with INR_{VAR} in our cohort, an association that remained significant after adjustment for other variables. Higher BMI was associated with lower INR_{VAR} in African-Americans. Vitamin K is a fat soluble vitamin and if the dietary intake of vitamin K was lower or more variable in African-Americans it is possible that vitamin K in fat could protect against INR fluctuation under those circumstances. While there were no significant differences in INR_{VAR} , TTR tended to be higher in European-Americans compared to African-Americans. In addition to genetic factors, these variables would be affected by diet, adherence to medication and other factors.

Overall, this study sought to explain the variance observed in stability of the INR during long-term warfarin treatment using genetic and clinical factors. The total amount of variation explained by all factors was approximately 30% in African-Americans, and 5% in European-Americans. One reason for the difference may be that we did not study factors that contribute substantially to variability in European-Americans.

Our study had several limitations; we utilized a retrospective case-only design and thus were not able to prospectively study variables of interest such as diet or other clinical and environmental factors. African-American subjects represented a relatively small cohort and thus we had limited statistical power to detect small effects. Also, we could not measure adherence to warfarin but this should not be differentially affected by genotype.

In conclusion, we report for the first time an association between genetic variants in the vitamin K pathway other than *VKORC1* and *CYP2C9* on anticoagulation stability (INR_{VAR}) and intensity (TTR) in patients on long-term warfarin. The effects were modest and much of the variability in the stability of anticoagulation in patients receiving chronic warfarin remains unexplained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Words and Abbreviations

INR	International Normalized Ratio
TTR	Time in therapeutic range
EMR	Electronic Medical Records
INR_{VAR}	INR Variability
MAF	Minor Allele Frequency

SNP	Single Nucleotide Polymorphisms
VUMC	Vanderbilt University Medical Center

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Figure 1. INR Variability and *GGCX* Variant Genotypes in African Americans

Footnote Data shown for *GGCX* variants rs11676382 and rs699664 and depicted as median (solid dark line) and interquartile range (box) and 1.5 times the interquartile range (whiskers).

Rs11676382; GG = 0, GC = 7, CC = 131 ($p = 0.009$)

Rs 699664; CC = 21, CT = 63, TT = 56 ($p = 0.02$)

Table 1**Cohort Characteristics**

	European-Americans (N = 943)	African-Americans (N = 140)	Overall (N = 1083)
Age (years)	67.0 (45.0, 83.8)	60.5 (32.0, 79.0)	67.0 (43.0, 83.0)
Gender (female)	42.7	57.1	44.6
BMI (kg/m ²)	28.2 (22.2, 38.0)	30.3 (22.5, 43.1)	28.4 (22.2, 38.3)
Current smokers	9.8	21.4	11.3
Amiodarone use	8.3	8.6	8.3
Enzyme inducers	2.7	2.9	2.7
Indication for warfarin			
Atrial fibrillation	56.5	31.4	53.3
Thrombosis	22.4	49.3	25.9
Stroke	5.4	5.0	5.4
Orthopedic	0.2	0.7	0.3
Other	17.3	15.0	17.0

Continuous variables are shown as 10th, 50th and 90th percentiles. Categorical variables are shown as percentages

Table 2

Association between variants and INR variability

Variant (SNP)	Gene	European Americans		African Americans	
		Effect (95% CI)	P-value	Effect (95% CI)	P-value
rs339097	<i>CALU</i>	-	-	1.33 (0.76, 2.33)	0.32
rs1799853	<i>CYP2C9</i> (*2)	0.98 (0.80, 1.20)	0.82	0.76 (0.27, 2.10)	0.59
rs1057910	<i>CYP2C9</i> (*3)	1.09 (0.82, 1.45)	0.54	2.76 (0.67, 11.30)	0.16
rs9332131	<i>CYP2C9</i> (*6)	-	-	1.19 (0.33, 4.25)	0.79
rs7900194	<i>CYP2C9</i> (*8)	-	-	1.59 (0.81, 3.12)	0.18
rs28371685	<i>CYP2C9</i> (*11)	-	-	0.45 (0.14, 1.43)	0.18
rs2108622	<i>CYP4F2</i> (*3)	0.85 (0.74, 0.99)	0.03	0.85 (0.49, 1.47)	0.56
rs2292566	<i>EPHX1</i>	1.03 (0.84, 1.25)	0.80	1.14 (0.71, 1.83)	0.60
rs11676382	<i>GGCX</i>	1.13 (0.90, 1.42)	0.28	4.16 (1.45, 11.97)	0.009
rs699664	<i>GGCX</i>	0.97 (0.85, 1.12)	0.72	1.50 (1.07, 2.08)	0.02
rs9923231	<i>VKORC1</i>	1.03 (0.89, 1.18)	0.70	0.88 (0.51, 1.55)	0.67
rs2359612	<i>VKORC1</i>	1.02 (0.89, 1.17)	0.78	1.05 (0.70, 1.57)	0.81

INR variability data were log transformed prior to conducting simple (without clinical data adjustments) linear regression analyses. Effect estimates and confidence intervals were exponentiated and results shown should be interpreted as the ratio of the geometric mean or the median associated with a one minor allele change. Blank entries for variants in European Americans are for SNPs represented only in African Americans.

Table 3

Association between variants and time in therapeutic range

Variant (SNP)	Gene	European Americans		African Americans	
		Effect (95% CI)	P-value	Effect (95% CI)	P-value
rs339097	<i>CALU</i>	-	-	-2.17 (-11.28, 6.94)	0.64
rs1799853	<i>CYP2C9</i> (*2)	-1.79 (-4.83, 1.25)	0.25	-11.48 (-28.00, 5.05)	0.18
rs1057910	<i>CYP2C9</i> (*3)	-2.90 (-7.16, 1.35)	0.18	-13.62 (-36.57, 9.33)	0.25
rs9332131	<i>CYP2C9</i> (*6)	-	-	4.53 (-16.19, 25.26)	0.67
rs7900194	<i>CYP2C9</i> (*8)	-	-	-2.90 (-13.85, 8.04)	0.60
rs28371685	<i>CYP2C9</i> (*11)	-	-	-2.50 (-21.50, 16.50)	0.80
rs2108622	<i>CYP4F2</i> (*3)	0.92 (-1.26, 3.10)	0.41	5.25 (-3.64, 14.13)	0.25
rs2292566	<i>EPHX1</i>	-1.06 (-4.02, 1.9)	0.48	-0.07 (-7.82, 7.69)	0.99
rs11676382	<i>GGCX</i>	1.31 (-2.11, 4.73)	0.45	-23.19 (-40.48, -5.89)	0.01
rs699664	<i>GGCX</i>	-0.38 (-2.49, 1.73)	0.72	0.06 (-5.44, 5.55)	0.98
rs9923231	<i>VKORC1</i>	1.56 (-0.55, 3.67)	0.15	0.20 (-8.91, 9.32)	0.97
rs2359612	<i>VKORC1</i>	1.33 (-0.77, 3.43)	0.22	-1.42 (-7.94, 5.11)	0.67

P-values for SNP associations with TTR were calculated using simple linear regression (without clinical data adjustments). Effect estimates and confidence intervals capture the additive change in the mean percent TTR associated with a one minor allele change. Blank entries for variants in European Americans are for SNPs represented only in African Americans.