Influence of *CYP2C9* and *VKORC1 1173C/T* Genotype on the Risk of Hemorrhagic Complications in African-American and European-American Patients on Warfarin

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Abstract

The association of *CYP2C9* and *VKORC1 1173C/T* genotype and risk of hemorrhage among African Americans and European Americans is presented. This association was evaluated using Cox proportional hazard regression with adjustment for demographics, comorbidity, and time-varying covariates. Forty-four major and 203 minor hemorrhages occurred over 555 person-years among 446 patients (60.67±15.6 years, 50% men, 227 African Americans). The variant *CYP2C9* genotype conferred an increased risk for major (hazard ratio (HR) 3.0; 95% confidence interval (CI): 1.1–8.0) but not minor (HR 1.3; 95% CI: 0.8–2.1) hemorrhage. The risk of major hemorrhage was 5.3-fold (95% CI: 0.4–64.0) higher before stabilization of therapy, 2.2-fold (95% CI: 0.7–6.5) after stabilization, and 2.4-fold (95% CI: 0.8–7.4) during all periods when anticoagulation was not stable. The variant *VKORC1 1173C/T* genotype did not confer a significant increase in risk for major (HR 1.7; 95% CI: 0.7–4.4) or minor (HR 0.8; 95% CI: 0.5–1.3) hemorrhage. The variant *CYP2C9* genotype is associated with an increased risk of major hemorrhage, which persists even after stabilization of therapy.

Thromboembolic disorders are significant contributors to morbidity and mortality.[1] Although the efficacy of warfarin in the treatment and prevention of thromboembolic disorders is proven,[2-4] it is vastly underutilized,[5,6] with difficulties in management[5,7] and risk of complications being the main deterrents.[7,8] Recognition of genetic regulation of warfarin response has fueled

efforts aimed at quantifying this influence, but past efforts have focused on a few cytochrome *P450* 2C9 (*CYP2C9*) alleles in largely white populations.[9-13] Outcome definitions varied significantly, with several studies addressing risk of over-anticoagulation,[12,13] whereas others analyzed hemorrhagic complications retrospectively.[9-11] Exclusion of underrepresented ethnic groups and inability to address factors such as concurrent medications and comorbid conditions[9-13] limit the generalizability of study results. Although these studies have enhanced our understanding of the association of *CYP2C9* and hemorrhagic complications, a prospective study in racially representative population is lacking. A recent report indicates the significance of *1173C/T* polymorphism in the vitamin K epoxide reductase complex 1 (*VKORC1*) gene in explaining variability in warfarin dose requirements among both European-American and African-American patients.[14] However, the influence of *VKORC1* polymorphisms in risk of hemorrhagic complications is lacking.

Herein we present the association of *CYP2C9* and *VKORC1 1173C/T (rs9934438)* genotypes and risk of hemorrhagic complications among African Americans and European Americans on warfarin therapy.

RESULTS

All patients meeting eligibility criteria (*n*=526) were asked to participate. The age, gender, and racial makeup of patients declining participation (*n*=36, 17 African Americans, 21 women) did not differ from that of patients enrolled. Four hundred and ninety patients (mean age, 60.6 years; SD, 15.6 years) were recruited between August 2003 and August 2006. African-American patients comprise 48% of the cohort and men 51.3% with an average of 14.9 (±10.7) months of follow-up accrued.

CYP2C9 genotype was determined for 446 patients. Two patients, one Hispanic and one African-American man with a previously unreported polymorphism (*G1078A*) in the same region as the *CYP2C9*3* mutation, were excluded from the analysis assessing the Hardy—Weinberg equilibrium (HWE). The distribution of *CYP2C9* alleles differed by race (*P*<0.0001) but not by gender (*P*=0.13, data not shown). The frequency of variant *CYP2C9* genotype (Table 1) is significantly higher in European Americans than African Americans (29.8 vs 9.7%, *P*<0.0001). *CYP2C9* genotype frequencies were found to be in HWE among European Americans (*P*>0.45) and African Americans (*P*>0.75).

VKORC1 1173C/T was determined for 441 patients (Table 1). The variant *VKORC1* genotype is more frequent among European Americans compared to African Americans (and T allele, 62.5 vs 19.6%, *P*<0.0001). *VKORC1 1173C/T* frequencies were found to be in HWE among both European Americans (exact statistic *P*=0.69) and African Americans (exact statistic, *P*=0.99).

There were no significant differences in gender, body mass index, indication for therapy, comorbidity, concomitant medications, level of physical activity, or smoking status by *CYP2C9* genotype among study participants (Table 2). Patients with the variant *CYP2C9* genotype consumed more alcohol (*P*=0.018) and vitamin K-rich foods (*P*=0.008) and had a higher frequency of *VKORC1 1173C/T* variant. The occurrence of minor or major hemorrhagic complications did not differ by race (*P*=0.66 and 0.5, respectively), gender (*P*=0.16 and 0.39, respectively), or across *VKORC1* genotype (*P*=0.38 and 0.75, respectively). Patients with variant *CYP2C9* genotype experienced major (*P*=0.0024) but not minor (*P*=0.15) complications more frequently than the wild-type genotype (Table 3).

Forty-four major (in 37 patients) and 203 (in 115 patients) minor hemorrhages were encountered during 554.84 years of follow-up resulting in an overall incidence rate of 7.93

(95% confidence interval (CI): 5.8–10.6) per 100 patient-years for major and 36.6 (95% CI: 31.8–41.9) per 100 patient-years for minor hemorrhage, respectively.

The incidence rate for minor hemorrhage was not significantly higher for patients with variant *CYP2C9* genotype than wild-type genotype, as indicated by the unadjusted incidence rate ratio (IRR: 1.24, 95% CI: 0.9–1.7). The incidence rate of major hemorrhage was significantly higher among patients with variant *CYP2C9* genotype than those with the wild-type genotype (15.7 vs 5.8 per 100 patient-years, *P*=0.0015, Table 3), resulting in an unadjusted IRR of 2.73 (95% CI: 1.5–5.0). The incidence rate for minor or major hemorrhage was not significantly different for patients with variant *VKORC1 1173C/T* compared to wild-type genotype (IRR: 0.98, 95% CI: 0.7–1.3 and 1.2, 95% CI: 0.7–2.1, respectively).

Major hemorrhage, fatal in four patients, most commonly involved the gastrointestinal, genitourinary, or dermatologic systems. Major hemorrhage occurred over the duration of follow-up; with two of the 47 (4.3%) events occurring within 1 month, nine (17.0%) within 3 months, 17 (36.2%) within 6 months, and 28 (59.6%) within 1 year of therapy initiation. The mean international normalized ratio (INR) at the time of event was 4.5±3.8 (median 3.4, range: 1.3–21.0) with 20 events (42.5%) occurring at INR of <3. At the time of the major hemorrhage, patients with the variant genotype had lower median INRs (2.9 (range: 1.1–8.2) vs 4.1 (range: 1.3–21.6), *P*=0.31) and lower median warfarin dose (22.0 mg/week (range: 7–47.5) vs 35 mg/ week (range: 10–77.5), *P*=0.0009). There was no difference in the use of medications that interact with warfarin by genotype (*P*>0.4) at the time of the event. Time to first major hemorrhage was significantly shorter for patients with variant *CYP2C9* genotype than wild-type genotype (log-rank *P*=0.004, Figure 1a) but not for patients with variant *VKORC1* genotype (*P*=0.5, Figure 1b).

Hazard ratios (HRs) from covariate-adjusted Cox proportional hazard (PH) models are presented in Table 4. The variant *CYP2C9* genotype confers an increased risk of major (HR 3.0; 95% CI: 1.1–8.3, Figure 1c) but not minor (HR 1.3; 95% CI: 0.8–2.1) hemorrhage. Variant *VKORC1 1173C/T* genotype did not confer a significant increase in risk for major (HR 1.7; 95% CI: 0.7–4.4) or minor (HR 0.8; 95% CI: 0.5–1.3) hemorrhage. We recognize that European American and African Americans have distinct genetic ancestry. The genetic heterogeneity introduced by combining these groups in the analyses could potentially result in a spurious gene—hemorrhage association.[15,16] Therefore, we also conducted analysis stratified by race. The risk conferred by the variant *CYP2C9* genotype did not differ across race for minor hemorrhage. For major hemorrhage, the risk was higher among European-American (HR 3.9; 95% CI: 1.1–13.7) than African-American patients (HR 2.8; 95% CI: 0.5–17.3, Table 4), although not significantly different (*P*=0.21 for *CYP2C9* race interaction). The interaction of *VKORC1* and *CYP2C9* assessed in an epistatic model was found to be statistically nonsignificant (*P*=0.48). Therefore, further analyses assessed the association of *CYP2C9* genotype and hemorrhage with adjustment for *VKORC1* genotype and race.

The effect of variant *CYP2C9* genotype on risk before attaining first INR in the target range could not be evaluated, as only one event occurred in this time period. The variant *CYP2C9* genotype conferred a fivefold increased risk of major hemorrhage before first stabilization and continued to confer a 2.2-fold risk even after anticoagulation is first stabilized. As only four events occurred during periods of stable therapy, the risk conferred by the variant *CYP2C9* genotype could not be assessed. During periods where anticoagulation was not stable, patients with variant *CYP2C9* genotype were at a 2.4-fold higher, although statistically nonsignificant, risk of major hemorrhage. The variant *CYP2C9* genotype conferred an increased risk at INRs below (HR 3.6; 95% CI: 0.5–27.9), within (HR 5.6; 95% CI: 1.8–16.9), and above (HR 2.6; 95% CI: 0.5–13.8) the target range. However, a statistically significant increase was observed only at INRs within the target range.

The risk conferred by the variant *CYP2C9* genotype was greater in patients experiencing major hemorrhage without pathology (*e.g*., active ulcer) and drug interactions (HR 4.7; 95% CI: 0.9–24.1) than those with significant pathology (HR 3.4; 95% CI: 0.7–16.5). Among patients experiencing major hemorrhage, six patients also had end-stage renal disease. These patients have a high level of comorbidity and multiple factors increasing the risk of hemorrhage. Therefore, the association of variant *CYP2C9* genotype with major hemorrhage was reassessed after their exclusion. In patients without end-stage renal disease, the variant *CYP2C9* genotype confers a higher risk (HR 3.7; 95% CI: 1.4–9.6, *P*=0.007) unchanged by correction for dependence (HR 3.7;=95% CI: 1.3–10.7, *P*=0.016).

DISCUSSION

This study provides strong evidence of the increased risk of major hemorrhage among patients with a variant *CYP2C9* genotype throughout the duration of warfarin therapy after accounting for *VKORC1 1173C/T* genotype, demographics, and clinical covariates. To our knowledge, this is the largest prospective study aiming to define the association of variant *CYP2C9* genotype with major hemorrhage among both European Americans and African Americans.

Our study is the first to examine the effect of the *CYP2C9*5, CYP2C9*6*, and *CYP2C9*11* allele in a large African-American population in addition to *CYP2C9*2* and *CYP2C9*3*. *CYP2C9*2* and *CYP2C9*3* were the most common *CYP2C9* variant alleles in both racial groups, with observed frequencies consistent with previous reports.[17,18] On the basis of variant allele frequencies, the combined poor-metabolizer genotypes are significantly more frequent among European Americans than African Americans (29.8 vs 9.73%, *P*<0.0001). Inclusion of the *5, *6*, and *11* alleles in determining *CYP2C9* genotype resulted in a higher poor-metabolizer genotype frequency (9.7%) among African Americans than that reported previously.[19] The frequency of *VKORC1 1173C/T* variant was similar for European Americans (60%) but slightly higher for African Americans (19.6 vs 15.4%) than that reported by Schelleman *et al*.[14]

Among European Americans, the risk conferred by the variant *CYP2C9* genotype is consistent with previous reports.[9,10] To our knowledge, this is the first report of *CYP2C9* genotype—major hemorrhage association in African Americans. Population stratification is often cited as a potential confounder in genetic association studies.[15] For it to introduce confounding bias, three conditions must be met.[20] First, the allele frequencies must differ across race; second, the occurrence of disease (hemorrhage) must vary by race; third, the genetic marker should not be in the casual pathway. In our study, although *CYP2C9* allele frequencies show substantial differences across race (*P*<0.0001), the occurrence of major or minor hemorrhage does not. Therefore, population stratification does not bias the association of *CYP2C9* genotype and risk of hemorrhage in our study. This is further emphasized by the lack of significant differences in the race-specific HRs (*P*-value for gene×race interaction=0.27). The lower frequency of the variant genotype in African Americans suggests that the population-attributable risk of this allele may be lower among African Americans than European Americans.

The incidence rate for major hemorrhagic complications in this study was similar to that reported previously.[10,21] However, the incidence rate for minor hemorrhagic complications in our population was higher than that reported previously (14.5 per 100 patient-years; 95% CI: 13.7–16.22),[21] perhaps because of inclusion of patients with minor hemorrhage who required additional testing or clinic visits.

Several studies have reported an increased risk of hemorrhage among patients with a variant *CYP2C9* genotype. However, most have defined the event as "over-anticoagulation" rather than a hemorrhagic event.[13,22-28] Four studies measured the occurrence of hemorrhagic

events.[9-11,29] Of these, only three reported bleeding risk by genotype.[10,11,29] Aithal *et al.*[9] reported bleeding risk for patients in a predefined low-dose category (<1.5 mg/day) rather than by genotype. Ogg *et al.*[29] reported the influence of a sole variant, *CYP2C9*3* variant, on risk of bleeding. A recent meta-analysis[30] summarized the bleeding risk by genotype. Patients with at least one copy of a variant allele had an increased risk of bleeding compared with non-carriers for *CYP2C9*2* (relative risk (RR) 1.9, 95% CI: 1.2–3.2), *CYP2C9*3* (RR 1.8, 95% CI: 1.1–2.9), and *CYP2C9*2* or **3* (RR 2.3, 95% CI: 1.4–3.7). Our results of increased risk (HR 3.0, 95% CI: 1.1, 8.0) are consistent with these reports.

Previous studies have not been able to compare the influence of individual variant alleles on the risk of major hemorrhage because of the rarity of the event and limited sample size. In our study, patients with the *CYP2C9*3* variant had a higher, although not statistically significant, risk of major hemorrhage than those with *CYP2C9*2* variant (RR 1.7, 95% CI: 0.47–5.8, P=0.42). The small number of events prevented the determination of the risk of major hemorrhage conferred by the other variant alleles.

As reported by Higashi *et al.*,[10] patients with the variant genotype had a higher risk of major hemorrhage before stabilization of anticoagulation. However, this association could not be assessed in a multivariable model because of the small number (n=5) of events. The availability of longitudinal data enabled us to define periods of "stable and unstable" anticoagulation for each patient. Patients with the variant genotype continue to have a higher risk throughout the duration of therapy, including periods of "unstable" anticoagulation across the INR range after accounting for the effects of covariates. These findings highlight that the risk conferred by the variant genotype does not dissipate after stabilization of therapy.

This study has several strengths including a 2-year follow-up in a clinically well-characterized cohort, adjustment for time-varying covariates, drug interactions and comorbidity, objective documentation of incident complications, separation of the genotype—phenotype data until time of analyses, and event adjudication by an expert without knowledge of patients' genotype. Besides the large African-American subgroup, the ability to model time-varying covariates is a major strength. The inclusion of patients from initiation of therapy in this prospective study and the high participation rate (93.1%) minimize selection bias. The generalizability of our findings is further improved by the representativeness of the study cohort as indicated by the similarity in age, gender, and racial distribution as compared to patients treated at University of Alabama at Birmingham (UAB) from the Birmingham metropolitan area.

We also recognize limitations of this study. Documentation of vitamin K intake was based on patients' report using vitamin K inventory,[31] not quantified by assay/measurements. However, all measurements were used consistently among all participants; therefore, bias if any should be non-differential. We also recognize that drug response is influenced by multiple genes. At least one other gene, *VKORC1*, has been shown to influence significantly warfarin dose in European Americans.[25,32-36] Although it has been hypothesized that *VKORC1* may influence the risk of hemorrhage, all studies have aimed at quantifying its influence on risk of severe over-anticoagulation (defined as INR of 6 or higher).[25-27,37] Schalekamp *et al.*[37] found that among acenocoumarol users the possession of *CYP2C9*3* allele was associated with an increased risk of over-anticoagulation compared to the *CYP2C9*2* and **1* alleles. However, among phenprocoumon users both *CYP2C9*2* and *CYP2C9*3* were associated with an increased risk of over-anticoagulation.[27] Accounting for the effects of *VKORC1*, the risk of over-anticoagulation was increased by a combination of *VKORC1* and *CYP2C9* genotypes. Carriers of a combination of *CYP2C9* and *VKORC1* polymorphism had an increased risk of severe over-anticoagulation compared with subjects with no polymorphism or only one polymorphism among both acenocoumarol (HR 3.8, 95% CI: 1.6–9.0)[26] and phenprocoumon (HR 7.2, 95% CI: 2.1–24.7) users.[25]

One study by Reitsma *et al.*[38] suggests an increased risk of bleeding in the phenprocoumon users (crude odds ratio 2.6, 95% CI: 1.2–5.7), but not in acenocoumarol users (crude odds ratio 1.2, 95% CI: 0.6–2.3). Of note, this association is not consistent across the coumarin anticoagulants and did not account for other factors known to increase the risk of hemorrhage, such as *CYP2C9* genotype, INR, drug interactions, and so on. Our results indicate a higher (but not statistically significant) hemorrhagic risk of *VKORC1 1173C/T* variants after adjusting for *CYP2C9* genotype, clinical and demographic variables. Results of ongoing investigations will help better define the influence of *1173C/T* and other *VKORC1* variants (which may differ by race) on the risk of hemorrhagic events among patients on chronic warfarin therapy.

Results of this prospective study provide evidence of an association of variant *CYP2C9* genotype and risk of major hemorrhage in both African-American and European-American patients after accounting for the effects of *VKORC1 1173C/T*, age, gender, warfarin dose, INR, drug interactions, vitamin K intake, and comorbidity. Compared to patients with the wild-type genotype, patients with the variant *CYP2C9* genotype exhibit an increased risk of major hemorrhage, which persists even after stabilization of anticoagulation therapy.

METHODS

Patients ≥20 years of age, initiated on warfarin with a target INR range of 2–3 and ≥2 years anticipated treatment duration were eligible. The study is being conducted at the UAB enrolling patients from the anticoagulation clinic at The Kirklin Clinics (TKC-AC) and the Jefferson Clinic PC, Jefferson County Health System (CGH-JC) under the approval of the respective Institutional Review Boards. At both clinics, patient care is managed via a physician-approved prescriptive authority protocol that provides a standardized approach to warfarin dose adjustments based on INR results, management of over-anticoagulation and under-anticoagulation, and frequency of follow-up.[39] A detailed medical, lifestyle, and medication history was documented. Information collected included indication for therapy, comorbid conditions, medications, smoking, alcohol use, education, income, medical insurance, and physical activity. Number of servings of foods rich in vitamin K consumed per week was used to document vitamin K intake.[31]

Time-dependent covariates

The risk of hemorrhage is influenced by factors that are likely to change over time including warfarin dose, INR, medications, vitamin K[31] and alcohol intake,[40] and level of physical activity.[41] As patients initiated on warfarin tend to be older and may have physical limitations, we created a simple five-point scale: wheelchair bound, uses walker/cane, ambulatory without assistance, physically active (moderate exercise 30 min 3 times/week), and moderately physically active (moderate to intense exercise 4–5 times/week).

Drug interactions can influence warfarin dose and risk of hemorrhage.[7,42] The increase in risk can result from concomitant use of drugs such as non-steroidal anti-inflammatory drugs or antiplatelet agents,[43] or drugs that alter warfarin pharmacokinetics, including *CYP2C9* inhibitors (*e.g.*, amiodarone), *CYP2C9* inducers (*e.g.*, rifampin), or *CYP2C9* substrates (*e.g.*, losartan).[44,45] Changes in these time-dependent covariates were documented at each visit.

Patient follow-up, event documentation, verification, and adjudication

During the 2-year follow-up, all complications were captured and verified through review of admissions and emergency department visits, and patient self-report. Only medically documented events were included in the analyses. The Alabama Center for Health Statistics was queried to verify cause of death for patients known to have expired to ensure inclusion of deaths due to hemorrhagic complications. All complications were documented by the study

nurse followed by an independent review by the principal investigator. Finally, all major hemorrhagic complications were reviewed independently by the Medical Director of the Anticoagulation Clinic. All genotype information was maintained separately until data analysis.

CYP2C9 genotype determination

Blood was collected in a Qaigen PAX gene tube and DNA was extracted using the PAX gene blood DNA extraction kits. *CYP2C9*2* genotyping was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism methodology,[46] and genotyping for *2, *3, *5, *6*, and *11* variants was performed using new pyrosequencing methodology.

Genomic DNA (10–30 ng) was amplified with 1 U Ampli Taq Gold (Applied Biosystems, Foster City, CA) in 40 μl reaction mixture containing 1× PCR buffer, 0.2 pmol/μl biotinylated forward primer, 0.2 pmol/μl reverse primer (Eurogentec, San Diego, CA) (Table 5), 2 mM final MgCl$_2$, and 0.5 mM final dNTPs. An initial denaturation of 95°C for 8 min is used followed by 95°C for 15s, 561C for 30s, and 72°C for 15s for 45 cycles. This reaction is followed by a final extension at 72°C for 5 min (GeneAmp PCR 9700 System, Applied Biosystems).

The entire biotinylated PCR product was mixed with 40 μl of 2×binding-washing buffer II (10 m$_M$ Tris—HCl, 2 $_M$ NaCl, 1 m$_M$ EDTA, and 0.1% Tween-20, pH 7.6) and immobilized with 3 μl (10 μg/μl) streptavidin-coated polystyrene beads (Amersham Biosciences, Piscataway, NJ). Samples were mixed at room temperature for 10 min. To achieve DNA strand separation, a Vacuum Prep Tool (Biotage, Foxboro, MA) was used; the PCR products were isolated through alkaline denaturation and wash steps (Biotage). Beads are released into wells of a PSQ sequencing plate (Biotage) containing 20 pmol of the appropriate sequencing primer (Table 5). The reactions annealed at 90°C for 5 min and cooled at room temperature for 10 min. Substrates and enzymes as well as dNTP from the manufacturer's kit (Biotage) were placed in a cartridge (supplied by Biotage) and then placed into the PSQ 96 MA Pyrosequencer (Biotage) for sequence determination. These methods allow the determination of the six alleles with four PCRs (Table 5).

VKORC1 1173C/T (rs9934438) genotyping was conducted (at The Broad Institute Center for Genotyping and Analysis) using the Sequenom iPLEX technology, which relies on matrix assisted laser desorption/ionization-time-of-flight mass spectrometry. In brief, PCR is performed across the region of the single-nucleotide polymorphism with a high-fidelity polymerase followed by a primer extension that generates allele-specific products with a unique mass that can be separated on mass spectrometry. Primers and the multiplex assay are designed with the Sequenom SPECTRO-DESIGNER and IPLEX software, respectively.

Classification of hemorrhagic outcomes

The review of hemorrhagic complications included objective documentation (*e.g*., endoscopy) of complication site (*e.g*., gastrointestinal), gravity of the event (requiring transfusion, surgical intervention, and so on), and laboratory findings (INR, hemoglobin/hematocrit, and so on) at the time of the event. In the absence of evidence of bleeding, isolated elevated INRs were not classified as hemorrhagic events.

Hemorrhagic complications were classified as minor or major using the scheme detailed by Fihn *et al*.[47] Minor hemorrhages included mild nosebleeds, microscopic hematuria, mild bruising, and mild hemorrhoidal bleeding. The "no additional testing/visit" criteria were relaxed, as these patients have multiple medical problems, heightened health concerns, and frequently access medical care. This modification was afforded by the prospective nature of

the study, ascertainment of incident complications, and adjusting for comorbid conditions and changes in time-dependent covariates.

Serious, life-threatening and fatal bleeding episodes as defined by Fihn *et al.*[47] were combined into one end point; "major hemorrhage" as these events are infrequent, demand medical/ surgical intervention, and may carry grave prognosis. Any hemorrhage resulting in discontinuation of warfarin was classified as a major hemorrhage. To explore the influence of underlying comorbidity (*e.g.*, ulcers) and drug interactions (*e.g.*, non-steroidal anti-inflammatory drugs, *CYP2C9* inhibitors),[8] major hemorrhagic complications were further classified as those occurring in the absence or presence of concurrent pathology or drug interactions.

Analysis

Patients were categorized as wild type (*CYP2C9*1/*1* homozygotes) and variant (≥1 variant alleles) with the former serving as the referent group. The HR and 95% CI for the association between *CYP2C9* genotype and hemorrhage was evaluated using the counting process format in the Cox PH model.[48,49] This format allows individuals to contribute more than one event for the hemorrhage outcome. Valid CIs were obtained by correction of dependence using robust variance estimation.[50] Departures from the PH assumption were assessed by examination of the log—log survival curves, statistical testing of Schoenfeld residuals, and using linear regression to evaluate the relationship between time and covariate-specific Schoenfeld residuals.

The PH models included *CYP2C9* genotype, age, race, gender, body mass index, *VKORC1 1173C/T*, and comorbid conditions. Changes in medications, vitamin K and alcohol intake, and level of physical activity, warfarin dose, and INRs were included as time-varying covariates. Models with interaction terms were fit to assess the potential effect modification of the genotype by each covariate. All interaction terms were statistically nonsignificant (full model, all *P*>0.05) and therefore excluded. Both statistically significant and clinically relevant covariates were included in the models.

Patients were followed from initiation of therapy until the date of event (if warfarin was discontinued) or the end of the follow-up, when all data were right censored. All patients withdrawn from the study were censored at the time of the last clinic visit. To understand the effect of recent initiation of therapy on risk of hemorrhage, analyses restricted by time to attainment of first target INR, time to first stabilization of anticoagulation therapy, and duration of therapy after first attainment of stable anticoagulation (three INRs in target range without dose change) were conducted. The HWE assumption was assessed with the χ^2 test of independence and exact test.[51] All analyses were conducted at a non-directional α-level of 0.05.

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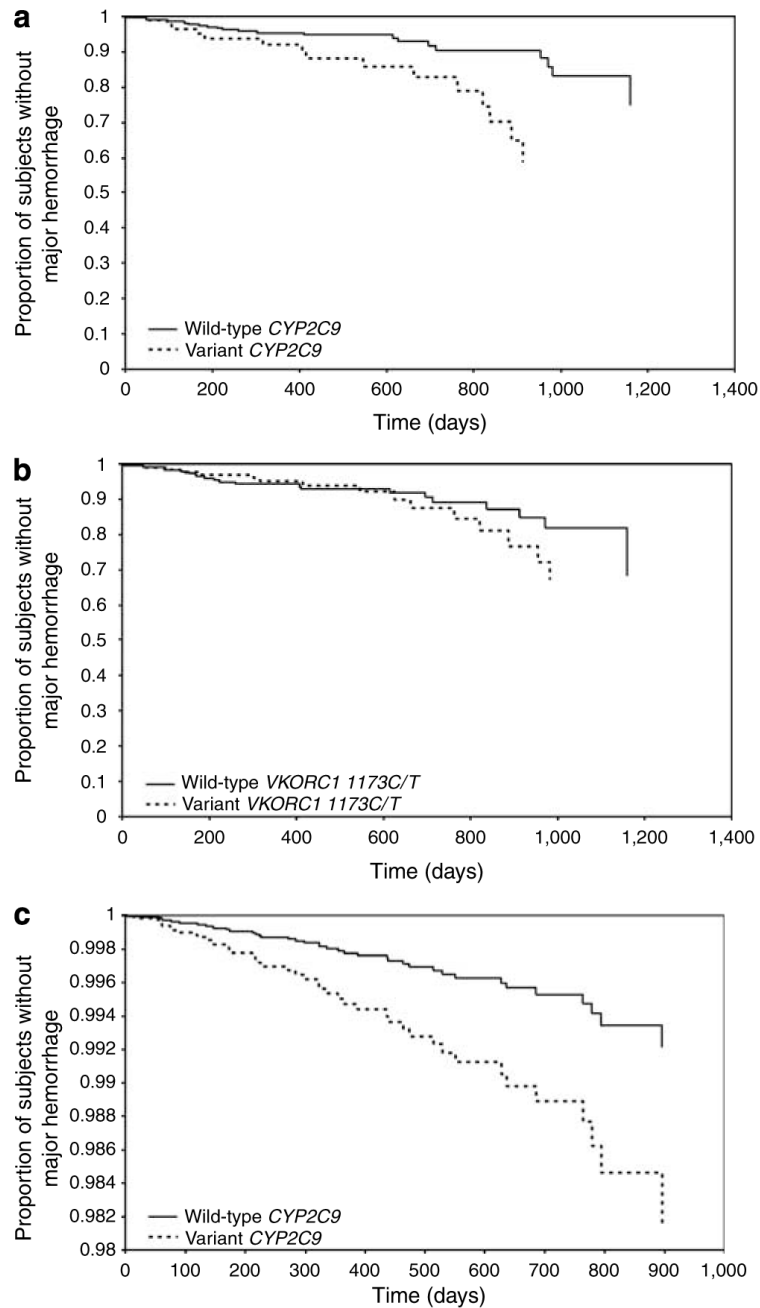
Figure 1.
Time to major hemorrhage by *CYP2C9* and *VKORC1* genotype. Kaplan—Meier curves for time to major hemorrhagic complication analyses plotted for (**a**) *CYP2C9* wild-type vs variant genotype and (**b**) *VKORC1 1173C/T* wild-type vs variant genotype. (**c**) Estimated survival curve from Cox PH model adjusted for *VKORC1 1173C/T* genotype, age, warfarin dose, gender, body mass index, INR, alcohol intake, vitamin K intake, number of comorbid conditions, and drug interactions (note: y axis starts at 0.98).

Table 1

CYP2C9 and *VKORC1 1173C/T* genotype distribution in participants of the POAT cohort[a]

Genotype CYP2C9	All participants N=444 No. of positive (%)	African Americans N=226 No. of positive (%)	European Americans N=218 No. of positive (%)
*1/*1	357 (80.405)	204 (90.265)	153 (70.183)
*1/*2	45 (10.135)	5 (2.212)	40 (18.349)
*1/*3	26 (5.855)	7 (3.097)	19 (8.715)
*1/*5	3 (0.676)	3 (1.327)	0 (0.000)
*1/*6	0 (0.000)	0 (0.000)	0 (0.000)
*1/*10	0 (0.000)	0 (0.000)	0 (0.000)
*1/*11	5 (1.126)	5 (2.212)	0 (0.000)
*2/*2	3 (0.676)	0 (0.000)	3 (1.376)
*2/*3	3 (0.676)	0 (0.000)	3 (1.376)
*3/*6	1 (0.225)	1 (0.442)	0 (0.000)
*5/*6	1 (0.225)	1 (0.442)	0 (0.000)

VKORC1	N=441	N=225	N=216
CC	262 (59.4)	181 (80.4)	81 (37.5)
CT	150 (34.0)	42 (18.7)	108 (50.0)
TT	29 (6.6)	2 (0.9)	27 (12.5)

*V denotes variant allele (2, *3, *5, *6, *10, or *11).

[a] Subjects recruited during the interval August 2003 to August 2006. Analysis excludes one Hispanic patient and one African-American patient with the *G1078A* mutation. *VKORC1 1173C/T* genotype determined for 441 patients.

Table 2

Demographic and clinical characteristics of patients participating in the POAT cohort by variant *CYP2C9* genotype

Variable	CYP2C9 genotype		P-values
	Wild type (*N*=358)	Variant (*N*=88)	
Age (years)	60.1 (±16.0)	62.9 (±14.0)	0.14
BMI	29.8 (±7.5)	28.4 (±7.0)	0.11
Follow-up time			
Mean (±SD)	14.5 (10.7)	16.4 (10.5)	0.15
Median (range)	12.0 (0.0–47.0)	15.0 (1.0–38.0)	-
Average alcohol intake[a]	1.3 (3.2)	2.3 (3.7)	**0.018**
Average level of physical activity[b]	3.37 (0.9)	3.5 (0.8)	0.30
Average vitamin K intake[c]	1.7 (1.0)	2.0 (1.2)	**0.008**
Average warfarin dose (mg/day)			
Mean (±SD)	5.8 (±2.53)	4.4 (±1.92)	**<0.0001**
Median (range)	5.3 (1.3–20.9)	4.3 (1.1–10.4)	-

Variable	CYP2C9 genotype		P-values
	N(%)	N(%)	
Race			
African American	204 (57.0)	23 (26.1)	**<0.0001**
European American	153 (42.7)	65 (73.9)	-
Hispanic	1 (0.3)	0 (0.0)	-
Gender			
Female	186 (51.0)	37 (42.0)	0.10
Male	172 (48.0)	51 (58.0)	-
Indication[d,e]			
Arterial thromboembolism	152 (42.5)	44 (50.0)	0.20
Venous thromboembolism	148 (41.3)	29 (32.9)	0.15
None	62 (17.3)	16 (18.2)	0.85
Comorbidity[f]			
Low (<2 comorbid conditions)	119 (33.2)	23 (26.1)	0.11
Moderate (2–4 comorbid conditions)	159 (44.4)	50 (56.8)	-
High (>44 comorbid conditions)	80 (22.4)	15 (17.1)	-
Medications[d,g]			
CYP2C9 inducers	5 (2.0)	0 (0.0)	0.26
CYP2C9 inhibitors	124 (34.6)	28 (31.8)	0.61
CYP2C9 substrates	139 (38.8)	27 (30.7)	0.16
NSAID	51 (14.2)	11 (12.5)	0.67

Variable	CYP2C9 genotype		P-values
	N(%)	N(%)	
Antiplatelet agents	197 (55.0)	58 (65.9)	0.06
Smoking status[h]			
Current	53 (14.8)	11 (12.5)	0.82
Past	134 (37.4)	31 (35.2)	-
Never	163 (45.5)	43 (48.9)	-
			-
VKORC1 1173C/T[i]			
CC	219 (62.0)	43 (48.9)	**0.024**
Any T	134 (38.0)	45 (51.1)	-

BMI, body mass index; NSAID, non-steroidal anti-inflammatory drug. *CYP2C9* genotype (wild type denotes ***1/*1**, V denotes at least one variant allele (**2*, **3*, **5*, **6*, **10*, or **11*)). One patient with a previously unreported polymorphism (*G1078A*) in the same region as the *CYP2C9*3* mutation was categorized as variant.

[a]Average alcohol intake is defined as number of alcoholic beverages (beer, wine, whiskey, liquor) consumed per week.

[b]Average level of physical activity: we created a simple five-point physical activity scale: wheelchair bound (1), uses walker/cane (2), ambulates without assistance (3), physically active (4), and consistent exercise (5). The level of physical activity is then averaged over the study period.

[c]Average vitamin K intake is defined as the equivalent number of servings of foods rich in vitamin K consumed per week.

[d]Individual patients can have more than one indication for warfarin therapy listed and can be on multiple medications.

[e]Arterial thromboembolism includes patients with myocardial infarction, stroke, or transient ischemic attack. Venous thromboembolism includes patients with deep vein thrombosis or pulmonary embolism. None includes patients with no prior thromboembolic events (*e.g.*, atrial fibrillation, cardiomyopathy, low ejection fraction).

[f]Level of comorbidity is calculated based on number of comorbid diseases in each individual patient. Comorbid conditions include hypertension, hyperlipidemia, atrial fibrillation, congestive heart failure, coronary artery disease, diabetes mellitus, malignancy, renal insufficiency, renal failure, peptic ulcer disease, and history of hemorrhage.

[g]Medications: *CYP2C9* inhibitors include amiodarone, fluconazole, fluvastatin, isoniazid, episodic use of antibiotics such as metronidazole and sulfamethoxazole. *CYP2C9* inducers include phenobarbital, carbamazepine, and rifampin. *CYP2C9* substrates include ibuprofen, diclofenac, losartan, irbesartan, glipizide, tolbutamide, fluvastatin, fluoxetine, and so on. NSAIDs include ibuprofen, naproxen, piroxicam, diclofenac, and so on. Antiplatelet agents include aspirin, clopidogrel, and dipyridamole.

[h]Missing in 11 patients.

[i]*VKORC1 1173C/T* assessed in 441 patients. All significant *P*-values (*P*<0.05) are in bold.

Table 3

Incidence rate of hemorrhagic complications stratified by *CYP2C9* and *VKORC1 1173C/T* genotype (per 100 patient-years)

	CYP2C9 genotype			
	Total (*N*=446)	Wild type (*N*=358)	Variant (*N*=88)	*P*-values
Follow-up, person-years	**554.84**	**434.16**	**120.68**	—
Major hemorrhage				
Patients (*n*, %)	37 (8.3%)	22 (6.1%)	15 (17.0%)	**0.0024**
Number of events	44	25	19	
Incidence rate	7.93 (5.8–10.6)	5.67 (3.8, 8.4)	15.74 (9.8, 24.1)	**0.0015**
Minor hemorrhage				
Patients (*n*, %)	115 (25.8%)	87 (24.3%)	28 (31.8%)	0.15
Number of events	203	151	52	
Incidence rate	36.6 (31.8, 41.9)	34.8 (29.6, 40.7)	43.1 (32.5, 56.1)	0.182

	VKORC1 1173C/T genotype[a]			
	Total (*N*=441)	"CC" (*N*=262)	Any "T" (*N*=179)	*P*-values
Follow-up, person-years	**551.9**	**339.4**	**212.5**	—
Major hemorrhage				
Patients (*n*, %)	37 (8.4%)	21 (8.0%)	16 (8.4%)	0.75
Number of events	44	25	19	
Incidence rate	8.0 (5.8, 10.7)	7.4 (4.8, 10.9)	8.9 (5.4, 13.9)	0.52
Minor hemorrhage				
Patients (*n*, %)	113 (25.6%)	65 (24.8%)	48 (26.8%)	0.38
Number of events	201	125	76	
Incidence rate	36.5 (31.6, 41.8)	36.8 (30.7, 43.9)	35.8 (28.2, 44.8)	0.84

CYP2C9 genotype (wild type denotes *1/*1, V denotes at least one variant allele (*2, *3, *5, *6, *10, or *11). Minor hemorrhagic complications included mild nosebleeds (lasting less than 30 min), microscopic hematuria, mild bruising, and mild hemorrhoidal bleeding. Major hemorrhagic complications include serious, life-threatening and fatal bleeding episodes as defined by Fihn *et al.*[47] Among patients with variant CYP2C9 genotype, nine major hemorrhages occurred in patients with *1/*3, eight in *1/*2, one in *2/*3, and one in *5/*6.

[a]*VKORC1 1173C/T* determined for 441 patients. All significant *P*-values (*P*<0.05) are in bold.

Table 4

Adjusted hazard ratios and 95% CI for the association of variant *CYP2C9* genotype and hemorrhagic complication among POAT participants[a]

Hemorrhagic event	Adjusted HR[b]		Adjusted HR with robust variance estimation[c]	
	HR (95% CI)	P-values	HR (95% CI)	P-values
Minor hemorrhage	1.3 (0.89, 1.88)	0.16	1.3 (0.79, 2.14)	0.3
African Americans	1.5 (0.81, 2.76)	0.21	1.5 (0.75, 2.93)	0.26
European Americans	1.2 (0.73, 1.82)	0.55	1.2 (0.65, 2.04)	0.63
Major hemorrhage[c]	**3.0 (1.22, 7.54)**	**0.017**	**3.0 (1.12, 8.03)**	**0.032**
Stratified by race				
African Americans	2.8 (0.56, 14.79)	0.21	2.8 (0.37, 15.95)	0.25
European Americans	3.9 (1.1, 14.26)	**0.039**	3.9 (1.16, 13.67)	**0.03**
Stratified by stage of anticoagulation therapy				
Before attaining first target INR	Undefined	—	Undefined	—
Before first stabilization of therapy	5.3 (0.47, 58.70)	0.17	5.3 (0.43, 64.67)	0.19
After first stabilization of therapy	2.2 (0.72, 6.57)	0.16	2.2 (0.73, 6.50)	0.16
During all periods of unstable therapy	2.4 (0.91, 6.39)	0.08	2.4 (0.77, 7.38)	0.13
During all periods of stable therapy	Undefined	—	Undefined	—
Stratified by INR at time of event[d]				
INR<2	3.6 (0.47, 27.76)	0.21	3.6 (0.46, 27.9)	0.22
INR 2–3	5.6 (1.8, 16.9)	**0.02**	5.6 (1.19, 26.28)	0.03
INR>3	2.6 (0.50, 13.81)	0.26	2.6 (0.41, 16.79)	0.31
Stratified by concurrent pathology±drug interactions at the time of the event				
Without pathology±drug interactions	4.7 (0.93, 23.53)	0.06	4.7 (0.91, 24.07)	0.065
With pathology±drug interactions	3.4 (1.06, 11.08)	0.039	3.4 (0.71, 16.52)	0.12

BMI, body mass index; CI, confidence interval. Minor hemorrhagic complications included mild nosebleeds (lasting less than 30 min), microscopic hematuria, mild bruising.

[a]Target INR range for all patients in the study was 2.0–3.0. Only one event occurred before first attainment of INR of 2.0. Five events occurred before first stabilization of anticoagulation therapy. Thirty-six events occurred after initial stabilization of therapy. Thirty-nine events occurred during all periods where anticoagulation therapy was not stable. Four events occurred during all periods of stable anticoagulation therapy. All significant *P*-values ($P<0.05$) are in bold.

[b]Adjusted for age, gender, race, BMI, *VKORC1 1173C/T*, vitamin K and alcohol intake, warfarin dose, interacting drugs, number of comorbid conditions, and INR at the time of the event.

[c]All major hemorrhagic complications were adjudicated by the Director of the Anticoagulation Clinic blinded to genotype. Major hemorrhagic complications include serious, life-threatening and fatal bleeding episodes as defined by Fihn *et al.*[47] Major hemorrhagic complications were further classified as those occurring in the absence (major without pathology) or presence (major with pathology) of other contributing factors (medical comorbidity and concomitant drugs).

[d]INR at the time of event was <2 in seven patients, between 2 and 3 in 13 patients, and >3 in 24 patients with major hemorrhage.

Table 5

PCR sequencing primers for pyrosequencing *CYP2C9* alleles

PCR primers	bp size[a]	2C9 allele	Sequencing primer
Exon 3			
F: B-5′-AAACAGAGACTTACAG AGCTC-3′	381	*2	5′-GGGCTTCCT CTTGAAC-3′
R: 5′-CTAACAACCAGACTCAT AATG-3′			
Exon 5			
F: B-5′-CAGAGCTTGGTATATGGT ATG-3′	323	*6, *10	5′-AAGCTTTTGT TTACATTTT-3′
R: 5′-TCGTAAACACAGAACTAGT CAAC-3′			
Exon 7			
F: B-5′-CTGAATTGCTACAACAAA TGTG-3′	314	*11	5′-TTGCATGC AGGGGCT-3′
R: 5′-GATACTATGAATTTGGGAC TTC-3′	155	*3, *5	5′-GCTGGTGG GGAGAAG-3′
F: B-5′-TGCACGAGGTCCAGA GAT-3′			
R: 5′-GATACTATGAATTTGGG ACTTC-3′			

B, biotin-labeled primer; F, forward primer; PCR, polymerase chain reaction; R, reverse primer.

[a]
bp indicates PCR fragment base pair size. 2C9 allele: cytochrome *02C9 single-nucleotide polymorphism.*