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Influence of CYP2C9 Genotype on warfarin dose among African American and European Americans

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Abstract

Background—Cytochrome P450*2C9* (*CYP2C9*) plays a vital role in drug metabolism. There has been an increased effort to identify polymorphisms within the gene and determine their clinical consequences. However, most of these efforts have focused on populations of European descent. Herein we report the influence of *CYP2C9* genotype on warfarin dose among European American and African American patients. We also identify two new mutations; one in the coding region and one in the non-coding region of the *CYP2C9* gene.

Methods—Patients (≥20 years of age) are enrolled after obtaining medical, lifestyle and concomitant medication history. Changes in International Normalized Ratio (INR), warfarin dose, co-medications, diet, physical activity and the occurrence of complications are documented. *CYP2C9* genotype was determined using PCR-RFLP and pyrosequencing. Differences in genotype frequencies and HWE assumptions were assessed using χ^2 statistics and exact tests. The genotype dose association was evaluated using multivariable linear regression.

Results—This report includes 490 patients (mean age 60.6 ± 15.6, 51.3% men). African American patients comprise 48.9% of the cohort with mean follow-up of 13.5 (±10.6) months. Both the *CYP2C9* *2 and *3 allele were more frequent in European Americans (11.24%, 5.1%) compared to African Americans (1.1% and 1.8%). *CYP2C9* *5 (0.9%), *6 (0.4%), and *11 (1.1%) variants were only observed in African Americans. The variant genotype is more frequent among European Americans compared to African Americans (29.8% vs. 9.73%, $p<0.0001$). Warfarin dose was significantly related to *CYP2C9* genotype ($p<0.0001$) both in univariate and multivariate analyses. Multivariable race-specific analyses highlight the contribution of *CYP2C9* genotype among European American but not among African American patients.

Conclusion—The variant *CYP2C9* genotype is more frequent among European Americans compared to African Americans. Among African Americans the variant genotype frequency is higher than previously reported. *CYP2C9* genotype predicts warfarin dose in European Americans, but not in African Americans.

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Keywords

CYP2C9; Pyrosequencing; African American; European American; Warfarin Dose

Introduction

Cytochrome P450s are a superfamily of oxygen-activating enzymes involved in the metabolism of a gamut of endogenous and exogenous substrates.[1] Although the annotation of the human genome has revealed the presence of some 57 human P450 genes, less than a dozen play a critical role in the hepatic clearance of drugs. [2,3]Cytochrome P450*2C9* (*CYP2C9*) has drawn considerable attention for several reasons. [4] First, within the *CYP2C* subfamily *CYP2C9* is the largest contributor to the hepatic microsomal P450 content. [5] Second, it is involved in the metabolism of up to 15% of all drugs that undergo phase I metabolism, second only to Cytochrome P450*3A4*. [2] Third, it plays a vital role in drug metabolism serving as the principal metabolic pathway for clinically important drugs such as warfarin, tolbutamide, phenytoin, etc. [6] It has been implicated in causing adverse drug reactions and drug-drug interactions that pose unique therapeutic management problems, especially for *CYP2C9* substrates which have low therapeutic index (e.g. warfarin and phenytoin). [7,8]Fourth, the gene coding for *CYP2C9* exhibits significant polymorphism. [9]Some data suggest that up to 40% of Caucasian populations are carriers of alleles that encode for a partially defective enzyme which exacerbates adverse drug reactions and drug-drug interactions involving *CYP2C9* substrates, especially those with intrinsically low margin of safety. [4,10]

Recognition of polymorphisms with *CYP2C9* spearheaded efforts to determine their clinical consequences. However, most of these efforts have focused on populations of European descent. [11–18]Herein we report *CYP2C9* allele frequencies among European American and African American patients and evaluate the influence of CYP2C9 on warfarin dose stratified by race. We detail genotyping methodology and describe study design and cohort characteristics at enrollment. We also describe two new mutations in the CYP2C9 gene (one coding change and one noncoding change).

Methods

Study Setting

The Pharmacogenetic Optimization of Anticoagulation Therapy (POAT) is an ongoing prospective cohort study aiming at defining the influence of *CYP2C9* polymorphisms on the dose of warfarin and risk of warfarin related complications during a 2-year follow-up period. As part of the study protocol *CYP2C9* genotype was assessed in all participants.

The study is being conducted at the University of Alabama at Birmingham (UAB) enrolling patients from the anticoagulation clinic at The Kirklin Clinics (TKC-AC) and the Jefferson Clinic P.C., 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 International Normalized Ratio (INR) results, management of over-anticoagulation and under-anticoagulation and frequency of follow-up.

Inclusion and Exclusion

Patients ≥20 years of age are identified at the initiation of chronic warfarin therapy. Patients are considered eligible if the intended duration of anticoagulation therapy was at least 2 years, the target INR range was 2–3 and are to be managed at one of the two anticoagulation clinics.

Data Collection

A structured interview form is used at the time of enrollment to obtain a detailed medical history. Information on self-reported race, indication for therapy, demographics, height and weight, concomitant medications and co-morbid conditions is documented. Lifestyle and socioeconomic data included smoking, alcohol use (number of alcoholic drinks per week) [19–21], education, annual household income, medical insurance, compliance, level of physical activity[22,23], and dietary vitamin K intake (number of servings of foods rich in vitamin K per week). [24] All patients are followed for up to two years from initiation of therapy at monthly intervals. At each visit changes in factors influencing warfarin response are documented.

Blood collection, DNA extraction and genotyping methodology

Approximately 8 ml of blood is collected in a PAXgene tube (Qaigen Inc., Valencia, CA) at the time of patient consent. DNA is extracted using the PAX gene blood DNA extraction kits and stored at 2–8 °C.

Cytochrome P450 *2C9* (*CYP2C9*) genotype determination

Although the initial aim was to determine *1, *2 (C430T, R144C), *3 (A1075C, I359L), *5 (C1080G, D360E) and *6 (818delA (frameshift) variants of *CYP2C9*, evolving evidence indicates the *11 (C1003T, R335W) variant has reduced metabolic efficiency. [25,26] Therefore, this variant was also included in this study. The *10 allele (A815G, E272G) is a rare allele reported in European Americans. [26] Although *CYP2C9*.10 protein *in vitro* did not indicate altered catalytic activity, the genotype was readily determined in the pyrosequencing reaction of the *6 allele, and therefore included. All of these alleles are documented on the Home Page of the Human Cytochrome P450 *(CYP)* Allele Nomenclature Committee (http://www.cypalleles.ki.se). These pyrosequencing methods allow for the determination of six alleles with four PCR reactions and four sequencing reactions.

Pyrosequencing

Genomic DNA (10–30 ng) is amplified with I unit Ampli Taq Gold (Applied Biosystems, Foster City, CA) in 40 μl reaction containing 1X PCR buffer, 0.2 pm/μl biotinylated forward primer, 0.2 pm/μl reverse primer (Eurogentec, San Diego, CA) (Table 1), 2 mM final MgCl2, and 0.5 mM final dNTPs. An initial denaturation of 95 °C for 8 minutes is used followed by 95 °C for 15 sec, 56 °C for 30 sec and 72 °C for 15 sec for 45 cycles. This reaction is followed by a final extension at 72 °C for 5 min (GeneAmp PCR 9700 System, Applied Biosystems, Foster City, CA).

The entire biotinylated PCR product is mixed with 40 μl of 2× Binding-Washing buffer II (10 mM Tris-HCL, 2 M NaCl, I mM EDTA and 0.1% Tween 20, pH 7.6) and immobilized with 3 μl (10 ug/ul) streptavidin-coated polystyrene beads (Amersham Biosciences, Piscataway, NJ). Samples are mixed at room temperature for 10 minutes. To achieve DNA strand separation, a vacuum prep tool (Biotage, Foxboro, MA) is used and PCR products are isolated through alkaline denaturation and wash steps (Biotage, Foxboro, MA). Beads are released into wells of a PSQ™ sequencing plate (Biotage, Foxboro, MA) containing 20 pmoles of the appropriate sequencing primer (Table 1). The reactions annealed at 90°C for 5 min and cooled at RT for 10 min. Substrates and enzymes as well as dNTP from the manufacturer's kit (Biotage, Foxboro, MA) were placed into a cartridge (supplied by Biotage) and placed into the PSQ 96 MA Pyrosequencer (Biotage, Foxboro, MA) for sequence determination. These methods allow the determination of the six alleles with four PCR reactions (Table 1).

*CYP2C9**2 was determined with one PCR reaction amplifying exon 3 and a single sequencing primer. The *CYP2C9**6 and *CYP2C9**10 alleles were determined with a second PCR reaction amplifying exon 5 and one sequencing primer. The *CYP2C9**3, *CYP2C9**5, and *CYP2C9**11 alleles were determined with two PCR reactions for exon 7, each with a separate sequencing primer.

Polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) was also used to assess the *CYP2C9**2 variant in 293 patients as described by Sullivan–Klose et al [27] with some modifications. Each 25 μL reaction contained 1X Thermopol Buffer II (New England Biolabs, Beverly, MA), 3 mM magnesium sulfate, 50 μM dNTPs, 0.02 U/μl standard Taq DNA polymerase (New England Biolabs, Beverly, MA), 100ng genomic DNA, and a 0.25 μM of forward (5′-TACAAATACAATGAAAATATCATG-3′) and reverse 5′-CTAACAACCAGACTCATAATG-3′) primer for CYP2C9*2. Thermocylcing conditions (touch down protocol) consisted of 94°C for 5 minutes followed by 10 cycles of 94°C for 30seconds, 55°C → 50°C (touch down protocol, −0.5°C per cycle) for 30 seconds and 72°C for 30 seconds. This is followed by 20 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 15 seconds with a 5 minute extension step at 72°C. Ten μls of the *CYP2C9**2 amplicon were digested overnight at 37°C using the restriction endonuclease Ava II under manufacturer recommended directions (New England Biolabs, Beverly, MA). The product digests are then separated by electrophoresis through a 2.5% agarose gel and visualized by staining with ethidium bromide.

Statistical Methods

Dose was defined in two ways: the average maintenance dose required to maintain anticoagulation for the duration of therapy and stable dose at which therapy was considered stabilized (3 consecutive INRs in target range, with these INR measurements encompassing a period of at least 2 weeks, with a maximum difference between the mean daily dosages of 10%). The distribution of dose was marginally skewed to the right therefore log transformation was done to attain normality. The association of dose and CYP2C9 was evaluated using linear regression after accounting for the effects of covariates. Genotypes are categorized into three groups: homozygous wild type [*1/*1], heterozygotes (*1/Variant [*V] or homozygotes [*V/*V]) for the variant allele. The homozygous wild type is considered the referent group for all analyses. Student's t-tests are used to test differences for continuous variables and the χ^2 test of independence to assess the differences for categorical variables between racial groups. All analyses are performed using SAS version 9.1 (SAS Institute, Cary NC) at a non-directional α level of 0.05.

The assumption of Hardy Weinberg Equilibrium (HWE) is tested using the χ^2 test of independence. We also tested the HWE assumption using the "HARDY" genetic analysis software (http://linkage.rockefeller.edu/soft/list2.html#h) to obtain exact test statistics. This program uses the Markov Chain Monte Carlo (MCMC) algorithm for association in two-dimensional contingency tables, and for testing Hardy-Weinberg equilibrium. [28]

Results

All patients meeting eligibility criteria (n=526) were approached to participate in the study after consent of the treating physician. Thirty-six (6. 9%) patients (17 African Americans, 19 European Americans, 15 men, 21 women) declined participation for various reasons. The age, gender and racial makeup of patients declining participation did not differ from that of those who agreed to participate. The age, gender and racial makeup of patients enrolled in the study was similar to that of patients initiated on warfarin therapy at UAB, residing in the five-county Birmingham metropolitan area. A majority (94.4%) of patients resided within the five-county Birmingham metropolitan area, with 81% residing in the Jefferson County. The gender and

racial makeup of participants from Jefferson County is similar to that reported of its residents in the 2000 US census. [29]

This report includes 490 patients (mean age 60.6 ±15.6, range 26 to 93 years) followed from initiation of chronic warfarin therapy. Patients were recruited between August 2003 and August 2006. African American patients comprise 48.9% of the cohort and men 51.3% with an average of 13.5 (±10.6) months of follow-up accrued.

Table 2 displays the socio-demographic and selected lifestyle characteristics of study participants by race. The mean age was significantly higher in European American compared to African American patients (63.5 vs. 57.5 years, $p<0.0001$). There were no significant differences in gender distribution, height, weight and body mass index (BMI; kg/M^2). More European American patients were light drinkers (alcohol intake: 1–7 drinks/week) ($p<0.0001$) and were more physically active ($p=0.002$) compared to African American patients. European American patients were more likely to have medical insurance (90.8% vs. 73.6%, $p<0.0001$), higher education ($p<0.0001$) and higher income ($p<0.0001$). European American patients were less likely to have smoked or to engage in smoking at the time of enrollment ($p=0.005$).

The clinical characteristics of patients enrolled (n=488) by race are presented in Table 3. As of August 2006 there is no difference in the duration of follow-up accrued by race ($p=0.89$). The prescribed target INR for all patients was 2–3. Stroke ($p=0.007$) and venous thromboembolism (both venous and pulmonary, $p= 0.002$ and 0.0004 respectively) were more common indications for warfarin therapy in African American patients, while atrial fibrillation was a more common indication in European Americans. There were no significant differences by site of thromboembolism except venous thromboembolism, which was more prevalent in African American compared to European American patients ($p< 0.0001$). This finding was consistent with the higher prevalence of prior history of venous thromboembolic event in the African American patients ($p=0.013$). Multivariable race-specific analyses highlighting the contribution of FVL mutation to the risk of venous thromboembolic events in European American ($p = 0.03$) but not in African American patients ($p = 0.95$) have recently been reported. [30]

The prevalence of individual comorbid conditions differed across racial groups. More European American patients had a prior history of myocardial infarction ($p=0.04$), undergone coronary artery bypass grafting or percutaneous coronary angioplasty ($p=0.001$), hyperlipidemia ($p=0.001$), and malignancy ($p=0.002$) compared to African American patients. The prevalence of end stage renal disease ($p<0.0001$) was significantly higher and renal insufficiency marginally higher ($p=0.07$) in African American patients compared to European American patients. European American patients had higher number of comorbid conditions at baseline compared to African American patients ($p=0.046$).

Five patients refused to submit a blood sample after enrollment and *CYP2C9* genotype has not been determined for 39 patients. *CYP2C9**2 variants were assessed using both PCR-RFLP and pyrosequencing. Initial pyrosequencing results for *CYP2C9**2 were indeterminate for six samples due to low signal strength (n=2), failed dispensation (n=1) and potentially new single nucleotide polymorphisms (n=3). Repeat pyrosequencing confirmed *CYP2C9* genotype as *1/*1 for three samples. Pyrosequencing called the *CYP2C9* genotype as *2/*2 for eleven samples. However, eight samples were graded as “check” or “fail” by the pyrosequencing software. Resequencing of these samples for exon 3 [27] confirmed the genotype as *1/*2 heterozygote in all eight samples. Three samples were genotyped unequivocally as homozygous for *CYP2C9**2 and were considered “pass” by the software. All three pyrograms were identical and PCR-RFLP confirmed the genotype as *2/*2 homozygote, as did resequencing of one of the samples.

For the *CYP2C9**2 variant the concordance between pyrosequencing and PCR-RFLP methodologies was 99.3%. The discordant results involved two European Americans with a previously unreported non-coding change (C429T) in the region of the *2 mutation (C430T). The *CYP2C9* genotype for these two patients was determined to be *1/*2 by PCR-RFLP (Figure 1, sample #81) but a new mutation at base pair 429 was detected by pyrosequencing. C429T was noted as an extra peak on the pyrogram (the incorporation of an A instead of the expected negative dispensation on the antisense strand) and a decrease in the height of the preceding G peak. This change was confirmed as C429T on the sense strand by genomic sequencing. Figure 2 shows the predicted histograms and resulting pyrograms for *CYP2C9* exon 3 in individuals genotyped as *1/*1 (Panel 1), *1/*2 (Panel 2) and *1/C429T (Panel 3). Sequencing of genomic DNA confirmed this C429T non-coding change as a silent mutation (D143D); therefore the *CYP2C9* genotype in these two patients was designated *1/*1 for all further analyses.

Figure 3 shows the predicted histograms and resulting pyrograms for *CYP2C9* exon 7 in individuals presenting with *1/*1 (Panel 1), *1/*3 (Panel 2), 1/*5 (Panel 3), and *1/C1078T (Panel 4). Panel 4 depicts the pyrogram from an African American man who was found to carry a previously unreported polymorphism, confirmed by DNA sequencing, to represent a G1078A mutation in the same region as the *CYP2C9**3 mutation. The resulting codon change 'GAC to AAC' would lead to the substitution of D360N in the *CYP2C9* protein. This patient, in addition to two Hispanic patients was excluded from the analysis assessing the HWE.

Of the variant alleles tested only the *CYP2C9**2 (11.24%), and *CYP2C9**3 (5.1%) alleles were observed among European Americans. Therefore the inferred frequency of *CYP2C9**1 allele is 83.7%. Of the variants tested, *CYP2C9**2 (1.1%), *CYP2C9**3 (1.8%), *CYP2C9**5 (0.9%), CYP2C9*6 (0.4%), and *CYP2C9**11 (1.1%) were observed among African Americans. Therefore the inferred frequency of *CYP2C9**1 allele is 94.7%. The prevalence of variant *CYP2C9* genotypes (Table 4) is significantly higher in European Americans compared to African Americans (29.82%% vs. 9.73%, p <0. 0001). The distribution of *CYP2C9* genotype differed by race (p <0.0001) but not by gender (p = 0.13, data not shown). CYP2C9 genotype frequencies were found to be in HWE among European Americans (all p-values >0.45) and African Americans (all p-values >0.75).

Table 4 summarizes the average maintenance dose by *CYP2C9* genotype. *CYP2C9* significantly influenced warfarin dose among European Americans (p=0.0007) but not African Americans (p=0.59). Dose difference among patients with *CYP2C9*5, *6,* and **11* alleles could not be determined due to the rarity of these variants. Restricting analysis to *CYP2C9**2 and **3* alleles did not change the magnitude of the dose-genotype associations. Combining genotype into groups according to the presence of variant alleles did not alter the dose-genotype associations among European Americans or African Americans (Table 5).

Although the variability in dose explained by *CYP2C9* genotype decreased slightly from the univariate to the multivariable model, the significance of the dose-genotype association remained unchanged. In the multivariable analysis *CYP2C9* genotype accounted for 3.5% variability in average warfarin dose (p<0.0001) and 5.6% for stable warfarin dose (p<0.0001) which was largely due the influence of *CYP2C9* among European Americans. The variability in warfarin dose explained by *CYP2C9* genotype was 6.4% (average dose) and 7.9% (stable dose) among European Americans and 1% African American (Table 6). European American, but not African American patients with a variant *CYP2C9* genotype required significantly lower warfarin dose compared to their wild-type genotype comparators.

Discussion

Although the efficacy of warfarin is proven, it is vastly underused with difficulty in management of therapy and fear of complications being the main deterrents. *CYP2C9,* the primary enzyme involved in the metabolism of warfarin, is known to be polymorphic. Although the influence of these polymorphisms has been documented in Caucasians, their influence in African Americans is not extensively documented. To our knowledge, the 226 African Americans who comprise 50% of the POAT cohort represent the largest population of African Americans genotyped for *CYP2C9*. Inclusion of the recently discovered variants [26,31,32] in the genotyping provides a robust estimate of the *CYP2C9* allele frequencies in this racially underrepresented group.

As previously reported, the variant *CYP2C9* genotype is more common in European Americans compared to African Americans (29.8% vs. 9.73%). Inclusion of rare alleles such as *CYP2C9**5, *CYP2C9**6 and *CYP2C9**11 resulted in the variant genotype frequencies to be higher among African Americans (5.3%) than previously reported (3.7%) for this population. [9,33] As previously reported CYP2C9*2 and *CYP2C9**3 were the most common poor-metabolizer *CYP2C9* alleles, among both European American and African American patients, although the frequency of these alleles was higher in the former group. The allele frequencies for *CYP2C9**2 and *CYP2C9**3 are consistent with those reported previously.[9,34]

African American populations contain known or putative poor-metabolizer alleles (*CYP2C9**5, *CYP2C9**6 and *CYP2C9*11*) which are rarely found in European Americans. As reported by Dickmann et al [31] the *CYP2C9**5 allele was observed only in African American patients. However the frequency of the *CYP2C9**5 allele was lower in our study, 0.9% versus 1.7% reported previously. This difference is probably due to differences in racial admixture which is known to vary among African Americans residing within different geographic regions of the US. [35–37] Our study is the first to examine the frequencies of the *CYP2C9**6 and *CYP2C9**11 alleles in a large African American population. *CYP2C9**6 (818delA, frameshift) contains a premature stop codon, resulting in a null allele that dramatically decreases the metabolism of phenytoin and warfarin. [31,32]Based on studies with recombinant *CYP2C9* [25,26] *CYP2C9**11 is predicted to have greatly diminished catalytic activity, but has not received extensive clinical study. One study [38] reported significantly lower warfarin dose requirements in patients with the *CYP2C9**1/*11 genotypes compared to those with *CYP2C9**1/*1 genotype. The rarity *CYP2C9**6 (0.4%) and *CYP2C9**11 (1.1%) allele did not facilitate evaluation of the dose-genotype association.

We identified two new mutations, one new non-coding change (C429T, Figure 2) in the same region as *CYP2C9*2* mutation. This C429T change, a silent noncoding mutation (D143D) was noted in 2 (of 218 participants, 0.92%). This noncoding change is important because it destroys the AvaII site commonly used in RFLP analysis of the *CYP2C9*2* allele, invalidating this test. This example discrepancy between the two genotyping methodologies demonstrates the superiority of pyrosequencing over RFLP genotyping tests.

Amino acids 359 and 360 lie within one of the predicted substrate recognition sites of the CYP2 family of enzymes.[39] This area seems to be a “hot spot” for mutations in *CYP2C.* The new D360N coding change found in one African American in this study represents a new mutation within this substrate recognition site. Several defective alleles which arise from mutations in this region greatly affect the affinity and catalytic activity of the *CYP2C9* enzyme resulting in large variability in dosage requirements of *CYP2C9* substrates. [40]These alleles include *CYP2C9**3 (I359L)[27], *CYP2C9**4 (I359T) (found in Japanese), [41]*CYP2C9**5 (D360E) in African-Americans. [31] A Y358C mutation has also been reported to the NCIdbSNP homepage (rs1057909). Newer crystal structures show that the hydrophobic substrate pocket

includes the adjacent amino acids L362 and L366 [42] which limit access for warfarin to the heme group. [43] The proximity of I359 and D360 to these amino acids can potentially result in defective alleles. Although rare, these alleles can produce clinically meaningful changes in warfarin dose and toxicity.

This study confirms the association of *CYP2C9*2* and *CYP2C9*3* polymorphisms on warfarin dose among European Americans after accounting for the effects of multiple potential covariates. It also confirms recently findings by Kealey et al [33] on the lack of significant influence of *CYP2C9*2* and *CYP2C9*3* on warfarin dose in African Americans. Our findings further extend this lack of association after inclusion of CYP2C9**5, *6 and *11* among African American patients. Although African American patients with 2 variant alleles required lower doses (Table 5), trend tests were not statistically significant, probably due to the small sample size in this subgroup.

We also recognize limitations of the present study. Documentation of vitamin K intake was based on patient report using vitamin K inventory [24] not quantified by assay measurements. However, all measurements were used consistently among all participants; therefore, bias if any should be non-differential. Although the focus of the current study was to define the association of *CYP2C9* genotype on warfarin dose and risk of complications we recognize that drug response is influenced by multiple genes.[44] [45,46] At least one other gene; Vitamin K Epoxide Reductase (*VKORC1*) has been consistently shown to significantly influence warfarin dose in European Americans [14,15,47,48] and recently among African Americans.[49] Expansion of genotyping efforts, within this prospective cohort, to include other genes along the warfarin pharmacodynamic and pharmacokinetic pathways will facilitate gene-gene interaction studies and help tailor warfarin therapy based on genetic, clinical and demographic characteristics. Inclusion of underrepresented racial groups such as African Americans will help identify genetic and non-genetic determinants of drug response and quantify their influence in this subgroup where the risk of thromboembolic diseases and the resultant mortality is disproportionately high. [50]

Conclusion

The variant *CYP2C9* genotype is more frequent among European Americans compared to African Americans. Among African Americans the variant genotype frequency is higher than previously reported. CYP2C9 genotype predicts warfarin dose in European Americans, but not in African Americans.

Executive Summary

- ▪This prospective study documents the frequencies of CYP2C9 polymorphisms (*2, *3, *5, *6, *11) in European Americans and African Americans.
- ▪The variant genotype is more frequent among European Americans (29.8%) compared to African Americans (9.7%).
- ▪CYP2C9 polymorphisms influenced warfarin dose requirements in European Americans but not African Americans in univariate and multivariable analyses.
- ▪CYP2C9 genotype predicts warfarin dose in European Americans, but not in African Americans.
- ▪Further studies are needed to identify other genetic and non-genetic predictors of warfarin dose in African Americans.

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Abbreviations

CYP2C9
: Cytochrome P4502C9

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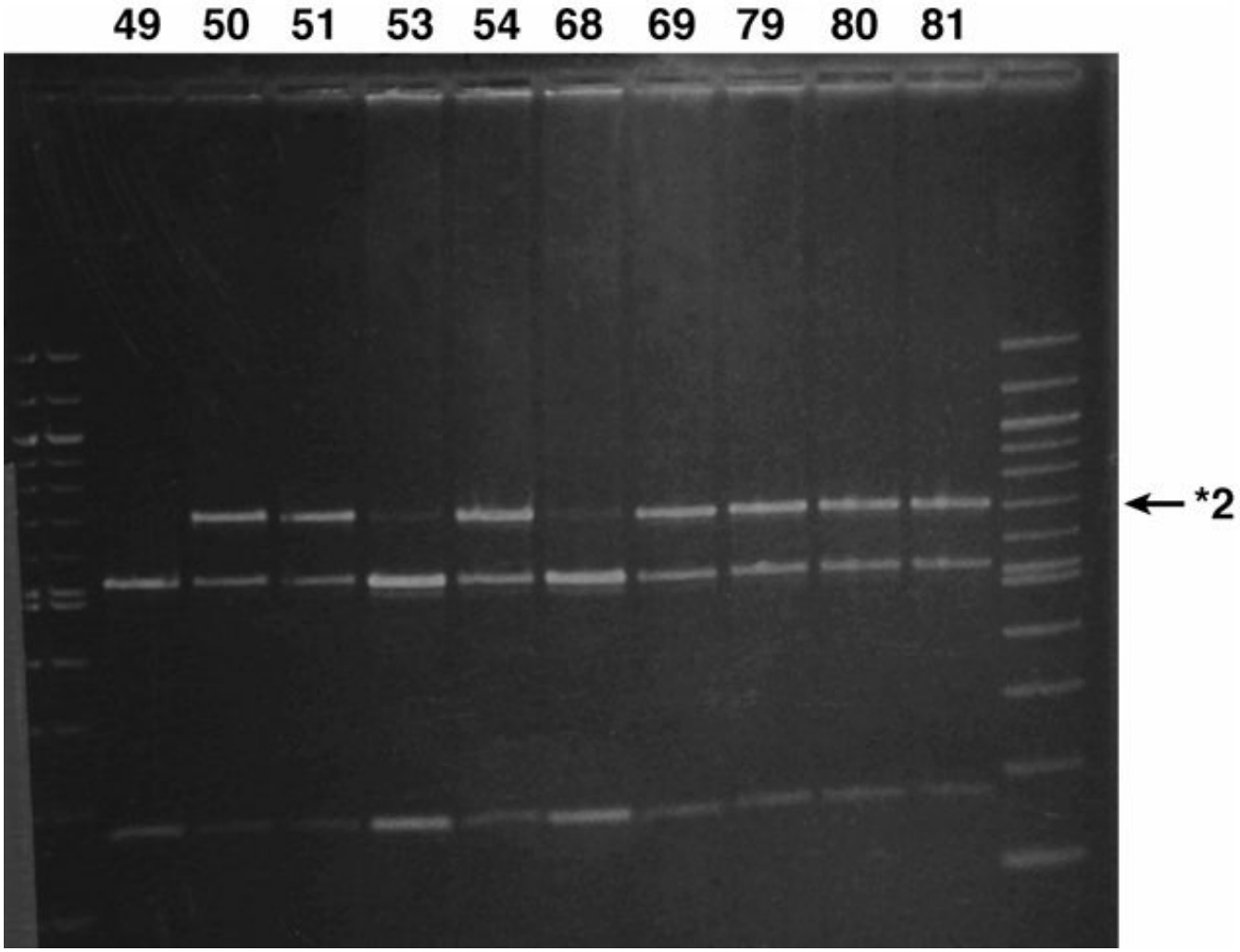

Figure 1.
CYP2C9 *2 variant as assessed by PCR-RFLP as described by Sullivan Klose et al. [27]The first and last lane contains molecular weight markers (100 base pair ladder). The remaining 10 lanes correspond to the Ava II digested PCR products from patients 49, 50, 51, 53, 54, 68, 69, 79, 80 and 81 separated by electrophoresis through a 2.5% agarose gel and visualized by staining with ethidium bromide. The CYP2C9 genotype for patients 49, 53 and 68 is CYP2C9*1/*1 and CYP2C9*1/*2 for patients 50, 51, 54, 69, 79, 80 and 81. The genotype for patient #81 (second last lane) is falsely determined to be *1/*2 due to a previously unreported non-coding change at base pair 429. The genotype for patient 81 was CYP2C9*1/*1 by pyrosequencing (Figure 2)

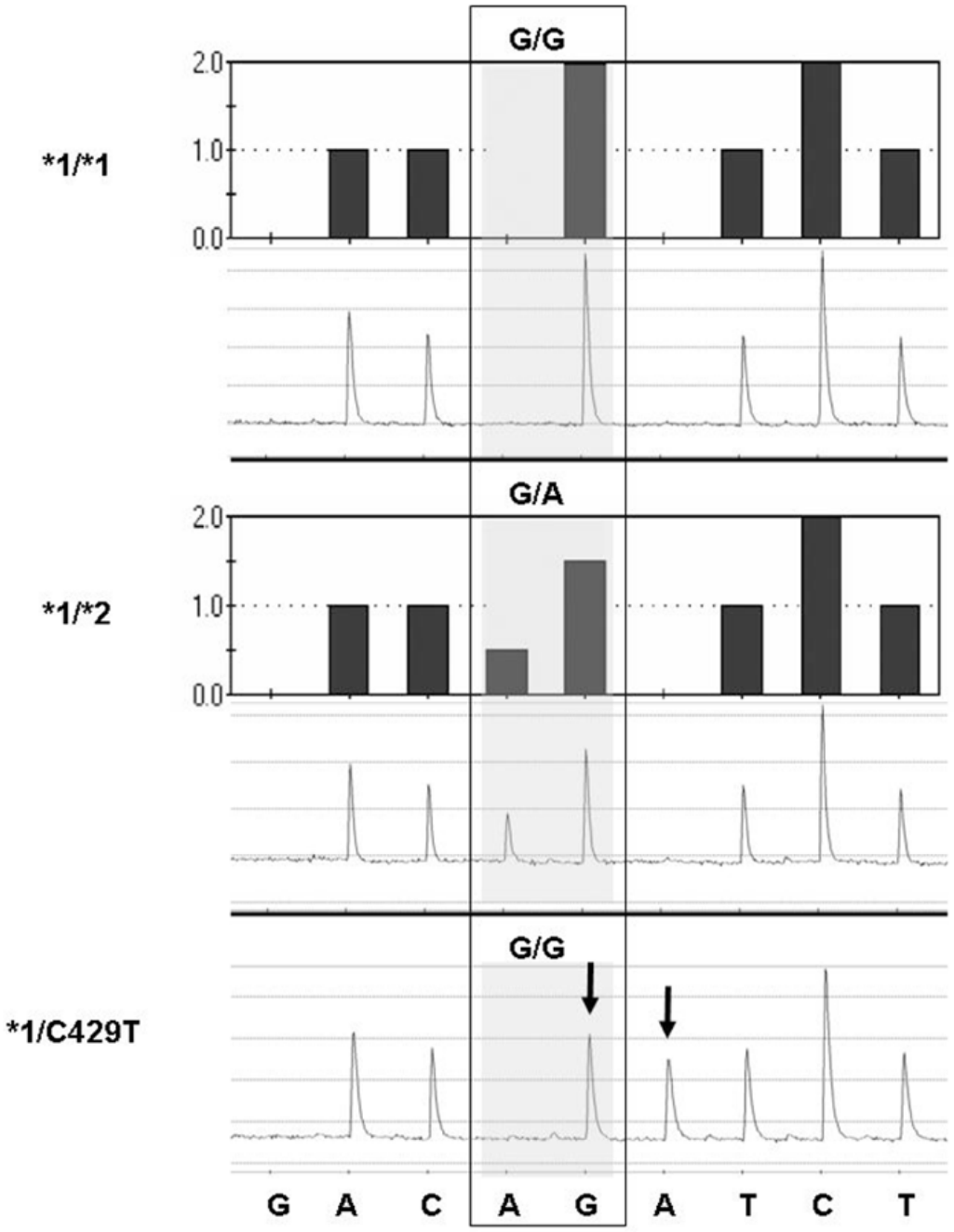

Figure 2.

Predicted histograms and actual pyrograms showing the CYP2C9*2 genotyping test. The reverse strand was sequenced; the sequence at the bottom refers to the nucleotide dispensation. While the dispensation order is G(negative)AC[AG]A(negative)TCT, the actual sequence analyzed (reverse strand) is AC[**A**/G]GTCCT (variant nucleotides in brackets with the mutant nucleotide in bold). The predicted histogram is shown at the top, and the actual pyrogram is shown below. The shaded boxes indicate the regions in which the two variable nucleotides were dispensed, with the base designation of the sample at base pair 430 at the top. The actual genotype of the sample is indicated at the left. Samples are from individuals genotyped as CYP2C9*1/*1 (top panel), CYP2C9*1/*2 (middle panel) and the bottom panel shows a

pyrogram from patient #81 with an abnormal pyrogram (see arrows) with the disappearance of a G and the appearance of an A at bp 429 on the antisense strand (confirmed by sequencing as heterozygous for a noncoding mutation 429C>T on the sense strand).

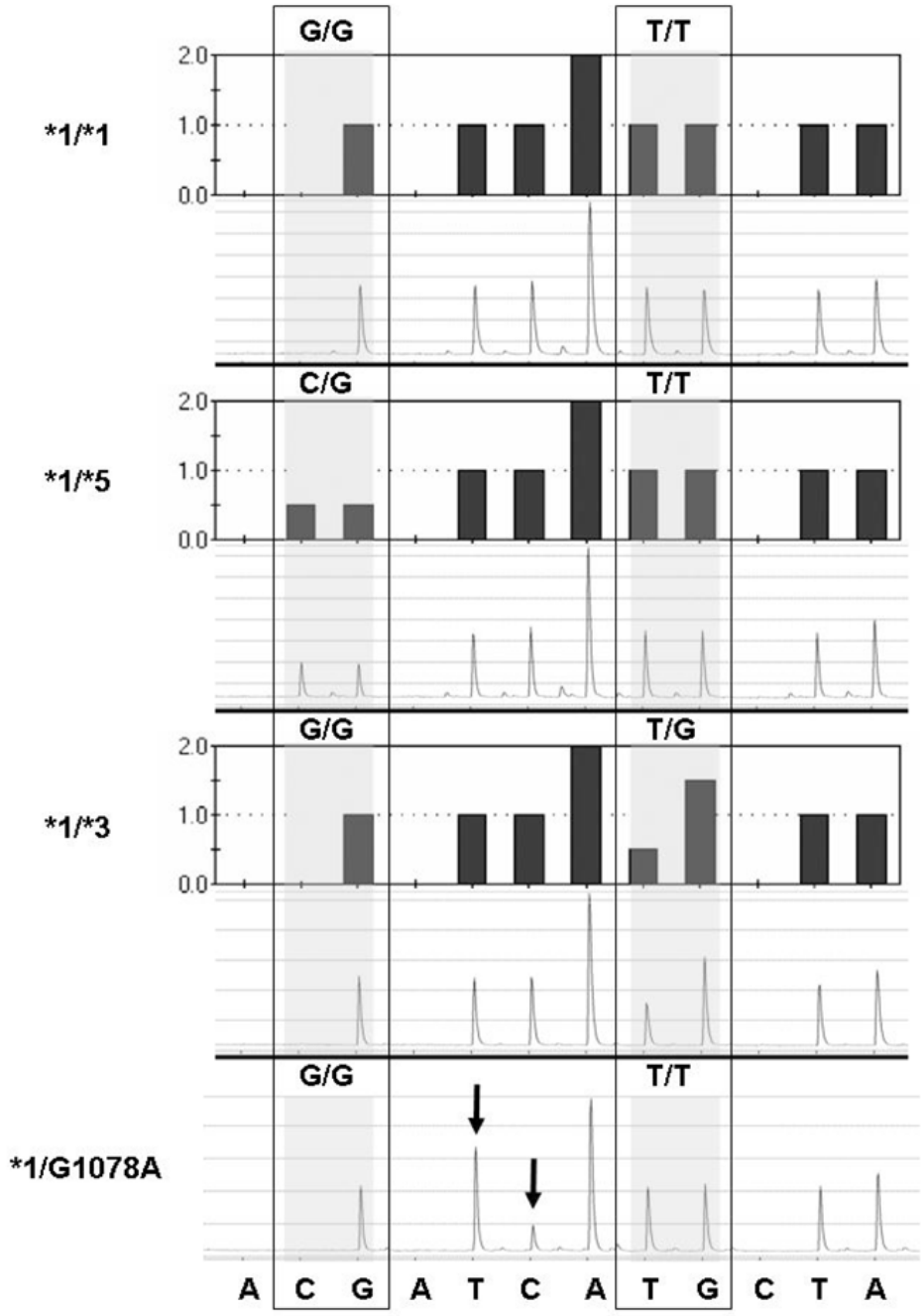

Figure 3.

Histograms and pyrograms showing CYP2C9*5 and CYP2C9*3 genotyping tests. The sequence at the bottom refers to the nucleotide dispensation. While the dispensation order is A(negative)[CG]A(negative)TCA[TG]C(negative)TA, the actual sequence (reverse strand) analyzed is [**C**/G]TCAA[T/**G**]GTA (variant nucleotides in brackets with mutant nucleotides in bold). The shaded boxes indicate the variable region in which two nucleotides were dispensed, with the base designation of each allele. Pyrograms shown are examples of individuals homozygous for CYP2C9*1/*1 or heterozygous for CYP2C9*1/*5 and CYP2C9*1/*3. The final pyrogram is from individual # 43 who shows an abnormal pyrogram (see arrows) with the appearance of an extra T at bp 1078 and the disappearance of a C

(antisense strand), confirmed by sequencing of the genomic DNA as a G1078A mutation on the sense strand producing a new coding change D360N).

Table 1

PCR sequencing primers for Pyrosequencing CYP2C9 alleles

PCR primers[a]	bp[b] size	2C9 allele	Sequencing primer
Exon 3			
F: B-5′-AAACAGAGACTTACAGAGCTC-3′ R: 5′-CTAACAACCAGACTCATAATG-3′	381	*2	5′-GGGCTTCCTCTTGAAC-3′
Exon 5			
F: B-5′-CAGAGCTTGGTATATGGTATG-3′ R: 5′-TCGTAAACACAGAACTAGTCAAC-3′	323	*6,*10	5′-AAGCTTTTGTTTACATTTT-3′
Exon 7			
F: B-5′-CTGAATTGCTACAACAAATGTG-3′ R: 5′-GATACTATGAATTTGGGACTTC-3′	314	*11	5′-TTGCATGCAGGGGCT-3′
F: B-5′-TGCACGAGGTCCAGAGAT-3′ Reverse primer same as above	155	*3,*5	5′-GCTGGTGGGGAGAAG-3′

[a] F: forward primer; R: reverse primer, B: biotin labeled primer

[b] bp indicates PCR fragment base pair size

2C9 allele: cytochrome P4502C9 single nucleotide polymorphism

Table 2

Socio-demographic and lifestyle characteristics of the POAT study participants.

Characteristic	African American (n=239)	European American (n=249)	p-value
Age, mean (SD)	57.5 (15.8)	63.5 (14.8)	**<0.0001**
Height (inches)	67.6 (4.1)	68.2 (6.4)	0.20
Weight (lbs)	196.0 (51.2)	193.2 (51.7)	0.55
Body Mass Index, mean (SD)	30.2 (7.4)	29.3 (7.5)	0.25
	N (%)	**N (%)**	
Gender			
Female	126 (52.7)	111 (44.6)	0.07
Male	113 (47.3)	138 (55.4)	
Alcohol (drinks per week)[1]			
0	185 (77.4)	150 (60.2)	**<0.0001**
1–7	34 (14.2)	83 (33.3)	
>8	19 (8.0)	16 (6.4)	
Smoking Status[1]			
Current	48 (20.0)	23 (9.2)	**0.005**
Past	83 (34.7)	95 (38.2)	
Never	104 (43.5)	123 (49.4)	
Level of Physical Activity[1]			
Wheelchair bound	14 (5.9)	7 (2.8)	**0.003**
Uses Walker/Cane	39 (16.3)	24 (9.6)	
Ambulates without assistance	46 (19.2)	43 (17.3)	
Physically active	137 (57.3)	163 (65.5)	
Consistent/Intensive exercise	1 (0.42)	11 (4.4)	
Education			
< High School	73 (30.5)	21 (8.4)	**<0.0001**
High School	110 (46.1)	90 (36.1)	
College	55 (23.0)	106 (42.6)	
Graduate School	1 (0.4)	32 (12.9)	
Annual Household Income[1]			
<15,000	96 (40.2)	31 (12.5)	**<0.0001**
15,000–25,000	73 (30.5)	29 (11.6)	
25,000–50,000	65 (27.2)	84 (33.7)	

Characteristic	African American (n=239)	European American (n=249)	p-value
50,000–100,000	3 (1.2)	84 (33.7)	
>100,000	1 (0.4)	21 (8.4)	
Clinic			
The Kirklin Clinic	151 (63.2)	231 (92.8)	**<0.0001**
Cooper Green Hospital*	88 (36.8)	18 (7.2)	
Medical Insurance			
Medicare	66 (27.6)	103 (41.4)	**<0.0001**
Medicare Medicaid	5 (2.1)	0 (0.0)	
Medicaid	14 (5.9)	9 (3.6)	
Private	91 (38.1)	114 (45.8)	
None	63 (26.3)	23 (9.2)	

Mean (SD) displayed for continuous variables and frequency counts (column percent) for categorical variables

1 Information on missing for smoking (n=13), level of physical activity (n=3), alcohol (n=2), income (n=1)

* 2 Hispanic patients excluded, all significant p-values **bolded**

Private insurance includes various private insurance plans such as Blue Cross Clue Shield, Aetna, Travelers, etc.

Table 3

Clinical characteristics of the POAT study participants.

Characteristic	African American (n=239)	European American (n=249)	p-value
Follow-up accrued (months, Mean ± SD)	13.5 (10.5)	13.6 (10.7)	0.89
Indication for warfarin therapy*			
Deep Vein Thrombosis	84 (35.1)	56 (22.5)	**0.002**
Pulmonary Thromboembolism	41 (17.1)	17 (6.8)	**0.0004**
Recurrent Venous Thromboembolism	20 (8.4)	14 (5.6)	0.23
Atrial Fibrillation	76 (31.8)	147 (59.0)	**<0.0001**
Valvular Heart Disease	26 (10.9)	40 (16.1)	0.09
Low Left Ventricular Ejection Fraction	40 (16.7)	29 (11.6)	0.11
Cardiac Thrombus	10 (4.2)	12 (4.8)	0.73
Myocardial Infarction	62 (25.9)	80 (32.1)	0.13
Transient Ischemic Attack	15 (6.3)	20 (8.0)	0.45
Stroke	53 (22.2)	32 (12.8)	**0.007**
Peripheral Vascular Disease	27 (11.3)	38 (15.3)	0.20
Site of thromboembolism**			
Arterial	99 (41.1)	112 (45.0)	0.43
Venous	118 (49.4)	76 (30.5)	**<0.0001**
Both	25 (10.5)	19 (7.6)	0.27
None	34 (14.2)	44 (17.7)	0.30
Comorbidity			
History of Myocardial Infarction	8 (3.3)	19 (7.6)	**0.04**
History of CABG/PTCA	25 (10.5)	53 (21.3)	**0.001**
Cardiomyopathy	14 (5.9)	16 (6.4)	0.79
Coronary Artery Disease	74 (31.0)	90 (36.1)	0.22
Congestive Heart Failure	60 (25.1)	51 (20.5)	0.22
Hypertension	103 (43.1)	99 (39.8)	0.45
Hyperlipidemia	49 (20.5)	84 (33.7)	**0.001**
Diabetes Mellitus	83 (34.7)	75 (30.1)	0.28
Malignancy	23 (9.6)	49 (19.7)	**0.002**
Prior Hemorrhage	21 (8.8)	11 (4.4)	**0.051**
Renal insufficiency	43 (18.0)	30 (12.0)	0.07
End Stage Renal Disease	28 (11.7)	7 (2.8)	**<0.0001**
Number of Comorbid Conditions			
Low (0 or 1)	81 (33.9)	64 (25.7)	**0.046**
Medium (2 to 4)	112 (46.9)	117 (47.0)	
High (5 or more)	46 (19.2)	68 (27.3)	

2 Hispanic patients excluded, significant p-values **bolded**

* patients with orthopedic surgery excluded due to short (3–6 month) treatment duration, patients with mechanical heart valve and hypercoagulable state excluded due to higher intensity of anticoagulation required

** Arterial thromboembolism includes patients with MI, Stroke & TIA. Venous thromboembolism includes patients with DVT & PE. Both include patients with venous and arterial events. None includes patients with no thromboembolic events (e.g. Atrial Fibrillation),

Table 4

CYP2C9 genotype and average warfarin dose (mg/day ± SD) distribution among participants of the POAT cohort.

Genotype	All Participants N=444		African Americans N=226		European Americans N=218	
	N (%)	Dose	N (%)	Dose	N (%)	Dose
*1/*1	357 (80.4)	5.7 (± 2.5)	204 (90.3)	6.1 (± 2.6)	153 (70.2)	5.3 (± 2.4)
*1/*2	45 (10.1)	4.7 (± 2.0)	5 (2.2)	6.6 (± 1.59)	40 (18.3)	4.5 (± 2.0)
*1/*3	26 (5.8)	4.1 (± 1.4)	7 (3.1)	5.0 (± 1.0)	19 (8.7)	3.8 (± 1.4)
*1/*5	3 (0.7)	6.3 (± 1.9)	3 (1.3)	6.3 (± 1.9)	0 (0.0)	-
*1/*11	5 (1.1)	5.8(± 2.0)	5 (2.2)	5.8(± 2.0)	0 (0.0)	-
*2/*2	3 (0.7)	2.9 (± 1.6)	0 (0.0)	-	3 (1.4)	2.9 (± 1.6)
*2/*3	3 (0.7)	1.8 (± 0.6)	0 (0.0)	-	3 (1.4)	1.8 (± 0.6)
*3/*6	1 (0.2)	2.0	1 (0.4)	2.0	0 (0.0)	-
*5/*6	1 (0.2)	4.0	1 (0.4)	3.0	0 (0.0)	-

Subjects recruited during the intervalof August 2003 – August 2006

Analysis excludes Hispanic patients (n=2)

Genotype frequencies present as number of participants with genotype and (percent)

Average daily warfarin dose (mg/day ± SD) analysis included 437 patients

Table 5

Warfarin daily dose stratified by CYP2C9 genotype

Dose (mg/day)	CYP2C9*1/*1	CYP2C9*1/*V	CYP2C9*V/*V	p-value
Average Maintenance	5.3 [4.0–6.8]	4.4 [3.0–5.9]	2.12 [1.8–3.2]	<0.0001
African American	5.8 [4.2–7.5]	5.3 [4.7–6.3]	3.0 [2.0–4.0]	0.16
European American	4.9 [3.7–6.3]	4.0 [2.8–5.7]	2.1 [1.5–2.4]	<0.0001
Stable Dose	5.0 [3.9–6.4]	4.3 [2.9–5.7]	2.1 [1.5–2.8]	<0.0001
African American	5.0 [3.9–6.6]	5.0 [4.3–6.2]	-[1]	0.71
European American	5.0 [3.9–6.4]	1.0 [2.9–5.3]	2.1 [1.5–2.8]	0.0003

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

Median daily dose (interquartile range) presented. All p-values are based on Kruskal-Wallis Test

[1]two patients were not stabilized at the time of data analysis

Analysis excludes Hispanic patients (n=2)

Table 6

Percent variation in warfarin log-dose explained by variant CYP2C9 genotype

	Average Maintenance Dose			Stable Dose		
	Model	**CYP2C9**	**p**	**Model**	**CYP2C9**	**p**
All patients	27.0%	3.5%	<0.0001	25.7%	5.6%	<0.0001
Daily Dose (mg)		5.3 vs. 4.0	<0.0001		5.3 vs. 4.1	<0.0001
European Americans	19.4%	6.4%	<0.0001	21.6%	7.9%	0.0003
Daily Dose (mg)		4.9 vs. 3.7	<0.0001		5.2 vs. 3.8	<0.0001
African Americans	28.0%	1.0%	0.092	33.6%	1.0%	0.25
Daily Dose (mg)		5.6 vs. 5.3	0.22		5.4 vs. 5.0	0.16

Warfarin daily dose is predicted dose for wild type versus variant genotype. Analysis for stable dose included 232 patients

Adjusted for age, gender, BMI, drug interactions, average alcohol intake, average vitamin K intake, number of comorbid conditions

CYP2C9 genotype effects are present for variant genotype (*1/*V and ***V**/*V) versus ***1**/*1* genotype. V denotes variant allele (2, *3, *5, *6, *10, or *11).