

Warfarin Dosing and the Promise of Pharmacogenomics

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Abstract: Due to its narrow therapeutic index and substantial inter-patient variability in clinical response, warfarin represents an ideal drug candidate to benefit from the promise of pharmacogenomic-guided dosing strategies. Consistent with *in vitro* data, clinical studies have demonstrated that *CYP2C9* polymorphisms significantly influence warfarin pharmacokinetics by reducing (S)-warfarin metabolic clearance, consequently lowering maintenance dose requirements and increasing the risk over-anticoagulation during the initiation phase of therapy. Recent data suggest that polymorphisms in genes encoding several pharmacodynamic determinants of the coagulation cascade may also influence warfarin's anti-thrombotic dose-response. Of these, *VKORC1* polymorphisms account for a significant proportion of the inter-individual variability in warfarin dose requirements in all populations evaluated. Collectively, these data suggest that assessment of genetic polymorphisms affecting both warfarin pharmacokinetics and pharmacodynamics could help to predict warfarin dose requirements in patients. Therefore, the promise of pharmacogenomic-guided dosing as a useful strategy to improve clinical outcomes with warfarin therapy appears credible and warrants further investigation.

Key Words: Warfarin, pharmacogenomics, polymorphism, *CYP2C9*, *VKORC1*.

INTRODUCTION

Warfarin, named after the initials of its patent holder The Wisconsin Alumni Research Foundation, is a synthetic analogue of the natural product dicoumarol with potent anti-thrombotic properties [1]. Warfarin sodium is the most commonly prescribed oral antithrombotic agent for the prophylaxis and treatment of arterial and venous thromboembolic events [2]. Due to its narrow therapeutic index, the full benefits of warfarin therapy are often not attained due to both underutilization and suboptimal dosing. For example, while warfarin has been shown to reduce ischemic stroke risk by 65% in patients with atrial fibrillation [3], warfarin is prescribed in only half of these patients who are appropriate candidates for anticoagulation [4-6] even though their risk of ischemic stroke is 5-fold higher compared to those in normal sinus rhythm [7, 8]. The underutilization and suboptimal use of warfarin often results from concerns regarding the safe administration of a drug with a slow onset of action, narrow therapeutic index, substantial pharmacokinetic and pharmacodynamic variability [9, 10], and propensity for drug and food interactions, which necessitate regular therapeutic drug monitoring *via* the International Normalized Ratio (INR).

Warfarin initiation doses are often prescribed empirically and typically range from 2.5 to 10 mg/day with peak effects usually observed after approximately 5 days. Intense INR monitoring and dose adjustments are particularly necessary during the initiation phase of therapy, which can take several weeks to establish a patient's optimal maintenance dose requirement (often ranging anywhere from 1 to 20 mg/day) [2]. However, even with INR monitoring, outcomes achieved during routine clinical practice are substantially inferior to those obtained in clinical trials [11]. Moreover, it is well established that stroke intensity and mortality rates are

significantly higher in atrial fibrillation patients with sub-therapeutic INRs [12]. Although recent data from the SPORTIF trials suggest that anticoagulation specialty clinics can significantly improve the proportion of time in which patients maintain a therapeutic INR to approximately 67% [13, 14], additional strategies are needed to maximize "therapeutic" dosing. The recent failure of the direct thrombin inhibitor ximelagatran due to liver toxicity suggests that novel approaches for improving utilization of warfarin therapy should continue to be explored while the pharmaceutical industry attempts to discover and develop additional oral antithrombotic agents [15].

Warfarin appears to represent an ideal candidate to benefit from the promise of pharmacogenomic-guided dosing strategies due to its narrow therapeutic index and substantial inter-patient variability in clinical response. Numerous polymorphisms in genes associated with both warfarin disposition (pharmacokinetics) and response (pharmacodynamics) have been identified. For example, elimination of the more potent (S)-warfarin enantiomer is almost exclusively dependent on *CYP2C9*-mediated hepatic metabolism [16]. Numerous single nucleotide polymorphisms (SNPs) in *CYP2C9* contributing to inter-patient pharmacokinetic variability have been identified and significantly correlate with warfarin dose requirements [17]. Warfarin's antithrombotic effects occur through interference with the coagulation cascade through competitive inhibition of a vitamin K epoxide reductase multiprotein complex (VKOR) responsible for regeneration of reduced vitamin K. Multiple variants in the genes encoding vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and other mediators in the coagulation cascade have been identified and also correlate with warfarin dose requirements [18]. The development of personalized dosing schemes based on an understanding of the genomic and non-genomic factors that influence the warfarin dose-response relationship offers the potential to significantly improve efficacy and safety of warfarin therapy in patients. This review will exam-

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ine the critical *in vitro* and *in vivo* data published to date describing the influence of genetic variation on warfarin pharmacokinetics, pharmacodynamics and dose requirement, and will provide insight into the development of future genomic-guided dosing strategies with the ultimate goal of improving clinical outcomes.

CYP2C9 PHARMACOGENETICS – PHARMACOKINETIC DETERMINANT OF WARFARIN RESPONSE

Warfarin is formulated as a racemic mixture. (R)-Warfarin undergoes oxidative metabolism by CYP1A2 and CYP3A4, while (S)-warfarin is predominantly cleared *via* CYP2C9-mediated 6'- and 7'-hydroxylation. Since it is well established that (S)-warfarin enantiomer has approximately 3-5 times more anti-thrombotic activity compared to (R)-warfarin, CYP2C9-mediated clearance has proven to be a major pharmacokinetic determinant of warfarin response in humans [19, 20].

The gene coding for the CYP2C9 protein has been mapped to chromosome 10q24.2 [21, 22] and multiple polymorphisms have been identified. Currently, at least 24 of these encode for amino acid substitutions and represent true CYP2C9 alleles, as designated by the Human CYP Allele Nomenclature Committee (<http://www.imm.ki.se/CYP-Palleles/cyp2c9.htm>). The most common allele is designated as CYP2C9*1, and is considered the wild-type allele [23-25]. The Arg144Cys amino acid change in exon 3 (CYP2C9*2 allele) and the Ile359Leu amino acid change in exon 7 (CYP2C9*3) were the first nonsynonymous CYP2C9 variants discovered [23-25]. Subsequently, alleles CYP2C9*4 through *24 have been discovered across multiple populations, each encoding a nonsynonymous change in CYP2C9 [26-35]. Discovery of these CYP2C9 variants has stimulated numerous *in vitro* and *in vivo* investigations designed to evaluate the functional relationship between CYP2C9 genotype, CYP2C9 metabolic activity, warfarin disposition and warfarin dose requirement. However, the vast majority of these investigations have evaluated the functional significance of the CYP2C9*2 and *3 variant alleles. Comprehensive reviews describing the results of such studies with warfarin and other CYP2C9 substrates have been previously published [17, 36-42].

Population Frequencies of CYP2C9 Alleles

Population genotyping studies have characterized the frequency of the CYP2C9*1, *2 and *3 alleles in individuals of distinct ethnic background, and these data have been comprehensively reviewed [17, 42]. Briefly, the variant CYP2C9*2 and *3 alleles appear to be prevalent in White populations at frequencies of 10-15% and 5-10%, respectively [17, 42]. However, in African and Asian populations approximately 2-4% and 0% carry the CYP2C9*2 allele, while 1-2% and 1-4% carry the CYP2C9*3 allele, respectively [17, 42]. The population frequencies of the more recently identified CYP2C9 alleles have not been as rigorously evaluated; however, the majority of the CYP2C9*4 through *24 variants have been predominantly identified in Asian and African populations at frequencies <5% [26-35]. These allele frequencies remain to be confirmed in larger populations.

***In Vitro* Metabolism**

Multiple *in vitro* investigations evaluating the functional effects of the CYP2C9*1, *2 and *3 alleles have demonstrated significantly lower intrinsic clearance of warfarin in recombinant cell systems expressing the variant CYP2C9*2 and in particular the *3 variant allele compared to wild-type [43, 44]. These studies have been reviewed in detail [17, 40]. The reduced substrate binding capacity associated with the CYP2C9*3 variant leads to lower enzymatic activity across a broad range of substrate concentrations rather than solely lowering maximum catalytic threshold as observed with the CYP2C9*2 variant, suggesting that the CYP2C9*3 allele may be more functionally relevant *in vivo*. The Ile359Leu (*3), Ile359Thr (*4) and Asp360Glu (*5) substitutions are all located in a region coding for substrate recognition site 5 of the CYP2C9 protein, accounting for the lower binding capacity and lower intrinsic clearance observed with each variant relative to wild-type [45]. The CYP2C9*6 [28] and *15 variants [32, 33] introduce premature stop codons in the CYP2C9 protein and are devoid of CYP2C9 metabolic activity. The CYP2C9*11, *12, *14 and *16 variants have significantly lower intrinsic clearance of tolbutamide, whereas the CYP2C9*8 variant has significantly higher intrinsic clearance relative to wild-type CYP2C9 [29, 33]. Of these newly discovered alleles, warfarin metabolism has only been evaluated with the CYP2C9*11 allele. Although no differences in (S)-warfarin hydroxylation were observed in cells expressing the CYP2C9*11 variant compared to wild-type enzyme, this variant demonstrated significantly reduced enzyme stability and expression [46]. Future studies evaluating the impact of these newly discovered variants on warfarin metabolism *in vitro* are necessary, particularly since the metabolic impact of certain CYP2C9 variants appear to be substrate dependent [17].

***In Vivo* Metabolism and Dose Requirement**

Multiple retrospective investigations evaluating the effect of CYP2C9 genotype on warfarin maintenance dose requirement in anticoagulation patients with a stable and therapeutic INR have been completed and reviewed previously [17, 37-41]. A recent meta-analysis of these studies reported that individuals carrying two of the variant CYP2C9*2 and *3 alleles required 0.85 mg (17%) and 1.92 mg (37%) lower warfarin maintenance doses on average, respectively, compared to CYP2C9*1/*1 individuals. Those carrying one of either the CYP2C9*2 or *3 variant required 1.47 mg (27%) lower doses [47]. With the exception of CYP2C9*3/*3 where differences are substantial, more subtle but statistically significant differences in dose requirement are observed across the other genotype groups in most of the individual studies. For instance, two important early studies demonstrated that patients requiring ≤ 1.5 mg/day were significantly more likely to carry at least one variant allele (CYP2C9*2 or *3) compared to patients requiring >1.5 mg/day of warfarin, (odds ratio 6.21, 95% confidence interval 2.48-15.6) [48] and (odds ratio 3.85, 95% confidence interval 1.24-11.9) [49], whereas patients carrying two variant alleles demonstrated the greatest probability of the "low-dose" requirement. More recently, individuals heterozygous for the CYP2C9*11 variant, which was identified in ap-

proximately 2% of individuals of African descent, required approximately 33% lower warfarin maintenance doses compared to *CYP2C9**1/*1 individuals [46].

Consistent with the aforementioned *in vitro* metabolism studies, the underlying reason for these differences in warfarin sensitivity appears to be secondary to genotype-associated alterations in (S)-warfarin clearance. For instance, *CYP2C9* genotype (*CYP2C9**2 and *3 variants) significantly correlated with two human phenotypic measures of *CYP2C9* metabolic activity, (S)-warfarin oral clearance ($P < 0.0001$) and the plasma S:R warfarin ratio ($P < 0.0001$) in 93 Italian anticoagulation patients [50]. The most substantial differences in these phenotypic measures were observed in individuals with the *CYP2C9**3/*3 genotype. Similarly, significantly longer (S)-warfarin elimination half-life was observed in anticoagulation patients carrying at least one *CYP2C9**2 or *3 variant allele compared to wild-type individuals [51]. Additional studies have demonstrated similar results for the oral antithrombotic agent acenocoumarol, which is widely used outside the United States. A significant relationship exists between *CYP2C9* genotype and both acenocoumarol dose requirement and *CYP2C9*-mediated (S)-acenocoumarol clearance [52, 53]. Collectively, these pharmacokinetic studies demonstrate significantly lower *CYP2C9*-mediated clearances of (S)-warfarin and (S)-acenocoumarol in patients carrying the *CYP2C9**2 or *3 variant alleles, subsequently leading to lower maintenance dose requirements.

During the initiation of warfarin therapy, patients carrying a variant *CYP2C9**2 or *3 allele also appear to be significantly more likely to experience a major bleeding event, complications from bleeding or a prolonged hospitalization due to unstable anticoagulation compared to *CYP2C9**1/*1 individuals [48, 54]. Higashi *et al.* demonstrated that patients carrying a *CYP2C9**2 or *3 variant allele were at an increased risk of a supratherapeutic INR (INR > 4.0) and required a longer period of time to achieve stable dosing compared to wild-type individuals (median difference of 95 days) [55]. Moreover, these individuals were significantly more likely to experience a bleeding event during the initiation phase of therapy (hazard ratio 3.94, 95% confidence interval 1.29-12.06) and a serious or life-threatening bleeding event at any time during the course of warfarin therapy compared to *CYP2C9**1/*1 individuals [55]. Among patients initiated at similar warfarin doses, 64% of *CYP2C9**2 carriers ($P = 0.006$) and 67% of *3 allele carriers ($P = 0.012$) had a first INR drawn after warfarin initiation (day 4) that was > 3.0 compared to 33% of *CYP2C9**1/*1 individuals [56]. Interestingly, mean INR values were not significantly different across genotype by day 11 after appropriate warfarin dose adjustment, suggesting the greatest genotype-associated risk of supratherapeutic INRs was present during the initiation of therapy. Numerous case reports have also described supratherapeutic INRs and adverse bleeding events in patients with variant *CYP2C9* genotypes, particularly the *CYP2C9**3/*3 genotype [40]. These findings have been confirmed by a recent meta-analysis which reported that individuals carrying at least one *CYP2C9* variant allele are at significantly higher risk of bleeding compared to *CYP2C9**1/*1 individuals (*2 or *3 carrier: relative risk 2.26, 95% confidence interval 1.36-3.75) [47].

Although *CYP2C9* genotype has shown to account for a significant proportion of inter-patient variability in (S)-warfarin clearance and warfarin dose requirement, numerous other patient factors also contribute to variability in warfarin sensitivity. For instance, Loebstein *et al.* demonstrated that both age and *CYP2C9* genotype are independent predictors of warfarin dose requirement, such that the mean dose requirements for *CYP2C9**1/*1, *1/*2 and *1/*3 individuals were 7.9 ± 3.7 , 5.5 ± 2.6 and 4.4 ± 2.0 mg/day for patients ≤ 65 years old, and 5.3 ± 2.2 , 4.8 ± 2.1 and 2.2 ± 1.2 mg/day for patients ≥ 66 years old, respectively [57]. Moreover, *CYP2C9* genotype has been estimated to independently account for only 20% of the observed inter-individual variability in warfarin dose requirement when also considering other clinical factors such as age, body weight, gender and concomitant medication use [58, 59]. Importantly, these regression models have not considered inter-individual variability in pharmacodynamic determinants of warfarin's antithrombotic effect. Although *CYP2C9* genotype is independently associated with warfarin dose requirement *via* contribution to its pharmacokinetics, the contribution of variation in genes related to warfarin pharmacodynamics appear to account for additional inter-individual variability in response.

THE COAGULATION CASCADE - PHARMACODYNAMIC DETERMINANTS OF WARFARIN RESPONSE

Warfarin exerts its antithrombotic effect *via* inhibition of the vitamin K epoxide reductase complex (VKOR) and interruption of the vitamin K redox cycle. VKOR catalyzes the reduction of vitamin K epoxide to vitamin K and then to the hydroquinone form which is subsequently used as a cofactor for γ -glutamyl carboxylase (GGCX) [60]. The latter reaction is postulated to be the rate limiting step [61, 62]. GGCX catalyzes the γ -carboxylation and activation of coagulation factors II (prothrombin), VII, IX, X, and proteins C and S. The by-product of this carboxylation reaction is vitamin K 2,3-epoxide which is then recycled by VKOR to start the cycle anew. Warfarin inhibits vitamin K-dependent clotting factor synthesis *via* interruption of this redox cycle. Therefore, polymorphisms in the genes encoding VKOR and GGCX have been hypothesized to significantly influence warfarin pharmacodynamics. Detailed reviews on warfarin's mechanism of action have been published [2, 60, 63].

Vitamin K Epoxide Reductase Complex Subunit 1 (VKORC1)

Originally, VKOR was thought to comprise a multicomponent lipid and enzyme complex anchored to the endoplasmic reticulum. However, a single gene located on chromosome 16 which encodes a 18-kDa transmembrane protein in the endoplasmic reticulum responsible for VKOR activity was recently identified using family linkage and rodent genetics studies, and subsequently named vitamin K epoxide reductase complex subunit 1 (*VKORC1*) [64-66]. Once these studies established an association between *VKORC1* and both rare coagulation deficiencies and warfarin resistance, gene sequencing studies were conducted to identify common polymorphisms potentially contributing to the inter-individual variation in warfarin-sensitivity observed in patients [67-69].

The various SNP designations that have been used in different publications when referring to the same *VKORC1* polymorphism are described in Table 1. In each study to date, an important 1173C>T SNP in intron 1 of *VKORC1* was identified and subsequently found to be in strong linkage disequilibrium (LD) with other reported *VKORC1* SNPs associated with warfarin dose requirements. The impact of these *VKORC1* SNPs and associated haplotypes on warfarin maintenance dose requirements, the populations under study and the respective contribution of *CYP2C9* polymorphisms are depicted across studies in Table 2. Taken together, it is apparent that genetic variation in *VKORC1* and *CYP2C9* independently account for a significant proportion of the observed inter-individual variability in warfarin maintenance dose requirement.

D'Andrea *et al.* [67] were the first to identify common *VKORC1* SNPs affecting warfarin pharmacodynamics. In this Italian Caucasian population of 147 patients on therapeutic and stable warfarin regimens, common noncoding polymorphisms were identified in intron 1 (1173C>T) and in the 3' untranslated (UTR) region (3730G>A) of *VKORC1* (Table 1). Warfarin dose requirements were highest for homozygous carriers of 1173C and 3730A alleles, which were also the most prevalent in this population at 36.8% and 15.0%, respectively. However, only the 1173C>T variant in *VKORC1* was independently associated with warfarin dose requirement, accounting for 13.8% of the observed inter-individual variability. After adjustment for covariates, mean dose requirements for the *VKORC1* 1173C>T genotypes were 6.2, 4.8 and 3.5 mg/day for the C/C, C/T and T/T genotypes, respectively. *In vitro* expression studies indicated that the 1173C>T polymorphism does not affect mRNA processing. Therefore, another unidentified *VKORC1* SNP in LD with the 1173C>T was hypothesized to be responsible for the higher dose requirements associated with the 1173C allele in this population [67]. In comparison, the *CYP2C9**2 and *3 variants accounted for 21.5% of the observed variability, suggesting genetic variation in both the pharmacodynamic and pharmacokinetic pathways contributed to the observed inter-individual variability in warfarin dose requirement.

Rieder *et al.* [68] conducted a more comprehensive study to identify additional polymorphisms and haplotypes (genotype clusters) that affect patient response to warfarin in a population of 186 European-American patients on long-term

anticoagulation therapy. The entire 11-kb genomic region for *VKORC1* was sequenced, and 28 noncoding and one non-synonymous (5432G>T encoding Ala41Ser) SNPs were identified. Interestingly, the single individual heterozygous for the Ala41Ser variant had the highest warfarin maintenance dose requirement in this population (15.5 mg/day). Ten of the noncoding SNPs had frequencies greater than 5% and five of the seven SNPs that were associated with warfarin dose, including 1173C>T, were found to be in significant LD ($r^2 \geq 0.9$). Stepwise regression analysis demonstrated that these five *VKORC1* SNPs accounted for approximately 25% of the observed inter-individual variability in warfarin dose requirement. In contrast, the *CYP2C9**2 and *3 variant alleles predicted 10% of the observed variance.

The ten common noncoding SNPs were then used to infer nine haplotypes and genealogic analysis revealed two distinct and highly divergent haplotype groups (haplotype groups A and B) that were associated with different warfarin dose requirements. Four informative SNPs were identified that could distinguish the two haplotype groups, and the *VKORC1* 1173T and 1173C alleles were present on haplotype A and B, respectively. *VKORC1* haplotype group combinations A/A, A/B and B/B were associated with significantly different warfarin dose requirements (2.7±0.2, 4.9±0.2 and 6.2±0.3 mg/day, respectively) irrespective of *CYP2C9* genotype. These data provide strong evidence supporting the notion that genetic variation in *VKORC1* accounts for a significant proportion of the inter-individual variability in the pharmacodynamic effects of warfarin. To validate and extend these findings, a separate population of 368 European-American subjects were also genotyped for the four informative *VKORC1* SNPs and the *CYP2C9**2 and *3 polymorphisms. *VKORC1* haplotype and *CYP2C9* genotype accounted for 21% and 6% of the variability in warfarin dose requirement, respectively, and demonstrated that genetic variation in both pathways influence warfarin sensitivity. However, these data suggest that genetic variation in *VKORC1* may be a more substantial contributor than *CYP2C9* polymorphisms. Importantly, *VKORC1* RNA expression was found to be 3-fold higher for the high dose haplotype B group compared to the low dose haplotype A group in liver biopsies obtained from 53 European-Americans. These data suggest a direct association between levels of *VKORC1* expression and warfarin dose requirement, and provide a mechanistic explanation for the influence of genetic variation in *VKORC1* on the

Table 1. Common *VKORC1* Polymorphisms and the Respective Designations by Study

Reference	D'Andrea <i>et al.</i> [67]	Wadelius <i>et al.</i> [70]	Rieder <i>et al.</i> [68]*	Yuan <i>et al.</i> [69]	Veenstra <i>et al.</i> [71]	Aquilante <i>et al.</i> [73]
Population	Italian Caucasians	Swedish Caucasians	European American	Han Chinese	Hong Kong Chinese	American Caucasians
Intron 1 SNP	1173 C>T	Rs9934438	6484 T>C	1173 C>T	6484 (1173) T>C	-
5'UTR SNP	-	Rs9923231	3673 G>A	-1639 G>A	3673 (-1639) A>G	3673 G>A
3'UTR SNP	3730 G>A	Rs7294	-	3730 G>A	-	-

*African-American, Asian-American, and European-American were studied as a secondary population.

Table 2. *VKORC1* Genotype/Haplotype and Warfarin Dose Requirements by Study

Population	Genotype / Haplotype	Frequency %	Mean Warfarin Maintenance Dose (mg / X) ^[a]	<i>VKORC1</i> % Variability Accounted For	<i>CYP2C9</i> % Variability Accounted For	Notes	Reference
Italian Caucasian	1173 C/C	36.8	7.0	14	22	–	D'Andrea <i>et al.</i> [67]
	1173 C/T	46.9	5.1				
	1173 T/T	16.3	3.7 ^[b]				
European American Caucasians	HAP A/A	A = 36 ^[c]	2.7 ^[d]	21	6	Haplotype A = 1173T Haplotype B = 1173C	Reider <i>et al.</i> [68]
	HAP A/B		4.9				
	HAP B/B	B = 64 ^[c]	6.2				
Swedish Caucasians	HAP 1/2	59 ^[c]	35-40 / Wk	30	12	Haplotype 1/2 = 1173C Haplotype 3/4 = 1173T	Wadelius <i>et al.</i> [70]
	HAP 3/4	41 ^[c]	24-26 / Wk				
Hong Kong Chinese	HAP 1/1	77	2.93	31	8	Same assignment used by Reider <i>et al.</i> ; Clade A-Haplotype 1 Clade B-Haplotype 2	Veenstra <i>et al.</i> [71]
	HAP 1/7	20	4.96 ^[e]				
	HAP 7/7	3	6.50, 6.75 ^[f]				
Han Chinese	-1639 A/A	80	2.61	NR	NR	-1639 G>A in LD with 1173C>T	Yuan <i>et al.</i> [69]
	-1639 A/G+G/G	20	3.81 ^[g]				
European American Caucasians	-1639 G/G	25	4.53 ^[h]	15	17.5	-1639 G>A in LD with 1173C>T	Sconce <i>et al.</i> [72]
	-1639 G/A	56	3.83				
	-1639 A/A	19	2.23				
Primarily Caucasian ^[i]	3673 G/G	42.7	45.6 / Wk ^[j]	23.3/28.8 ^[l]	10.2/3.8 ^[m]	3673G>A	Aquilante <i>et al.</i> [73]
	3673 G/A	45.2	32.9 / Wk				
	3673 A/A	12.1	23.1 / Wk ^[k]				

Notes: [a] per day unless otherwise noted; [b] $p < 0.001$ vs. hetero/homozygous; [c] haplotype copy frequency; [d] $p < 0.001$ among the three haplotype combinations; [e] $p < 0.001$ vs. HAP 1/1; [f] $N = 2$; [g] $p < 0.0001$ vs. AA; [h] $p < 0.05$ vs. hetero/homozygous; [i] 91.1% white; [j] $p < 0.0001$ vs. hetero/homozygous; [k] $p < 0.0001$ vs. heterozygous; [l] partial r^2 for hetero/homozygous; [m] partial r^2 for heterozygous and homozygous(non*1) NR = not reported; Wk = week.

pharmacodynamic effects of warfarin independent of variability in warfarin clearance. To explore the ethnic diversity of the *VKORC1* polymorphisms, the authors subsequently evaluated the *VKORC1* haplotype distribution in three secondary populations of Asian-American, European-American and African-American descent [68]. Haplotypes A and B accounted for 99%, 96% and 62% of the haplotypes in these populations, respectively. Interestingly, the low dose haplotype A group was more frequent among Asian-Americans (89%) compared to both European-American (37%) and African-Americans (14%), suggesting genetic variation in *VKORC1* may partly explain the population differences in warfarin dose requirement often observed clinically.

Wadelius *et al.* [70] also evaluated the association between *VKORC1* haplotypes and warfarin dose requirement in 201 Caucasian subjects. Two common haplotypes, which were tagged by the 1173T allele and the -1639A allele of a -1639G>A SNP in the 5'UTR (rs9923231, Table 1), were associated with warfarin dose requirements of 24-26 mg/week compared to 35-40 mg/week for the haplotypes tagged by the 1173C and -1639G alleles. Similar to the findings by Reider *et al.* [68], genetic variation in *VKORC1* accounted for a significantly greater proportion of the variability in warfarin dose requirement compared to the *CYP2C9**2 and

*3 alleles (27.0% vs. 10.5%, respectively). Veenstra *et al.* [71] evaluated the contribution of *VKORC1* polymorphisms to warfarin dose requirement in a population of 69 Hong Kong Chinese. Similarly, patients were genotyped and then haplotype pairs were identified based on inferred low dose (H1) and high dose (H7) haplotypes. These haplotypes were tagged by the -1639A/1173T and -1639G/1173C alleles, respectively. Individuals carrying the H1/H1 ($n = 53$) and H1/H7 ($n = 14$) haplotype pairs required average maintenance doses of 2.93 ± 1.22 and 4.96 ± 1.53 mg/day ($P < 0.001$), respectively, while the $n = 2$ individuals with a H7/H7 haplotype pair required 6.5 and 6.75 mg/day. *VKORC1* haplotype, presence of the *CYP2C9**3 allele and age independently accounted for 31%, 7.9% and 21.5% of the variability in warfarin dose requirement, respectively.

Yuan *et al.* [69] evaluated the association between genetic variation in *VKORC1* and warfarin sensitivity in a Han Chinese population. Patients were categorized as either warfarin sensitive ($n = 11$, ≤ 1.5 mg/day), normal ($n = 95$, 1.6-5.9 mg/day) or warfarin resistant ($n = 5$, ≥ 6 mg/day). All warfarin sensitive and no warfarin resistant subjects were found to be homozygous carriers of the -1639A allele, which was in nearly complete LD with the 1173C>T polymorphism (i.e., the -1639A and 1173T alleles are located on the same haplo-

type). Individuals with the -1639 A/A genotype had significantly lower warfarin dose requirements compared to -1639G allele carriers (2.61 ± 1.10 vs. 3.81 ± 1.24 mg/day, respectively, $P < 0.0001$). In a population genotype frequency analysis, the -1639 A/A genotype was prevalent in 82.1% of Chinese individuals compared to 14.2% of a healthy Caucasian population, consistent with the lower average dose requirements often required to achieve therapeutic anticoagulation in Asian populations.

Importantly, the -1639G allele was predicted to abolish a putative E-box site in the *VKORC1* promoter [69]. Moreover, the -1639G allele was associated with higher *VKORC1* promoter activity using transfected HepG2 cells and a firefly luciferase reporter assay compared to the -1639A allele using *VKORC1* promoter constructs from patients with the -1639 G/G and A/A genotypes, respectively. Thus, higher basal *VKORC1* expression may explain the mechanism underlying the need for higher warfarin maintenance doses in individuals with the -1639G allele. Interestingly, the -1639G and 1173C alleles were in nearly complete LD in this Han Chinese population. Moreover, in the study by Reider *et al.* [68], the -1639G and 1173C alleles were both present on haplotype B, which was associated with significantly higher hepatic *VKORC1* expression and warfarin maintenance dose requirement. Although the study by D'Andrea *et al.* [67] did not evaluate the -1639G>A polymorphism, they demonstrated that the 1173C allele was associated with significantly higher warfarin dose requirements and this variant was likely not functionally relevant. Sconce *et al.* [72] also observed a significant association between the -1639G>A polymorphism and warfarin dose requirement in 297 Caucasian patients on stable warfarin regimens, with -1639 G/A and A/A individuals requiring significantly lower doses (Table 2). Similar results were also recently observed by Aquilante *et al.* [73]. Moreover, the -1639A allele has been associated with enhanced sensitivity to acenocoumarol in 222 healthy French volunteers, as measured by the factor VII ratio and percent INR change 24 hours after administration of a single 4 mg acenocoumarol dose [74]. Collectively, these findings regarding *VKORC1* haplotype-mediated associations in *VKORC1* expression and warfarin dose requirement appear to be consistent across populations, suggesting the functional polymorphism driving the observed haplotype effects may be -1639G>A. However, additional mechanistic studies will be required to confirm this observation.

γ -Glutamylcarboxylase (GGCX)

Current evidence suggests that common γ -glutamylcarboxylase gene (*GGCX*) polymorphisms do not significantly influence warfarin dose requirements [70, 75-77]. However, this may not hold true for all patient populations. Among the six *GGCX* polymorphisms identified in a small cohort of 45 Japanese subjects only a CAA insertion repeat in intron 6 was associated with altered warfarin sensitivity [75]. In this study by Shikata *et al.* [75], higher mean daily warfarin dose requirements were associated with increasing numbers of CAA insertion repeats (10, 11 or 13 repeats were present at frequencies of 62%, 34% and 3%, respectively). Dose requirements were approximately 2-fold higher in patients with either CAA 10/13 or 11/13 genotypes ($n=3$) and mar-

ginally higher for those with either CAA 10/11 or 11/11 genotypes ($n=27$) compared to the wild-type 10/10 genotype ($n=15$); although, these results were associated with a large degree of variability (Table 3). After correcting for the influence of polymorphisms in the genes encoding CYP2C9, proteins C and S, and coagulation factors II, VII, IX and X, the CAA insertion repeat accounted for 9% of the variability in warfarin dose requirement.

Wadelius *et al.* [70] also evaluated the influence of fourteen *GGCX* SNPs in 201 Caucasian patients. Nine *GGCX* SNPs were located in a region of high LD, had minor allelic frequencies of >30% and were used to infer five haplotypes. No statistically significant differences in warfarin dose requirement were observed among the five *GGCX* haplotypes. Only an intron 2 (rs12714145) polymorphism was significantly ($P=0.036$) associated with higher dose requirement; however, this variant independently accounted for only 3.3% of the observed variability. Subsequently, the effect of the CAA insertion repeat polymorphism was evaluated in this population, and 10, 11, 13, 14, 15 and 16 CAA repeat polymorphisms were identified [76]. In contrast to aforementioned findings by Shikata *et al.* [75], no differences in warfarin dose requirement were observed with either CAA 10/13 or 11/13 genotypes. Collectively, the *GGCX* insertion polymorphisms accounted for only 3.5% of the observed variability in warfarin dose requirement. Finally, a nonsynonymous SNP (8762G>A, Arg325Gln) in exon 8 did not influence dose requirement in an Israeli population [77]. Additional studies to confirm the minimal contribution of *GGCX* polymorphisms to warfarin dose requirement may be warranted given the paucity of available data.

Microsomal Epoxide Hydrolase, Glutathione S-Transferases and the VKOR Complex

Early investigations on the VKOR complex concluded that two phase II drug metabolism enzymes, microsomal epoxide hydroxylase (mEH) and glutathione S-transferases (GSTs) may be part of the VKOR complex [78]. However, the discovery of *VKORC1* and recent data suggests mEH is not a component of the VKOR complex [79, 80]. While it is still unclear if *VKORC1* is the sole determinant of vitamin K reductase activity, clinical studies have investigated the potential influence of genetic polymorphisms in the genes encoding mEH (*EPHX1*) and GST (*GSTA1*) on warfarin dose requirements [77]. Loebstein *et al.* studied the impact of SNPs in *EPHX1* (612T>C and 691A>G), *GSTA1* (631T>G and 567T>G) and *CYP2C9* in an Israeli population of 100 subjects [77]. The *EPHX1* 612T>C variant was associated with higher warfarin dose requirements, particularly in the 62 patients with a *CYP2C9* wild type genotype (Table 3). Compared to wild-type (C/C) individuals, heterozygous (T/C) and homozygous (C/C) carriers were significantly more likely to require >5 mg/day (odds ratio 2.0, 95% confidence interval 1.1-3.6) and >7 mg/day (odds ratio 3.14, 95% confidence interval 1.47-6.67) of warfarin, respectively, after adjusting for *CYP2C9* genotype. These data suggest that the *EPHX1* 612T>C polymorphism may be associated with higher warfarin dose requirements. The mechanism underlying this potential association between mEH and warfarin dose requirement remains unknown. In addition, the relative

Table 3. Polymorphisms in Other Genes Potentially Influencing Warfarin Pharmacodynamics and Warfarin Dose Requirements by Study

Protein	Polymorphism	Genotype	N	Mean Warfarin Dose (mg/day)	% Variability Accounted For	Reference
GGCX	Insertion repeat	(CAA) 10/10	15	NR	9	Shikata <i>et al.</i> [75]
		(CAA) 10 or 11/11	27			
		(CAA) 10 or 11/13	3			
Factor II	SNP	494 C/C	8	3.3	1	Shikata <i>et al.</i> [75]
		494 C/T	18	3.9		
		494 T/T	19	3.8		
Factor VII	SNP	-402 G/G	14	3.5	35	Shikata <i>et al.</i> [75]
		-402 G/A	22	3.9		
		-402 A/A	9	3.8		
Factor VII	Insertion/Deletion	D/D	263	38.0	1.3	Aquilante <i>et al.</i> [73]
		D/I+I/I	87	34.4 ^[c]		
Factor X	Insertion/Deletion	D/D	70	36.5	≤2.0	Aquilante <i>et al.</i> [73]
		I/D	182	37.1		
		I/I	98	37.6		
MEH ^[a]	SNP	612 C/C	32	6.0	19.6	Loebstein <i>et al.</i> [77]
		612 T/C	26	6.5		
		612 T/T	4	7.5 ^[b]		

Notes: [a] Carriers of wild-type CYP2C9 only; [b] Statistically significant vs. WT and Heterozygotes; [c] p=0.04 vs D/D; NR = not reported.

contribution of the *EPHX1* 612T>C polymorphism and *VKORC1* genotype/haplotype to inter-individual variability in warfarin response has not been evaluated. However, the 42% prevalence of the 612C variant allele suggests that incorporation of *EPHX1* genotype into future warfarin dosing algorithms may be warranted since presence of this variant may predict the need to initiate warfarin at *higher* doses. However, additional studies are required to confirm these findings in an independent population.

Coagulation Factors

Shikata *et al.* [75] examined the influence of polymorphisms in the genes encoding the vitamin K-dependent proteins (factors II, VII, IX, X, and proteins C and S) on warfarin sensitivity and dose requirement in 45 Japanese patients. Numerous polymorphisms were identified throughout these six genes; however, no significant associations were observed with warfarin dose requirement. The authors also examined the ratio of the INR to warfarin plasma concentrations (INR/Cp), termed the warfarin sensitivity index, which assessed the impact of these polymorphisms on warfarin pharmacodynamics independent of pharmacokinetic variability. Using this approach, two variants in the gene encoding factor VII (-402G>A, 746T>C) and one factor II variant (494C>T) were associated with lower and higher warfarin sensitivity, respectively (Table 3). The -402G>A polymorphism has been associated with increased in factor VII transcription [81] and accounted for 35% of the inter-individual variability in the warfarin sensitivity index ratio [75]. Geno-

types were combined and used to categorize 27 of the 45 patients into 5 groups to investigate the composite effect of these polymorphisms. When dose requirements were compared between a low-sensitivity (mean±SD INR/Cp = 2.14±0.31) group homozygous for the factor VII variant (-402 A/A), and a high-sensitivity (INR/Cp = 4.48±0.83) group homozygous for the factor II variant (494 T/T), the mean daily warfarin doses were 3.6±1.4 and 2.3±1.4 mg/day, respectively. However this difference was not statistically significant due to sample size limitations (N=3-5 in each group).

Aquilante *et al.* [73] also evaluated the influence of factor II, VII and X polymorphisms on weekly warfarin dose requirement in 350 patients on a stable warfarin regimen (Table 3). Individuals with one or two variant alleles of the nonsynonymous factor II Thr165Met SNP in exon 6 demonstrated a tendency for higher weekly warfarin dose requirements compared to wild-type individuals (39.2±18 vs. 36.4±15 mg/week, respectively, P=0.09); although, these differences did not attain statistical significance. A 10-bp insertion polymorphism in the factor VII promoter at position -323 was also associated with warfarin dose requirement [73]. Individuals with one or two insertion alleles required significantly lower doses than those with two deletion alleles (34.4±13 vs. 38.0±17 mg/week, respectively, P=0.04); however, this polymorphism only accounted for 1.3% of the inter-individual variability in warfarin dose requirement when adjusting for other genetic and non-genetic factors. However, these factor II and VII polymorphisms were not associ-

ated with warfarin dose requirements in the study by Shikata *et al.* [75]. Collectively, genetic variation in coagulation proteins does not appear to substantially contribute to warfarin pharmacodynamics; although, certain polymorphisms in factor VII have been associated with modest differences in warfarin dose requirement and certain pharmacodynamic indices. Additional studies will be necessary to further characterize these potential associations in independent populations.

CAN PHARMACOGENOMICS HELP PREDICT WARFARIN DOSE REQUIREMENTS?

A thorough understanding of the genetic and non-genetic factors influencing warfarin pharmacokinetics and pharmacodynamics is critical to account for the substantial inter-individual variability in the warfarin dose-response relationship. As discussed above, the *CYP2C9**2 and *3 polymorphisms significantly influence warfarin pharmacokinetics *via* their effect on (S)-warfarin metabolic clearance, consequently lowering dose requirements and increasing the risk over-anticoagulation and bleeding events during the initiation phase of therapy [17, 47, 55]. Similarly, *VKORC1* polymorphisms significantly alter warfarin pharmacodynamics and maintenance dose requirements [67-71]. The *VKORC1* 1173C>T and -1639G>A polymorphisms are the most commonly reported and may be as informative as the more extensive haplotypes described [68]. The variant 1173T and -1639A alleles are associated with significantly lower maintenance dose requirements compared to the wild-type 1173C and -1639G alleles, respectively; however, their influence on adverse event risk during the initiation of therapy remains to be evaluated. Importantly, these variant *VKORC1* alleles appear to have a higher penetrance in the low dose requirement phenotype characteristic of Asian populations compared to *CYP2C9* polymorphisms which are far less frequent [68, 82]. For example, the intensity of anticoagulation needed to prevent thrombotic events without hemorrhagic complications in elderly Japanese subjects is significantly lower (INR 1.5-2.6) than for other ethnicities [83, 84]. The source of this increased sensitivity to warfarin is unclear, but may be linked to pharmacodynamic covariates such as *VKORC1* polymorphisms which are significantly more frequent in Asian populations [68, 82]. Therefore, an assessment of both *CYP2C9* and *VKORC1* polymorphisms may be necessary to adequately account for the variability in dose requirements across populations. Polymorphisms in other genes related to warfarin pharmacodynamics, such as *GGCX*, *EPHX1*, factor II and factor VII have also been associated with warfarin dose requirements in certain populations [70, 73, 75, 77]; however, their relative contribution appears to be minor. Importantly, the influence of various genetic and non-genetic factors on warfarin pharmacokinetics, pharmacodynamics and maintenance dose requirements must be more extensively evaluated in order to more completely understand the influence of these factors on the observed variability in warfarin response.

Takahashi *et al.* evaluated population differences in warfarin dose requirements by developing a multivariable model which incorporates various pharmacokinetic (*CYP2C9*), pharmacodynamic (*VKORC1*) and demographic (age, gender, body weight) covariates known to influence warfarin

sensitivity [82]. When stratified according to *VKORC1* 1173C>T genotype, both Japanese and Caucasian patients with the C/C, C/T and T/T genotypes had mean maintenance doses of approximately 7, 5 and 3 mg/day, respectively, when the analysis was limited to individuals wild-type for *CYP2C9*, even though Caucasians had higher dose requirements than Japanese patients when evaluated independent of genotype (5.5 vs. 3.5 mg/day, respectively, $P<0.01$). Moreover, their multivariable model which included *VKORC1* genotype, *CYP2C9* genotype, age and body weight as covariates accounted for 57% of the overall inter-individual variability in warfarin daily dose requirements. The warfarin doses predicted by this model for each individual significantly correlated with their administered maintenance doses ($r=0.76$, $P<0.001$). Sconce *et al.* demonstrated that a multivariable model including age, height, *CYP2C9* genotype and *VKORC1* genotype accounted for 54.2% of the variability in warfarin dose requirement in 297 Caucasian therapeutically managed anticoagulation patients [72]. The predicted doses using this model also significantly correlated with the administered maintenance dose ($r=0.80$, $P<0.001$). Aquilante *et al.* evaluated the influence of various genetic (*CYP2C9*, *VKORC1*, factor II, factor VII) and non-genetic (age, body weight, smoking, concomitant medications, vitamin K intake) factors on weekly warfarin dose requirement in patients on stable warfarin regimens [73]. Their model accounted for 51.4% of the overall inter-individual variability in dose requirement in this predominantly Caucasian population. Importantly, over half of the variability was independently accounted for by the *VKORC1* -1639G>A polymorphism. Collectively, *CYP2C9* polymorphisms have been shown to account for approximately 6%-22% of the inter-individual variability in warfarin dose requirements in various populations, with the greatest contribution in Caucasian populations due to higher variant allele frequencies. However, *VKORC1* polymorphisms have accounted for approximately 14%-31% of this variability and appear to be the primary genetic factor contributing to warfarin sensitivity identified to date, irrespective of the population (Table 2).

Importantly, these models have not routinely identified genomic factors associated with high warfarin dose requirements, which may reflect the potential influence of other unidentified genetic variants. Both the Ala41Ser and Val66Met substitutions in *VKORC1* have been associated with warfarin resistance; however, these variants are extremely infrequent [68, 85]. Interestingly, homozygous carriers of the APOE*E4 gene encoding apolipoprotein E, which is responsible for the uptake of lipid soluble vitamin K, require approximately 60% higher weekly warfarin doses [86]. Polymorphisms in *EPHX1* [77], factor VII [75] and factor II [73] have also been associated with higher dose requirements; although, these effects are fairly modest and require confirmation in independent populations. Prospective assessment of multivariable models incorporating these polymorphisms appear warranted in order to more comprehensively predict an individual patient's inclusion in the low, normal or high dose requirement groups.

Lastly, the influence of *CYP2C9* and *VKORC1* polymorphisms on the extent and nature of various clinically relevant drug-drug interactions with warfarin have not been rigor-

ously evaluated. Visser *et al.* demonstrated that patients carrying a variant *CYP2C9**2 or *3 allele were significantly more susceptible to overanticoagulation (INR ≥ 6.0) after concomitant administration of warfarin and nonsteroidal anti-inflammatory drugs (relative risk 3.78, 95% confidence interval 2.02-7.09) compared to the risk observed in *CYP2C9**1/*1 individuals (relative risk 1.69, 95% confidence interval 1.05-2.69) (*P* for interaction=0.01) [87]. However, the potential clinical utility of pharmacogenomic information to predict the presence and severity of pharmacokinetic and/or pharmacodynamic drug-drug interactions with warfarin requires further investigation.

PROSPECTIVE GENOTYPING FOR WARFARIN DOSE INITIATION – A PROMISE FULFILLED

The ability to utilize pharmacogenomics in the development of individualized warfarin dosing regimens offers the potential to significantly improve the safe and effective use of this commonly prescribed oral antithrombotic agent. Underlying patient-specific factors coupled with environmental influences result in substantial inter-individual variability in dose requirement which, coupled with its narrow therapeutic index, often leads to extended periods of sub- or supra-therapeutic dosing and necessitates routine INR monitoring. The associated risks of adverse thrombotic or bleeding events, respectively, are particularly important for patients who fail to adhere to complex chronic warfarin regimens [88, 89]. However, once a maintenance dose is identified and antithrombotic stability is attained, clinicians are more effectively able to maintain a therapeutic INR and minimize these risks in adherent patients. The initiation phase of therapy, when a patient's chronic maintenance dose requirement is unknown, remains the dosing period with the greatest degree of uncertainty and risk of adverse bleeding events [90]. Moreover, practitioner concerns regarding the safe and effective use of warfarin in certain patients are largely responsible for its underutilization and suboptimal dosing. Pharmacogenomic-guided dosing strategies offer the potential to account for and minimize this variability in order to better predict a patient's dose requirement and more rapidly attain a therapeutic and stable INR.

It has been estimated that if one-half of atrial fibrillation patients who are not receiving stroke prophylaxis were initiated on warfarin and an additional one-half of patients currently receiving warfarin were optimally treated, then approximately 29,000 embolic events could be prevented annually for a cost savings of \$2.4 billion [91]. The cost benefits derived by extending warfarin therapy to untreated patients and optimizing therapeutic dosing would be enormous if the proposed utility of prospective pharmacogenomic-guided dosing is confirmed in prospective trials. Ultimately, demonstration of improved health outcomes will be needed in order to justify the costs of such strategies. It is also likely that validation *via* prospective examination of numerous clinical, economic and societal factors will also be required before routine implementation into clinical practice can occur [92, 93]; however, preliminary data suggest that incorporation of genotyping into warfarin dosing nomograms is feasible and acceptable in the clinical setting [94]. Regulatory acceptance of this paradigm shift is reflected by the recent

recommendation of the FDA Clinical Pharmacology Subcommittee to support prospectively guided warfarin dosing based on *CYP2C9* and *VKORC1* genotype [95].

SUMMARY

Current evidence suggests that the primary genetic factors that influence warfarin pharmacokinetics (*CYP2C9*) and pharmacodynamics (*VKORC1*) have been identified. Thus, we now have the means and impetus to prospectively validate multivariable models that incorporate an individual's genomic and non-genomic characteristics in order to better predict warfarin dose requirements, particularly prior to the initiation of therapy. Prospective, randomized, controlled clinical trials will be required to determine if these individualized pharmacogenomic-guided dosing nomograms significantly improve clinical outcomes and are cost-effective compared to traditional nomograms which rely on more empirical approaches and close INR monitoring. We believe the time is now for the promise of individualized therapy to prove its worth through prospective evaluation, and patients requiring oral antithrombotic therapy appear to be optimal candidates.

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