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Effect of Genetic Variants, Especially *CYP2C9* and *VKORC1*, on the Pharmacology of Warfarin

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Abstract

The genes encoding the cytochrome P450 2C9 enzyme (*CYP2C9*) and vitamin K-epoxide reductase complex unit 1 (*VKORC1*) are major determinants of anticoagulant response to warfarin. Together with patient demographics and clinical information, they account for approximately one-half of the warfarin dose variance in individuals of European descent. Recent prospective and randomized controlled trial data support pharmacogenetic guidance with their use in warfarin dose initiation and titration. Benefits from pharmacogenetics-guided warfarin dosing have been

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reported to extend beyond the period of initial dosing, with supportive data indicating benefits to at least 3 months. The genetic effects of *VKORC1* and *CYP2C9* in African and Asian populations are concordant with those in individuals of European ancestry; however, frequency distribution of allelic variants can vary considerably between major populations. Future randomized controlled trials in multiethnic settings using population-specific dosing algorithms will allow us to further ascertain the generalizability and cost-effectiveness of pharmacogenetics-guided warfarin therapy. Additional genome-wide association studies may help us to improve and refine dosing algorithms and potentially identify novel biological pathways.

Keywords

Warfarin; pharmacogenetics; polymorphisms; personalized medicine

Warfarin remains one of the most effective anticoagulants indicated for the treatment and prophylaxis of a range of prothrombotic cardiovascular, cerebrovascular, and hematologic conditions.[1-3] Although the cost-effectiveness and long-term safety of the latest generation of oral anticoagulants (e.g., dabigatran, rivaroxaban, apixaban) have become a subject of intense debate recently, warfarin continues to be the mainstay of anticoagulation after over six decades in clinical use.[4,5] Tens of millions of patients use warfarin worldwide, and there are over two million warfarin users in the United States with over 30 million prescriptions dispensed in 2004 alone.[6,7] Due to the wide interindividual variability and narrow therapeutic index of warfarin,[8-10] there has been considerable interest in identifying genetic and nongenetic variables that affect warfarin dose requirements.[11-14]

Initially developed to accelerate the rate with which therapeutic levels of anticoagulation were achieved, fixed-dose algorithms have successively been superseded by more sophisticated clinical algorithms based on patient demographics and other clinical variables[15]; however, the success in ameliorating variability has been modest. Recently, pharmacogenetic testing of candidate genes important in warfarin pharmacodynamics (e.g., vitamin K-epoxide reductase complex unit 1 [*VKORC1*]) and pharmacokinetics (e.g., the cytochrome P450 2C9 enzyme [*CYP2C9*]) have yielded encouraging results in dose initiation and titration,[16-18] with potential for cost savings and reduction of length of hospitalization.[19] Several pharmacogenetics-guided dosing algorithms incorporate clinical variables and patient demographics and have compared favorably with clinical data-only algorithms.[17,19-22] These studies have collectively contributed to the construction of a prototypical framework for translating genetics and physiologic phenotypes into clinical practice, to our improved understanding of human diversity, and to the advancement of genetics-guided personalized medicine.

Developed from decades of international collaboration, international normalized ratio (INR) as a standardized measurement of prothrombin time has served as the cornerstone for warfarin-related genetic studies and enabled comparisons between laboratories and different types of studies. Understanding how INR is derived is crucial because it represents the phenotype with which genes and variants are correlated. Concerted international efforts have recently revealed the differential contribution of genetic variants and transracial difference

in warfarin dose requirements across ethnic groups and populations.[12,23,24] For instance, although *VKORC1* and *CYP2C9* together with clinical variables and patient demographic information have been estimated to explain 40 to 50% of warfarin variability in individuals of European descent, their effects are often markedly less in warfarin users of Asian, African, Latin American, and other ancestry.[14,21,23,25,26] These ethnic differences are of clinical importance with scientific and public health implications, given the increasing admixture and migration of populations worldwide with globalization.[27,28]

In this review, we discuss the definition of INR as a phenotypic correlate; the published evidence for the major genetic determinants, *VKORC1* and *CYP2C9*, in influencing warfarin dose requirements in Europeans, Africans, Asians, and other ethnic groups; the clinical science and utility of *VKORC1* and *CYP2C9*; and other genetic variants that affect the pharmacology of warfarin based on evidence from candidate gene and genome-wide association studies (GWAS).

International Normalized Ratio

Studies designed to investigate genetic determinants of warfarin dose requirements, pharmacogenetics-guided dosing of warfarin, and genome-wide quantitative trait mapping of dose variations have taken advantage of INR as a standardized measurement of the anticoagulant response to warfarin. INR is the ratio of the prothrombin time of a patient (test) sample to a normal reference value (the mean of normal values, or Mean Normal PT, MNPT) raised to the power of the international sensitivity index (ISI): $(PT_{test}/PT_{reference})^{ISI}$. Prothrombin time is assayed by applying tissue factor-containing thromboplastin to plasma with the addition of excessive calcium to reverse the chelating effects of citrate in the sample; the time for a sample to clot is the prothrombin time. The ISI value is supplied by the manufacturer of an analytical system, and adjusts for reagent batch variability against a standardized panel of control reagents endorsed by the World Health Organization.[29] Periodic calibration of analytical instruments and the use of standardized assaying reagents are essential for reliable INR testing. Since its introduction in the 1970s, the normalization process has undergone several revisions.[23,30]

INR measures the extent of anticoagulation induced by warfarin. As a vitamin K antagonist, warfarin inhibits the production of functional forms of specific vitamin K-dependent clotting factors, including factors VII, IX, X, and II/prothrombin. Factor VII in the coagulation cascade is also the chief clotting factor activated by tissue factor in thromboplastin that triggers subsequent activation of factor IX, factor X, and prothrombin. Although INR is in general relatively robust, it represents an artificial surrogate measurement of warfarin anticoagulation reversible by exogenous tissue factor in vitro.

Warfarin Dosing in the Absence of Genetic Information

Following the development of standardized monitoring of anticoagulation with warfarin, it became apparent that fixed-dose loading regimens (e.g., 10 mg on each of the first three consecutive days followed by subsequent dose titration) were unreliable, often resulting in over-anticoagulation in up to 35% of patients and prolonged hospitalization in those under-anticoagulated. [31] Continual efforts led to the further refinement of algorithms and tailored

dosing schedules with later incorporation of patient demographics (e.g., age, weight) and clinical data (e.g., albumin levels) adaptable for use in inpatient or outpatient settings.[15,31-35] Adjusted-dose warfarin therapy was found to be superior to fixed-dose warfarin (plus aspirin) therapy, particularly evident in prospective, randomized clinical trials for the prevention of stroke in patients with atrial fibrillation.[1,36] Despite these advances, a considerable degree of uncharacterized variability still existed, and studies aimed at delineating these variables ensued.[13,37]

Of variables that affect warfarin dose variance, age accounts for 7% and its effects have been found consistent in different studies and validated across ethnic groups.[13,38-41] Flexible dosing protocols have been developed that adjust for age.[34] Nongenetic, complex environmental variables including drug and food interactions, serum vitamin K levels, and clinical factors (e.g., renal and hepatic function, presence of malignancy), among others, have been correlated in some studies.[37,42,43] Depending on the study population, clinical factors may explain up to 20% of warfarin dose requirements.[44] However, the common, large interindividual dose requirements (e.g., in one study ranging from 7 to 280 mg per week[45]) is not explained cumulatively by the majority of these uncommon though plausible factors with modest effect size. For instance, amiodarone can reduce warfarin dose requirement by approximately 18%[38] but is used by < 10% of warfarin users, and in a principal component analysis of 245 patients, of whom 21 took amiodarone, it was not found to be a statistically significant determinant.[13] Of 94 predictor variables analyzed in one study, age and pharmacogenetic variables were found to be the strongest determinants of warfarin dose requirements.[13]

Molecular Genetics, Polymorphisms, and Functions of *CYP2C9* and *VKORC1*

CYP2C9

Warfarin is a coumarin derivative that exists as a racemic mixture of *S*- and *R*-enantiomers. The former has about three times the potency of the latter, and the cytochrome P450 enzyme, CYP2C9, is responsible for metabolism of the *S*-enantiomer. [6,46,47] CYP2C9 is considered one of the most important P450 enzymes in the liver responsible for metabolizing xenobiotics and a host of clinically important drugs, including anti-inflammatory agents, oral hypoglycemic, oral anticoagulants, and diuretics.[48]

CYP2C9 is located on chromosome 10q24 spanning approximately 55 kb and contains nine exons.[49] Following reports that multiple cDNA sequences of *CYP2C9* were cloned to suggest a high level of polymorphism in the gene,[49] the search for allelic variants was spurred on. The convention of the nomenclature for *CYP2C9* is such that *(*number*) denotes the variant allele with reference to *CYP2C9*1*, the wild-type or major allele with the amino acid sequences (Arg144/Tyr356/Ile359/Gly417) first identified in Northern Europeans.[6,12] In this population, the allele frequency of *CYP2C9*2* (R144C; rs1799853) and *CYP2C9*3* was 12.5 and 8.5%, respectively.[50] Functional significance of the allelic variants *CYP2C9*2* in exon 3 and *CYP2C9*3* (I359L; rs1057910) in exon 7 was demonstrated in expression assays in vitro, providing evidence that both reduced-function variants resulted in impaired

metabolism of *S*-warfarin.[51,52] Respectively, enzymatic activity was reduced by approximately 30 and 80%,[17] and warfarin dose requirement was reduced by 14 to 20% and 21 to 49%.[12,53,54] Modeling data from 137 patients suggested that through effects on rates of drug clearance *CYP2C9*2* and *CYP2C9*3* predicted 58% of warfarin dose variation based on the measured plasma *S*-warfarin concentration after initiation of warfarin therapy.[55] Although both variants were also found to correlate with an increased risk of bleeding complications,[17,53,56-60] neither stability of INR nor likelihood of severe over-anticoagulation was associated.[54]

*CYP2C9*4* was first reported in the Japanese population and associated also with reduced warfarin dose requirements.[61] Studies on individuals of African descent revealed a host of other *CYP2C9* variants not (commonly) found in individuals of European descent, including *CYP2C9*5* (rs28371686), *CYP2C9*6* (rs9332131), *CYP2C9*8* (rs7900194), and *CYP2C9*11* (rs28371685), among others, that impact on warfarin dose requirements.[25,62-64]*CYP2C9*14* through *CYP2C9*19* were discovered in a cohort of Southeast Asians from Singapore.[65] There are at least 35 alleles of *CYP2C9* documented[6]; however, the effects of the majority of these alleles on warfarin metabolism and dose requirements are yet to be characterized at present.

VKORC1

Vitamin K is an essential lipid-soluble micronutrient required for maintaining the equilibrium of hemostasis and is available to humans from the gut microflora and dietary intake of certain plants.[66] As such, dietary variation, metabolic factors, and exposure to certain antibiotics and environmental factors can impact on warfarin anticoagulation.[67] Factors VII, IX, X, and prothrombin are vitamin K–dependent clotting factors that play central roles in the coagulation cascade as discussed earlier. Proper functioning of these factors requires posttranslational modification of glutamate side chains to γ-carboxyglutamate catalyzed by γ-glutamyl carboxylase (gene encoded by *GGCX*).[66] Reduced coagulant activity results when these proteins are partially γ-carboxylated or decarboxylated. [68,69] The process of γ-carboxylation is dependent on vitamin K, a cofactor that interchanges from the reduced (active) to the epoxide (inactive) state while glutamate is converted to γ-carboxyglutamate. Recycling of the epoxide (vitamin K-2,3–epoxide) to the reduced (vitamin K hydroquinone) form is catalyzed by vitamin K–epoxide reductase (VKOR) and inhibitable by warfarin (or other coumarin derivatives such as phenprocoumon).[70] The VKOR complex unit 1 (VKORC1) is an 18-kDa protein located in the endoplasmic reticulum[70] and is expressed abundantly in hepatocytes.[71]

Through positional cloning[72] and a siRNA gene knockdown approach,[73]*VKORC1* was localized to chromosome 16p11.2. *VKORC1* consists of 5,125 base pairs and comprises three exons.[70] Loss-of-function mutations in *VKORC1* can result in bleeding tendency, whereas warfarin resistance can also stem from mutations in *VKORC1*.[66,70,72,74] In exploring the functional significance of polymorphisms, studies have found expression of VKORC1 mRNA in several cell types and tissues, ranging from liver, myocardium, B lymphocytes to lung cancer cell lines.[21,70,71,73] Imbalance in allelic expression of VKORC1 (allelic mRNA:DNA) attributable to a regulatory polymorphism (3730A > G, rs9284) at the

3′-UTR of VKORC1 was demonstrated in human liver specimens.[71] Moreover, both the common, noncoding *VKORC1* single nucleotide polymorphisms (SNPs), – 1639G > A and 1173C > T, exhibited dose-dependent allele-specific effects on VKORC1 mRNA levels,[71] and *VKORC1* genotype is predictive of plasma *S*-warfarin concentration required to yield therapeutic INR[55] as well as the maintenance dose before initiation of therapy.[75] These findings extended the graded gene/haplotype-dose effect observed in the DNA transcript analysis of haplotype groups A (associated with low dose of warfarin) and B (associated with high dose of warfarin) incorporating the earlier SNPs that Rieder et al constructed in their study.[21] The – 1639G > A and 1173C > T polymorphisms are often tested interchangeably in pharmacogenetic studies due to their complete linkage disequilibrium (LD) with one another.[6,76] Several SNPs associated with warfarin resistance including the amino acid–changing (nonsynonymous) 106G > T (Arg36Tyr) variant[77] and others have been reported.[74,78,79]

CYP2C9 and *VKORC1* in Dose Initiation and Titration

The pharmacogenetic utility of *CYP2C9* and *VKORC1* during warfarin therapy has been examined in different clinical settings and populations over the last decade. Studies have established that *CYP2C9* and *VKORC1* genotypes conjointly influence warfarin dose requirements with impact on clinically significant endpoints and adverse events such as bleeding complications.[17,21,22,56,58,59,76,80-85] Several metrics have been used to measure the strength, accuracy, and safety of pharmacogenetic guidance including time to therapeutic INR, time to stable INR, percentage of time in the therapeutic range (%TTR), and percentage of out-of-range (%OOR) INR in comparison with fixed-dose (empirical) regimens.[16-18] However, a uniform standard of comparison between studies is lacking.

In a review published in 2009, Moyer et al summarizes well the literature on *CYP2C9* and *VKORC1* alleles and haplotypes, their prevalence across the three major races (individuals of European, Asian, and African descent), the mean dose requirements stratified by race and allele/haplotype, and the combined effects of *CYP2C9* and *VKORC1* on mean dose requirements as a guidance for dose prescription.[46] Since that time, tens of studies on subjects of different ancestry correlating with *CYP2C9* and *VKORC1* alleles have been published. The next section of this review article extends that summary and continues the discussion on multiethnic differences in allele distribution and warfarin dose requirements (see below).

To date, over a dozen pharmacogenetics-guided warfarin dosing algorithms incorporating clinical and demographic information have been published, and a host of others specify various modifications depending on the study objectives and populations examined.[42,86,87] Notable examples include the International Warfarin Pharmacogenetics Consortium (IWPC),[20] Warfarin Genetic study in Sweden (WARG),[26] CoumaGen,[16] http://www.warfarindosing.org,[42] Warfarin Regimen using A Pharmacogenetics-guided Initiation Dosing (WRAPID),[18] and Newcastle[22] algorithms. A pharmacogenetics-guided dosing algorithm consists of a regression equation for calculating the warfarin initiation dose, an institutional protocol for INR-guided dose adjustment during the maintenance phase, and a timeline for specifying the dose initiation, maintenance, and follow-up intervals. The

regression equation typically includes the following component variables: (1) patient demographics (e.g., age, height, weight), (2) clinical information (e.g., interacting medications, comorbidities), and (3) genetic information. These variable are factored into the calculation of the warfarin dose.[87]

The majority of warfarin dose prediction, algorithm development, modeling, and comparison studies are retrospective.[6,46,86,88-96] Putting algorithms and dose prediction modeling into practice, prospective studies are still scarce, but the numbers are increasing.[16-18,26,97-102] In the recently published prospective WRAPID study, 167 patients were initiated on warfarin for atrial fibrillation or venous thromboembolism using a newly developed pharmacogenetics algorithm incorporating patient demographics and clinical and genetic information (*VKORC1*, *CYP2C9*2* and **3*) for initial dose loading and maintenance dosing.[18] Although the study lacked a control group, it demonstrated the clinical utility and safety of pharmacogenetics guidance in determining loading and maintenance doses by the lack of genotype-specific differences in time to first therapeutic INR and risk of over-anticoagulation (INR > 4) even after adjusting for covariates.

The first prospective, randomized, controlled trial (CoumaGen) by Anderson et al compared standard empirical dosing versus pharmacogenetic guidance in 206 patients and concluded that pharmacogenetic-guided warfarin dose initiation more accurately and efficiently approximated stable doses.[16] A statistical significance in the primary end point of %OOR INR was not observed between the two arms (possibly due to insufficient power), but significance observed in exploratory analyses comparing wild-type and multiple variant carriers prompted the launch of the CoumaGen-II study.[17] The CoumaGen-II study comprised comparisons of primary end points at 30 days and up to 3months of %OOR INRs and %TTR between two groups in two arms. The first arm (*n*= 504 patients) compared a modified IWPC pharmacogenetics algorithm (PG-1) against one in which use of *CYP2C9* genotype information was deferred until after day 2 (based on the premise of the pharmacokinetics of warfarin) plus the use of a dose-revision algorithm (PG-2) with the goal of maximizing approximation of the initiation dose to the stable maintenance dose. The second arm (*n*= 2,343 patients) compared PG-1/PG-2 against the standard parallel controls consisting of patients being initiated on an empirical dose of warfarin (usually, 5 mg/d). Following initiation (from day 8 onwards), the institutional chronic anticoagulation clinic's warfarin maintenance dosing protocol was applied to both arms. The sufficiently powered study found no statistical difference in end points between PG-1 and PG-2 groups to suggest superiority of either algorithm, and thus, both were combined as a PG cohort in the second arm comparison against the standard dosing controls. The latter comparison saw highly significant differences in the prespecified primary end points (%OOR INRs and %TTR), as well as some secondary end points including average percent INRs ≥ 4 or ≤ 1.5, and average percent INRs ≤ 1.5.[17] As a metric of anticoagulation clinic quality (US national average of %TTR is approximately 50 to 60%), patient's compliance, longitudinal dosing stability, and, more importantly, a measure of therapeutic benefits (e.g., protection from stroke in patients with atrial fibrillation) derived from warfarin anticoagulation,[103] the %TTR conferring a > 10% (TTR from 58.4 to 68.9% at 30 days, and from 58.6 to 71.2%) improvement with pharmacogenetic guidance is remarkable. Clinical effectiveness of pharmacogenetic

guidance in dose initiation and titration has also previously been demonstrated in the prospective, nonrandomized Medco-Mayo study.[19] Intriguingly, there is a growing consensus that the lasting benefits of pharmacogenetic guidance extend beyond 1 week of warfarin therapy.[17,104] Other relatively small (sample size of around or fewer than 200 patients) prospective studies with or without randomization have reported mixed or negative results, possibly related to the study design, power, differences in patient populations, or other factors.[105,106] Several large, randomized clinical trials (e.g., COAG, GIFT, EU-PACT) approaching or exceeding 1,000 patients are ongoing[107] that should help to address questions not answered by available studies.

Interethnic Variability and Distribution of *CYP2C9, VKORC1* and Other Variants

Race and ethnicity are recognized determinants of warfarin dose requirements.[108-110] African ancestry generally confers the highest adjusted mean weekly warfarin dose (43 mg, range: 39 to 47 mg) compared with their European (36 mg, 34 to 39 mg), Latin American (31 mg, 25 to 37 mg), and Asian (24 mg, 21 to 27 mg) counterparts.[38] The African population is also the most genetically diverse and heterogeneous.[111]

Dose variability attributable to genetics is less well captured in non-Europeans, especially Asians and Africans.[24] Featuring *VKORC1, CYP2C9*2* and *CYP2C9*3* variants, and clinical information, the IWPC algorithm that was developed from an international panel of approximately 5,000 patients consistently performed well in individuals of European descent demonstrating approximately 50 to 65% or higher of dose variability with accuracy in the range of 10 to 20% of maintenance dose.[20,24,112] However, only about 30 to 40% (range: 8 to 46%) dose variance is explained in non-European populations depending on the study,[92,112-114] and suggests the inadequacy of *VKORC1*, *CYP2C9*2*, and *CYP2C9*3*, or missing pharmacogenetic information for non-Europeans.

Warfarin Users of African Descent

An analysis of *VKORC1* distribution has shown a haplotype A[21] frequency of 10.6% in African American (*n*= 273) and 35% in European-Americans (*n*= 302).[115] In general, *VKORC1* and *CYP2C9* variants account for upward of 30 to 40% of dose variance, even with incorporation of clinical information in individuals of African descent,[112,114-116] and depending on the number of *CYP2C9* variants included in the analysis.[25] In an analysis using the IWPC dosing algorithm that derived a 29% dose variance in a study population of African Americans,[112] addition of the *CYP2C9* variants *CYP2C9*6* (rs90449157), *CYP2C9*8* (rs90442184), and *CYP2C9*11* (rs90481096) (all rare in Europeans) improved the variance explained to 41%.[112] In a study of warfarin clinic subjects with discrepant therapeutic and algorithm-predicted doses, Scott et al demonstrated in one African American patient on a lower-than-expected dose (14.4 mg/wk) the failure of 15 published algorithms to accurately predict the required dose (a mean recommended dose of 41.8 mg/wk, range: 24.9 to 52.2 mg/wk).[25] The study pointed out the potential utility of *CYP2C9*8* (rs7900194; allele frequency of 0.047) in African American and recommended its inclusion in conjunction with *CYP2C9*2, CYP2C9*3, CYP2C9*5, CYP2C9*6*, and *CYP2C9*11*

(combined allele frequency of 0.133) for improved dose predictability.[25] Further support in using *CYP2C9*8* for warfarin dose prediction in Africans has come from a study on 213 South African blacks,[117] and another on 226 African American that illustrated improvement of the percent dose variance explained to approximately 30 to 36% with clinical variables and *VKORC1*– 1639G >A.[62]

Heterogeneity of the African race is evident in a study on 993 Africans that examined 14 SNPs in seven genes previously reported to influence warfarin dosage.[118] Comparing the different native African populations with migrant individuals of Asian and European ancestry in South Africa, *CYP2C9*2* was found to be monomorphic and *VKORC1* SNPs had low variant allele frequencies (0.03 to 0.04) in the Africans, indicating limited utility of those variants in South African blacks. Nonetheless, differences in allele frequencies of the panel of SNPs tested have enabled the differentiation of ancestral lineage among the African subpopulations, including native South Africans, Massai Kenyans, Luhya Kenyans, and Yoruba Nigerians; close affinity was unexpectedly observed between the latter two populations.[118] In a study of individuals with warfarin resistance, the *VKORC1* Asp36Tyr variant initially identified in Jewish individuals of Ethiopian (allele frequency of 15%) and Ashkenazi (4%) origins was reportedly common in 154 Ethiopian blacks (also with an allele frequency of 15%).[119] The great level of genetic heterogeneity, extensive population substructure, and less LD in the African population compared with other races[111] explains the difficulties in analyzing individuals of African descent, and demands close attention to their characterization.

Warfarin Users of Asian Descent

East Asians typically require on average about 3.0 to 4.0 mg/d, or about 21 to 28 mg/wk, of warfarin to achieve therapeutic INR.[38,120-122] A *VKORC1* haplotype analysis comparing five East Asian populations (Han Chinese from Taiwan and China, individuals from Indonesia, the Philippines, Thailand, and Vietnam) with a South Asian population (Indians residing in Taiwan) totaling 553 patients reported relatively close similarities in variant frequencies among East Asians, but significant differences were observed when compared with Indians.[123] Allele frequencies of the wild-type *CYP2C9*1* and the *VKORC1* 1173C > T variant found commonly in East Asians (allele frequencies of > 0.90 and 0.8 to 0.9, respectively) are similar among the Chinese,[23] Japanese,[120] and Koreans.[124] At the subpopulation level, the Bai, Tibetan, and Han Chinese ethnic groups also displayed similar *VKORC1* 3673G > A allele frequencies of 92.8, 90.2, and 90.8%, respectively, for the A allele.[125] An allele-specific, graded dose effect in accordance with the *CYP2C9* and *VKORC1* genotypes is seen in Asians as for the well-studied European populations.[23,126] Whereas the African populations have in general a repertoire of reduced-function *CYP2C9* variants (e.g., *CYP2C9*5, CYP2C9*6, CYP2C9*8, CYP2C9*11*) that influence warfarin dose response,[25,112,117] the relatively less heterogeneous East Asian populations have allele frequencies of > 90 to 95% at the wild-type *CYP2C*1*, and frequencies of 80 to 90% at the reduced-function *VKORC1* variants (e.g., 1173TT, – 1639AA) that could explain their overall lower warfarin dose requirements compared with their African and European counterparts. [110,124] By far the single factor that explains the greatest proportion of dose variation is *VKORC1*, estimated at 20 to 30% from genotyping studies and GWAS,[14] and is,

hence, a main source for the reduced dose requirement in East Asians. Collectively, patient, clinical, and genetic information account for 40 to 50% (mean: 48%, range: 33.2 to 76.8%, from 17 East Asian studies of dose variation explained.[86,102,120,124,126-137]

Multiethnic studies on Southeast Asians have shown greater similarity between Malaysians, Indonesians, Thais, and Chinese than with Indians.[27,123,138-140] For instance, *CYP2C9*2* is consistently absent in these Southeast Asian populations but present in up to 5.9% of Indians.[141,142] The dose variance explained by *CYP2C9* and *VKORC1* along with patient demographics and clinical data varied considerably from 15.4% in an Indonesian study,[143] 36.5% in a Malaysian study,[144] to 61% including *CYP4F2* in a multiethnic Singaporean study.[27]

Warfarin Users in Latin America, Other Regions, and Heterogeneous Populations

Apart from indigenous individuals native to Central and South America (Native Americans) in whom a unique frequency distribution of *VKORC1* SNPs is observed,[145] immigrant populations such as Brazilians and Argentinians of European descent have allele frequencies and profiles that follow their native European counterparts.[146,147] The direction of gene effects from allelic variants is congruent between the major populations and Hispanic Americans, Colombians, and Puerto Ricans.[148-151] These effects were also found consistent in the Turkish,[152,153] Lebanese,[154,155] Omani,[156] Iranian,[156] Egyptian,[157,158] and Jewish[97,159] populations. Several of these populations exhibit allele frequencies intermediate between the major races (Africans, Asians, and Europeans), and appear in line with the human migration patterns and the bottleneck of the founding of non-African populations some 50,000 to 100,000 years ago as evidenced by lower genetic diversity, a considerably higher level of LD, and more similar patterns of LD.[111]

Genomic Basis for *CYP2C9* and *VKORC1* as Determinants of Dose Variance and the Remaining Genetic Determinants

Over the past 5 years, advances in high-throughput technologies have made feasible the agnostic interrogation of SNPs genome-wide. Currently, two GWAS on warfarin dosage have been published with individuals of European ancestry.[11,14] Cooper et al[11] genotyped approximately 500K SNPs in 181 subjects as a discovery phase and then replicated the best signals with a p value of less than 10^{-4} in another 374 samples (Table 1). In the discovery phase, only SNPs in *VKORC1* reached the study's level of genome-wide significance ($p< 10^{-7}$), whereas polymorphisms in *CYP2C9* were genome-wide significant post-replication. The next best signal was rs2286461 near *FGFBP2* on chromosome 4, which had a p value of 6.6×10^{-7} in the discovery set. However, the joint discovery and replication analysis yielded a p value of 1.8×10^{-5}, suggesting that the effect in the discovery and replication sets were in opposite directions. The same was apparent for almost all the other regions that were selected for replication. One exception was rs216013 in the intron of *CACNA1C* on chromosome 12, which reached a p value of 8.6×10^{-7} in the joint analysis, with a p value of 9.2×10^{-5} in the discovery set. *VKORC1* and *CYP2C9* explained 25 and 9% of the warfarin dose variance, respectively, whereas inclusion of nongenetic information (age, gender, treatment with amiodarone, treatment with losartan, and weight) increased the

variance explained to 47%. This study[11] was underpowered to identify variants with small contribution to warfarin dose variance; for example, it had 80% power to detect a SNP association explaining 20% of the variance in warfarin dosing, which is almost the effect of *VKORC1*.

VKORC1 and *CYP2C9* were also statistically significant in another GWAS of Swedish individuals.[14] Takeuchi et al[14] applied a two-stage approach with 1,053 subjects in the discovery set and 588 in the replication set (Table 1). The study had 80% power to identify a genetic variant explaining at least 1.5% of the warfarin dose variance. The minor alleles of both *VKORC1* and *CYP2C9* reported SNPs had protective effects on warfarin dose (i.e., carriers of the minor alleles required less warfarin), and the variance explained was 28.3% and 7.5%, respectively. In the primary analysis, no other region reached the *a priori* defined level of genome-wide significance of 10^{-7}. In a secondary analysis, the authors fitted a multivariate regression adjusting for the effects of *VKORC1, CYP2C9*, age, and sex, identifying rs2108622 in exon 2 of *CYP4F2* on chromosome 19 at genome-wide level. The carriers of the minor allele had an increase of warfarin dose and thus an opposite effect of *VKORC1* and *CYP2C9* and explained around 1.5% of overall variance in the discovery set. The authors also investigated 2,530 copy number variants (CNVs); however, none of these were found to be associated with the warfarin dose.[14] Corroborating those findings, a focused genotyping study of CNVs in *CYP2C9*, *VKORC1*, *CYP4F2*, *GGCX*, and *CALU* from 178 multiethnic patient samples (Americans of European, African, Latino, and Asian descent), in addition to 350 additional samples testing for *CYP2C9* exon 8, found that all participants had two copies of the gene.[45]

Findings from GWAS on warfarin dose in Japanese individuals bore remarkable resemblance to those of their (European) counterparts.[160] Cha et al split their discovery set into subjects with high and low warfarin dose and treated the phenotype as binary. *VKORC1*, *CYP2C9*, and *CYP4F2* affected warfarin dose in the same direction as in the European samples (Table 1). The effects of these 3 loci reportedly explained approximately 43% of the phenotypic variance in a replication set.

Interestingly, a GWAS of acenocoumarol maintenance dose in Europeans identified culprits in the same three regions. The strongest signal was in rs10871454 in *STX4A* gene, which is in complete linkage disequilibrium (r^2= 1 and D′= 1) with rs9923231 in *VKORC1* (Table 1). All three effects had the same direction as per the warfarin GWAS and could explain similar portions of the variance of acenocoumarol maintenance dose, highlighting the importance of these loci in anticoagulation therapy using a dicoumarol/coumarin derivative. Furthermore, this study found rs1998591 in *CYP2C18* to have a genome-wide effect ($p< 5 \times 10^{-8}$), while accounting for ~ 1% of the acenocoumarol dose variance. Although published GWAS on warfarin dosage report no information on *CYP2C18*, polymorphisms in the same gene have been suggested to alter warfarin-related phenotypes in candidate gene studies.[130,161]

GWAS of warfarin dose have been relatively successful so far. Besides the strong effects in *VKORC1* and *CYP2C9*, there is evidence that more genes could play a role, although with small effects. *CYP4F2* is a notable example; because of its small contribution to variance explained (~ 1%), the underpowered GWAS by Cooper et al could not have identified it.

Evidence from GWAS in other traits suggests that larger discovery sets and meta-analysis of GWAS can increase power and help identify variants with (very) small effects.[162] Furthermore, none of the earlier GWAS has accounted for the effect of population stratification (differences in allelic frequencies between subpopulations) or reported the genomic inflation factor (λ).[163] Typically, population stratification can cause spurious associations; however, failure to account for population substructure can also cause loss of power to detect true effects.[164]

Conclusions and Implications

VKORC1 and *CYP2C9* in conjunction with patient demographics and clinical information explain about half of the dose variance in patients taking warfarin, particularly in individuals of European descent. Current evidence and existing pharmacogenetic and genomic data indicate that another one-half of dose variance is yet to be accounted for by genetic and nongenetic factors. Due to limitations of the previously performed GWAS, it remains unclear whether additional common, large-effect size genes similar to *VKORC1* and *CYP2C9* exist.

In the era of globalization, entry into health care systems is increasingly made by multiethnic and admixed populations, as evident in the patient population served by clinics and hospitals in major urban areas such as New York City,[64] Toronto,[28] Singapore,[123] and others. The development and advances in warfarin pharmacogenetic testing is already serving as a prototypical framework for future developments to follow. The priority will stand with how best to deliver health care and medicines safely and effectively to diverse, multiethnic populations in an individualized manner, and pharmacogenetics will likely be a major contributor.

Globalization and the Information Age have accelerated access to technology, and delivered direct-to-consumer personalized genomic testing.[165] Genetic/genomic data are already integrated with electronic medical record (EMR) system featuring capacity for clinical use.[112] Using warfarin pharmacogenetic testing as an example, one could imagine the one-off test results becoming available within 1 hour,[17,166] ready for use at the time of warfarin initiation based on a personalized dosing algorithm, and then the pharmacogenetic data being stored in an EMR until future use when the latest variables (e.g., advancing age, new current medications, comorbidities) are updated and the warfarin dose calculated and dispensed automatically at no additional cost. In a cost-effectiveness analysis in 2009, it was suggested that if warfarin pharmacogenetics guidance could reduce the OOR INR by 5 to 9%, then testing would be beneficial.[167] In the latest prospective, randomized, controlled trial, pharmacogenetic guidance was found to increase the OOR INR by 12%.[17]

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Table 1

Summary of GWAS on warfarin or related phenotype/substance

GWAS	Phenotype	Ancestry/population	Discovery sample size (*n*)	Replication sample size (*n*)	Number of variants analyzed	Replicated loci[a]
Cooper et al[11]	Daily maintenance warfarin dose (log-transformed)	European-Americans	181	374	538,629 (approx. 3M imputed)	rs9923231 (*VKORC1*); 6.2×10^{-13} / 4.7×10^{-34} rs4086116 (*CYP2C9*); 8.3×10^{-5} / 6.2×10^{-12}
Takeuchi et al[11,14]	Square root of mean warfarin dose	European (Swedish)	1,053	588	325,997 (approx. 2.2M imputed) 2,530 CNVs	rs9923231 (*VKORC1*); 1.6×10^{-122} / 2.7×10^{-181} rs1057910 (*CYP2C9**3); 2.6×10^{-55} / 2.6×10^{-79} rs1799853 (*CYP2C9**2); 1.7×10^{-28} / 1.10^{-31} rs2108622 (*CYP4F2*); 8.3×10^{-10} / 3.3×10^{-10}
Cha et al[160]	Discovery: Daily warfarin dose of 4 mg or more versus daily warfarin dose of 1 mg or less Replication: Warfarin dose	Asian (Japanese)	701 / 807	440	485,227 SNPs	rs9923231 (*VKORC1*); 8.65×10^{-31} rs10509680 (*CYP2C9*); 3.8×10^{-7} rs2108622 (*CYP4F2*); 8.7×10^{-6}/ 2.57×10^{-8}
Teichert et al[168]	Stabilized acenocoumarol dosage	European (Dutch)	1,451	287	Approx. 500,000 SNPs	*rs10871454 (*STX4A*)[b]; 2.0×10^{-12} rs4086116 (CYP2C9); 3.3×10^{-24} rs1998591 (*CYP2C18*); 1.9×10^{-9} / 4.9×10^{-12} rs2108622 (*CYP4F2*); 2.0×10^{-8} / 2.15×10^{-10}

Abbreviations: CNVs, copy number variants; GWAS, genome-wide association studies, M, million; SNPs, single nucleotide polymorphisms.

[a] *p* value in the discovery set are listed first and *p* value from the joint (discovery and replication) analysis second, where available.

[b] rs10871454 in *STX4A* is in perfect LD ($r^2 = 1$, $D' = 1$) with rs9923231 in *VKORC1*.