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## PREVALENCE OF COMBINATORIAL CYP2C9 AND VKORC1 GENOTYPES IN PUERTO RICANS: IMPLICATIONS FOR WARFARIN MANAGEMENT IN HISPANICS

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### Abstract

Polymorphisms in the cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) genes significantly alter the effective warfarin dose. We determined the frequencies of alleles, single carriers, and double carriers of single nucleotide polymorphisms (SNPs) in the *CYP2C9* and *VKORC1* genes in a Puerto Rican cohort and gauged the impact of these polymorphisms on warfarin dosage using a published algorithm. A total of 92 DNA samples were genotyped using Luminex® x-MAP technology. The polymorphism frequencies were 6.52%, 5.43% and 28.8% for *CYP2C9* \*2, \*3 and *VKORC1*-1639 G>A polymorphisms, respectively. The prevalence of combinatorial genotypes was 16% for carriers of both the *CYP2C9* and *VKORC1* polymorphisms, 9% for carriers of *CYP2C9* polymorphisms, 35% for carriers of the *VKORC1* polymorphism, and the remaining 40% were non-carriers for either gene. Based on a published warfarin dosing algorithm, single, double and triple carriers of functionally deficient polymorphisms predict reductions of 1.0–1.6, 2.0–2.9, and 2.9–3.7 mg/day, respectively, in warfarin dose. Overall,

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### Disclosures

Dr Ruaño is founder and President of Genomas, Inc. Dr Windemuth, Mr Kocherla, Ms Gorowski and Ms Bogaard are full-time employees of Genomas. Dr Seip is a consultant for Genomas.

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60% of the population carried at least a single polymorphism predicting deficient warfarin metabolism or responsiveness and 13% were double carriers with polymorphisms in both genes studied. Combinatorial genotyping of *CYP2C9* and *VKORC1* can allow for individualized dosing of warfarin among patients with gene polymorphisms, potentially reducing the risk of stroke or bleeding.

## Keywords

Warfarin; *CYP2C9*; *VKORC1*; Genotyping; Personalized Medicine

## INTRODUCTION

Warfarin is an oral anticoagulant considered as the standard-of-care therapy for many thromboembolic disorders.<sup>1–2</sup> More than 24 million prescriptions for warfarin were written in United States in 2007.<sup>3–4</sup> Warfarin is frequently associated with unpredictable responses, ranging from occult bleeding to hemorrhage, due in part to a narrow therapeutic index. Therefore, its activity has to be monitored by frequent blood testing for the international normalized ratio and adjustments are often necessary.<sup>2</sup>

Difference in successful outcomes during warfarin therapy is a multi-factorial issue.<sup>5–7</sup> The individual's unique genetic make-up also plays a cardinal role in the warfarin response. The first gene to be identified as affecting warfarin dose-response was *CYP2C9*. This gene encodes a particular isoform of *CYP450* enzyme, responsible for metabolizing S-warfarin. Approximately 25%–35% of the population have *CYP2C9* variants that lead to variably deficient enzyme activity. These variants can cause alterations in initial warfarin dose sensitivities, delays in achieving stable maintenance doses and increased bleeding complications.<sup>8–10</sup> Polymorphisms of *CYP2C9* include *CYP2C9\*2* and *CYP2C9\*3*, which are associated with reduced enzyme activity to 70% and 5% of the normal level, respectively.<sup>8, 9, 11–13</sup> The result is warfarin accumulation and possible hemorrhagic complications.<sup>14</sup> The variants have a significant impact on warfarin metabolism in several populations.<sup>8–10, 15–21</sup> The *CYP2C9* status by itself accounts for approximately 15%–20% of the variance in warfarin dose.<sup>13, 21–22</sup>

Warfarin exerts an anticoagulant effect through its inhibition of the *VKORC1* gene product.<sup>23–25</sup> Patients who are carriers of a common polymorphism in the *VKORC1* promoter sequence (–1639 G>A) require a lower warfarin maintenance dosage.<sup>26–27</sup> The –1639 G>A genotype and related haplotype can independently determine 20%–25% of warfarin dose variance.<sup>21, 27</sup> Together, the *CYP2C9* and *VKORC1* combinatorial genotypes may explain up to 45% of warfarin response variability.<sup>10, 16, 22, 27</sup>

Current approaches to warfarin induction fail to prevent adverse events. The major flaw of existing warfarin dosing algorithms is that they rely on trial-and-error after an initial warfarin dose of 2 to 10 mg, rather than being tailored to individual genetic and clinical factors. Using pharmacogenetic-based warfarin therapy, clinicians can now estimate *a priori* the therapeutic dose by genotyping their patients for single-nucleotide polymorphisms (SNPs) that affect warfarin metabolism or sensitivity. Hence, pharmacogenetic-based therapy could reduce medical expenses by preventing inpatients from being kept in the hospital until their therapeutic dose has been determined empirically.

In 2007, the Food and Drug Administration revised the warfarin label to include the impact of the *CYP2C9* and *VKORC1* polymorphisms in a pharmacogenomic subsection.<sup>28</sup> Major and fatal bleeding events occur at rates of 7.2 and 1.3/100 patient-years, respectively, according to

a meta-analysis.<sup>29</sup> If genotyping were performed before warfarin prescription, 85,000 serious bleeding events and 17,000 strokes could be avoided annually in the United States alone, saving more than \$1 billion in healthcare spending.<sup>30</sup> The US National Heart, Lung, and Blood Institute is currently sponsoring a prospective genotype-guided warfarin dosing protocol.<sup>31</sup>

The primary goal of this study was to determine the frequency of combinations of the *CYP2C9* and *VKORC1* deficient and null polymorphisms in a Puerto Rican cohort. The results were used for comparison to a cohort of cardiovascular outpatients at Hartford Hospital. The impact on population-wide dose adjustment of the observed prevalence of carriers for combinatorial genotypes is also discussed.

## MATERIALS AND METHODS

### Study Cohort

One hundred purified human DNA samples present in dried blood spots were used. The dried blood samples were supplied by the Puerto Rico Newborn Screening Program (PRNSP). The analysis was based on a controlled stratified-by-region representative sampling from the target population. Consequently, dried blood samples came from different medical centers in Puerto Rico and were randomly chosen.

### Laboratory Analysis

Genomic DNA samples were extracted and purified using Generation DNA Purification kit (QIAGEN Inc., CA, US) following the manufacturer's protocol. Extracted DNA was stored at  $-80^{\circ}\text{C}$  in TRIS-EDTA buffer. Quantification of DNA was performed by fluorescent staining of double-stranded DNA (PicoGreen<sup>®</sup> dsDNA Quantitation Kit, Molecular Probes, Eugene, OR, US). Fluorescent intensity was measured using a fluorescent micro-titer plate reader (POLARstar OPTIMA, BMG-LABTECH GmbH, Offenburg, Germany).

Genotyping of the *CYP2C9* and *VKORC1* genes at 12 variable sites, 5 SNPs in *CYP2C9* and 7 SNPs in *VKORC1*, was performed at Genomas (Laboratory of Personalized Health, Hartford, CT, US). The Tag-It<sup>™</sup> Mutation Detection assays (Luminex Molecular Diagnostics, Toronto, Canada) were utilized for genotyping. A full explanation of this assay can be found elsewhere.<sup>32,33</sup>

### Statistical Analysis

Allele frequencies (distribution) were determined in the Puerto Rican population for the loci of interest. Test for deviations from Hardy-Weinberg equilibrium (HWE) were used. Departure from HWE were estimated under the null hypothesis of the predictable segregation ratio of specific matching genotypes ( $P > .05$ ) by use of  $\chi^2$  goodness-of-fit test with one degree of freedom.

To explore the population-wide impact of polymorphisms on warfarin dose, we classified patients by *VKORC1* and *CYP2C9* combinatorial genotypes. Warfarin dose was estimated for each combinatorial genotype, using a published equation,<sup>16</sup> by assuming a reference idealized person, aged 55 years, male or female with BMI=27. Two values were generated for each combinatorial genotype, incorporating the respective correction factor for sex. The reduction in dose for each combinatorial genotype was calculated as the difference between the dose predicted for that combinatorial genotype and the dose predicted for the wild-type genotype.

## RESULTS

The results for the *CYP2C9* SNPs are shown in Table 1. The frequency of the alleles \*2 and \*3 were 6.52% and 5.43%, respectively. With respect to metabolic status, the \*3 allele is “highly deficient,” matching a phenotypically defined null metabolizer because this variant only has 5% of the normal metabolic function. Table 2 presents the SNP frequencies for the *VKORC1* marker. We tested for 7 different *VKORC1* SNPs, but only the –1639A promoter allele was observed, at 28.8% frequency.

The carrier prevalence calculated in this study sample, combining polymorphisms in both genes, is shown in Figure 1. The individuals without a polymorphism for either gene (non-carriers) accounted for 40% of the study population. The percentage of subjects with polymorphisms in only a single gene was 9% for *CYP2C9* and 35% for *VKORC1*. Double carrier patients with a single polymorphism in both genes accounted for 13% of the population. Triple carrier patients with a single polymorphism in *CYP2C9* and double in *VKORC1* accounted for 3.3%. Quadruple carrier patients with double polymorphisms in both genes were not detected in this study.

Figure 2 depicts the projected effect of *CYP2C9* and *VKORC1* combinatorial genotypes on the warfarin dose reduction, based on a published equation.<sup>16</sup> To estimate the effect of these genotypes on clinical dosing practices, we calculated the warfarin dosage for idealized individuals (both sexes) taken from a hypothetical population with the observed genotypic frequencies of our study population. The estimation procedure is based on combinatorial *CYP2C9/VKORC1* alleles, age, sex and physical attributes using a published algorithm.<sup>16</sup> The model yields stepwise dose reductions dependent on the number of polymorphisms.

In this study, 40.5% of patients were carriers of a single polymorphism in *CYP2C9* (9% \*1\*2 or \*1\*3) or *VKORC1* (31.5% GA) and, relative to non-carriers (40%), would require a dose decrease in the range of 1.0–1.6 mg/day. Double-carriers, single polymorphism in each gene (13%) or double in *VKORC1* (3.2%), accounted for 16.2% of the study population and would require a dose reduction in the range of 2.0–2.95 mg/day. Triple-carriers (*CYP2C9* \*1\*2 or \*1\*3, *VKORC1* AA) accounted for 3.3% of the population and would require a decrease in dose between 2.9–3.7 mg/day.

## DISCUSSION

The results from this study demonstrate that 60% of the study population was carrier of one or more polymorphisms resulting in deficient warfarin metabolism (*CYP2C9*) and/or sensitivity (*VKORC1*). This portion of the population is at increased risk of warfarin overdosing (bleeding) if given the standard dosage, indicating the need for genotype-guided dosing in the majority of Puerto Ricans. More than one third of the patients, 40%, are carriers of a single polymorphism, and about one fifth, 19.3%, are carriers of 2 or 3 polymorphisms. No subjects were found to be quadruple carriers, with double polymorphisms in both *CYP2C9* and *VKORC1* genes.

The prevalence of these polymorphisms was slightly lower when compared with an earlier study on unrelated cardiovascular outpatients, ranging 28–88 years old, that were enrolled in a study of dyslipidemias at Hartford Hospital.<sup>33</sup> In that population, 44.1% carried a single polymorphism and more than one quarter, 28.3%, carried two or three deficient alleles.<sup>33</sup> Although the ethnic composition was predominantly Caucasian, we consider that the observed differences would rather reflect the sampling of the population from cardiovascular (disease load) patients as opposed to newborns in the Puerto Rican random sample.

It is well-known that the prevalence of these allelic variants in people of African ancestry is lower than in Caucasians. Given recent estimates of European, Native American and African admixtures in Puerto Ricans that converge on a ratio of 60:20:20, respectively,<sup>34,35</sup> the observed lower frequencies for these alleles seem to be reasonable.

Notably, one sample from this study population carried the uncommon allele *CYP2C9\*6* (818delA), which is associated with decreased enzyme activity. *CYP2C9\*6* is commonly related to African ancestry.<sup>36–38</sup> All the *CYP2C9* genotypes were wild-type (WT) or heterozygous, and no homozygotes were found. Given the non-WT prevalence of approximately 25%, we would expect 1 or 2 homozygous (including “mixed” homozygotes such as \*2\*3) according to HW equilibrium, but finding none instead is not a statistically significant deviation.

No statistically significant deviations from HWE were found. HWE is applicable to classical population statistics but may have limited applicability to a population sample of diverse ethnogeographic ancestry. The Puerto Rican population is a three-way admixed population that experienced migration, which might lead us to expect increased heterozygosity. However, such deviations from HWE quickly subside within a single generation under the assumption of random mating. The size and geography of Puerto Rico do not favor isolated subpopulations, and social stratification is likewise not excessive. Considering that the primary admixing events (Spanish settlement for Caucasian influx and the slave trade for African admixture) are many generations past, observing no overt deviations from HWE stands to reason. Larger population samples and genotyping of several other genes are required to elucidate the population genetics of Puerto Rico. Such surveys are the subject of ongoing research by our group.

With regard to dosing algorithms, the combinatorial genotypes yield valuable information for potential DNA-guided adjustments in this population. Individuals with the greatest number of deficient polymorphisms will benefit most from this practice. The predicted warfarin dose reduction (5 mg/day as standard dosage) ranged from 1.6 to 3.7 mg/day for hypothetical patients having the observed combinatorial genotypes. This finding suggests that such a pharmacogenetic approach can then be recommended for reducing adverse events after warfarin administration in Puerto Ricans. Accordingly, risk-associated combinatorial genotype profiles may be assessed in patients with reported adverse events. Currently, our group is focused on conducting the corresponding clinical studies to develop a genomic-based warfarin-dosing algorithm for this population.

Although this report encompasses our initial observations, the multiplexed panel of 5 and 7 alleles for *CYP2C9/VKORC1* genes, respectively, and the population sample of 92 subjects address most common and clinically relevant variants for these two genes. Recent studies have demonstrated an increased risk of hemorrhage during long-term therapy in African Americans with *CYP2C9* minor variants.<sup>39–41</sup> In Asian populations, where the *VKORC1* haplotype predicting low warfarin dose phenotype is more frequent than in Caucasians,<sup>42,43</sup> DNA-guided warfarin dosing may also be clinically valuable.<sup>44,45</sup> Studies in Caucasian populations show that the *CYP2C9* polymorphisms are associated with a 2- to 3-fold increased risk of bleeding during warfarin induction,<sup>14,46–49</sup> but not during long-term therapy.<sup>50</sup> Evidence from various studies suggests that carriers of combinatorial polymorphisms have a higher risk of severe and life-threatening bleeding episodes due to warfarin-induced over-anticoagulation when compared with patients who carry polymorphisms in only a single gene.<sup>39,40</sup> The aggregate annual healthcare cost in the United States is \$1.15 billion.<sup>30</sup>

Whether to genotype an individual’s DNA before or during warfarin treatment, in order to improve clinical outcomes, is an evolving area for regulatory authorities and for the clinical community. Health care will be revolutionized by genotype-guided medicine in clinical



practice. The practice of personalized medicine will be increasingly dependent on defining unique, individual drug metabolizing status and target sensitivity by genotyping and tailoring therapy on an individualized basis. Discrepancies in warfarin management from the genotype-guided doses may increase the risk of overdosing and bleeding complications.<sup>46,47</sup> especially during the initiation of warfarin therapy.<sup>41,44</sup>

Besides typical covariates such as age, sex, body weight, etc., genotyping for combinatorial *CYP2C9* and *VKORC1* polymorphisms has the potential to become the standard-of-care in future warfarin management. Warfarin dosing represents a current working model for the practice of personalized health care. Genotype-guided pharmacotherapy holds great potential to enhance patient safety based on each individual's drug metabolism and sensitivity.<sup>51</sup>

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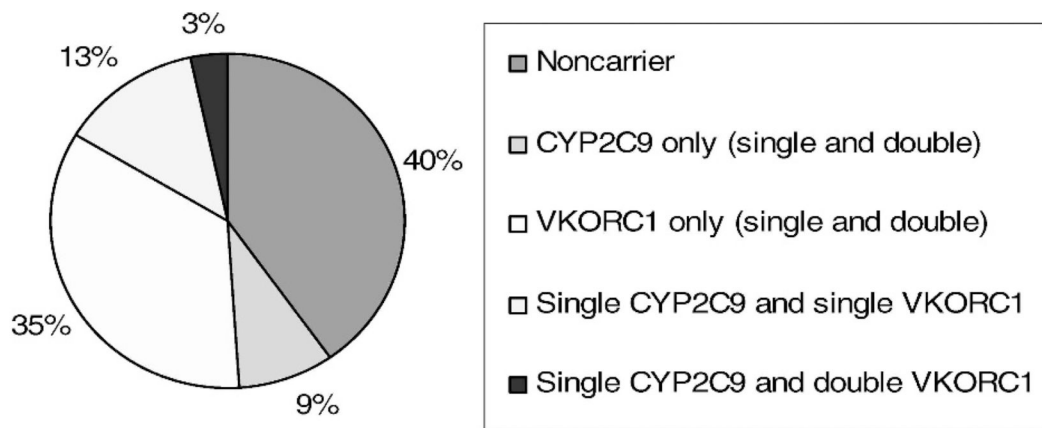
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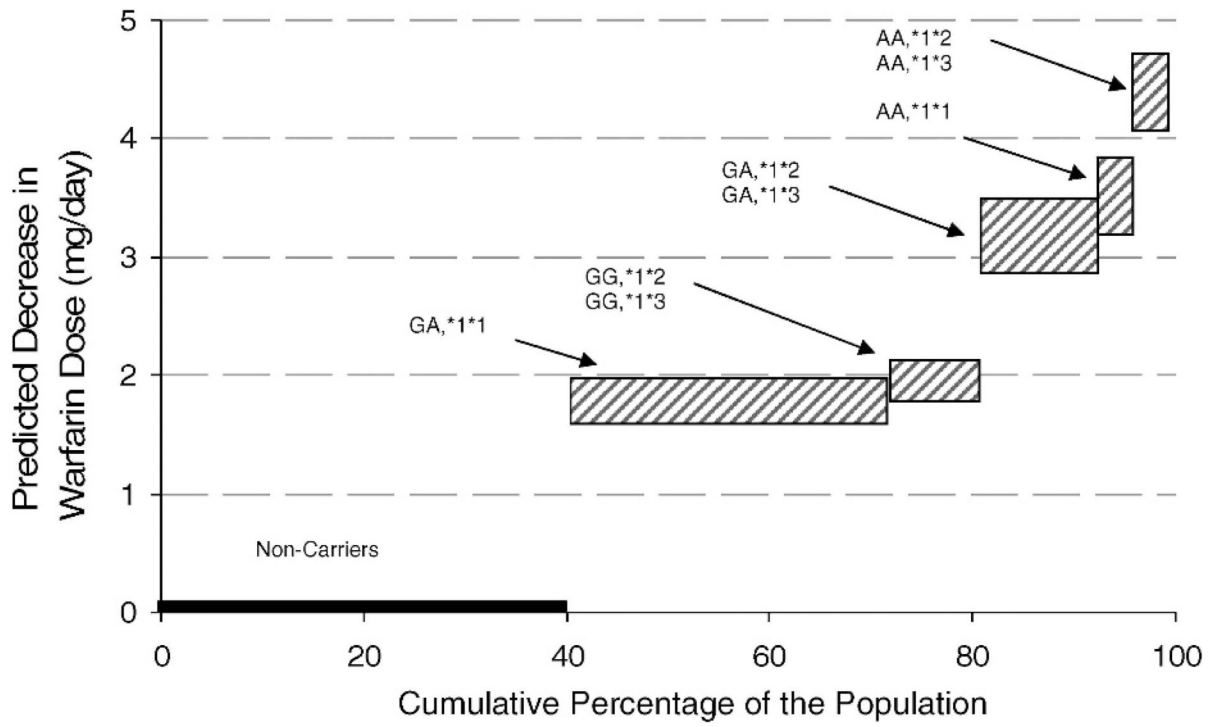
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**Fig 1.**

Carrier prevalence of *CYP2C9/VKORC1* polymorphisms in 92 Puerto Ricans: Eight *CYP2C9* (five \*1\*2; three \*1\*3) and thirty-two *VKORC1* only carriers (twenty-nine GA; three AA); twelve single *CYP2C9* and single *VKORC1* carriers (five \*1\*2/GA; six \*1\*3/GA; one \*1\*6/GA); three single *CYP2C9* and double *VKORC1* carriers (two \*1\*2/AA; one \*1\*3/AA). Thirty-seven non-carriers were found



**Fig 2.** Predicted mean decreases in warfarin dose (mg/day) in hypothetical individuals (55-yo, BMI=27, both genders) taken from a population with the same genotypic frequencies observed in Puerto Ricans. The hatched areas indicate ranges of predicted dose reductions based on a published algorithm<sup>16</sup>. The upper and lower bars represents the reductions for male and female, respectively

**Table 1**

*CYP2C9* alleles. Those observed among  $n=184$  from 92 samples are shown in bold

<i>Allele</i>	DNA Change	Amino-acid Change	Frequency (%)	Allele Count
*1	Reference	Reference	70.1	129
*2	<b>430C&gt;T</b>	<b>Arg144Cys</b>	<b>6.52</b>	<b>12</b>
*3	<b>1075A&gt;C</b>	<b>Ile359Leu</b>	<b>5.43</b>	<b>10</b>
*4	1076T>C	Ile359Tyr	0	0
*5	1080C>G	Asp360Glu	0	0
*6	<b>818delA</b>	<b>Frameshift</b>	<b>0.54</b>	<b>1</b>

**Table 2**

*VKORC1* alleles. Those observed among  $n=184$  from 92 samples are shown in bold

<i>Allele</i>	DNA Change	Amino-acid Change	Frequency (%)	Allele Count
<b>Reference</b>	<b>Reference</b>	<b>Reference</b>	<b>71.2</b>	<b>131</b>
<b>-1639</b>	<b>G&gt;A</b>	<b>Promoter</b>	<b>28.8</b>	<b>53</b>
85	G>T	Val29Leu	0	0
121	G>T	Ala41Ser	0	0
134	T>C	Val45Ala	0	0
172	A>G	Arg58Gly	0	0
1331	G>A	Val66Met	0	0
3487	T>G	Leu128Arg	0	0