Validation of Clinical Testing for Warfarin Sensitivity

Comparison of CYP2C9-VKORC1 Genotyping Assays and Warfarin-Dosing Algorithms

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Responses to warfarin (Coumadin) anticoagulation therapy are affected by genetic variability in both the CYP2C9 and VKORC1 genes. Validation of pharmacogenetic testing for warfarin responses includes demonstration of analytical validity of testing platforms and of the clinical validity of testing. We compared four platforms for determining the relevant single nucleotide polymorphisms (SNPs) in both CYP2C9 and VKORC1 that are associated with warfarin sensitivity (Third Wave Invader Plus, ParagonDx/Cepheid Smart Cyclor, Idaho Technology LightCycler, and AutoGenomics Infiniti). Each method was examined for accuracy, cost, and turnaround time. All genotyping methods demonstrated greater than 95% accuracy for identifying the relevant SNPs (CYP2C9 *2 and *3; VKORC1 -1639 or 1173). The ParagonDx and Idaho Technology assays had the shortest turnaround and hands-on times. The Third Wave assay was readily scalable to higher test volumes but had the longest hands-on time. The AutoGenomics assay interrogated the largest number of SNPs but had the longest turnaround time. Four published warfarin-dosing algorithms (Washington University, UCSF, Louisville, and Newcastle) were compared for accuracy for predicting warfarin dose in a retrospective analysis of a local patient population on long-term, stable warfarin therapy. The predicted doses from both the Washington University and UCSF algorithms demonstrated the best correlation with actual warfarin doses. (*J Mol Diagn* 2009, 11:216–225; DOI: 10.2353/jmoldx.2009.080123)

In 2007, the US Food and Drug Administration modified the package insert for warfarin (Coumadin) to include information on the relationship of safe and effective dosage to specific single nucleotide polymorphisms (SNPs) in two genes, cytochrome P450 2C9 (CYP2C9) and vita-

min K epoxide reductase complex subunit 1 (VKORC1). Warfarin binds to and inhibits vitamin K epoxide reductase (VKOR), encoded by VKORC1, thereby inhibiting activation of vitamin K-dependent clotting factors. A single common VKORC1 SNP -1639/3673 A>G defines VKORC1 haplotypes with increased sensitivity to warfarin (group A haplotypes, H1 and H2).^{1–9} CYP2C9 is responsible for metabolism of >90% of S-warfarin, the more active enantiomer of warfarin. Two common allelic variants of CYP2C9 with reduced enzymatic activity (CYP2C9*2 and CYP2C9*3) have been associated with reduced metabolism of warfarin, lower required doses of warfarin to achieve adequate anticoagulation, and increased risk of adverse events when beginning warfarin therapy.^{10–12}

As a result of increased awareness of the impact of these SNPs on warfarin dosage, it is likely that clinical laboratories will increasingly be asked to provide genotype information for the above genes. Thus, there is a need to evaluate both the analytic and clinical validity of genotyping to make dosing recommendations for warfarin. Several assays for genotyping are now commercially available. We evaluated four platforms for determining relevant SNPs in CYP2C9 and VKORC1: the Third Wave Invader Plus CYP2C9 and VKORC1 reagents, the Cepheid SmartCycler Rapid Genotyping Assay for CYP2C9 and VKORC1 (developed by ParagonDx), the Idaho Technology Warfarin Genotyping Reagents on the Roche LightCycler and the AutoGenomics Infiniti 2C9-VKORC1 assay (now marketed as Warfarin XP).

Several different dosing algorithms have been devised that incorporate VKORC1 and CYP2C9 genotype, in addition to demographic and clinical information, to predict optimal warfarin dose. We compared the accuracy of four published algorithms for determining warfarin dosage,

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herein called Wash U,¹³ UCSF,¹⁴ Louisville,¹⁵ and Newcastle,¹⁶ using retrospective data from a local patient population on long-term, stable warfarin therapy.

Materials and Methods

DNA Samples

Residual DNA from patients on warfarin therapy was generously provided by Darryl Stafford's laboratory (University of North Carolina). These samples were previously genotyped for SNPs in the *CYP2C9* and *VKORC1* genes using a combination of DNA sequencing and SNP genotyping by real-time polymerase chain reaction (PCR).⁶ We selected 20 samples that had a variety of combinations of SNPs at the sites to be genotyped to assess the accuracy of each platform for testing various genotype combinations. Each sample was analyzed by each method separately, following the manufacturer's instructions.

Genotyping Analysis

Four genotyping platforms were compared for *CYP2C9* and *VKORC1* genotyping: Invader Plus *CYP2C9* and *VKORC1* analyte-specific reagents from Third Wave Technologies (a subsidiary of Hologic, Inc., Madison, WI); Warfarin SimpleProbe Genotyping Reagents from Idaho Technology, Inc. (Salt Lake City, UT); Infiniti Warfarin XP Assay from AutoGenomics, Inc. (Carlsbad, CA); and the SmartCycler Rapid Genotyping Assay for *CYP2C9* and *VKORC1*, developed by ParagonDx (Morrisville, NC). Reagents and instrumentation for analysis were provided by the manufacturers.

The 20 residual DNA samples were analyzed on each platform. Genotype information was compared for each of the SNPs that are included on more than one platform: *CYP2C9* *2 and *3, and *VKORC1* –1639 (3673) and 3730 (9041). The SNPs detected by each assay are listed in Table 1. Because *VKORC1* –1639 (3673) has been shown to be in complete linkage disequilibrium with *VKORC1* 1173(6484),¹ the presence of either SNP was denoted as *VKORC1* –1639 for comparison. Each assay was run at least three times with a batch of six samples plus three controls (blank, *CYP2C9* *2/*3, and *VKORC1* –1639A/G) to represent a projected typical clinical run. Hands-on time was recorded using a stopwatch at each step for each assay and totaled for comparison.

Third Wave Invader Plus Assay

The Third Wave Invader Plus assay was performed per manufacturer instructions with an 18-cycle multiplex PCR on a PE9700 thermal cycler (Applied Biosystems, Foster City, CA), with the resultant amplicons used in three separate Invader reactions, one each for *CYP2C9* *2 and *3 and *VKORC1* –1639. Fluorescence related to both wild-type (reference) sequence and SNP was measured in each well by a GENios FL plate reader (Tecan, San Jose, CA) and the results loaded into an Excel spreadsheet provided by the company.

ParagonDx/Cepheid assay

The ParagonDx/Cepheid *CYP2C9/VKORC1* research use only (RUO) assay was performed per manufacturer instructions using two multiplex master mixes, each containing four primer sets and probes that were resuspended from lyophilized beads, one for *CYP2C9* *2 and *3 and the other for *VKORC1* 1173 and 3730. Real-time PCR analysis was performed on the SmartCycler (Cepheid, Sunnyvale, CA) and the results loaded into an Excel-based interpretation file provided by ParagonDx (formerly Gentris). A Food and Drug Administration cleared version of this assay without the *VKORC1* 3730 SNP is to be marketed by Cepheid but is not yet commercially available at the time of this publication.

Idaho Technology LightCycler Assay

The Idaho Technology SimpleProbe Warfarin assay was performed per manufacturer instructions. Reactions for each SNP tested (*CYP2C9* *2 and *3 and *VKORC1* –1639 A/G) were analyzed simultaneously on a Roche LightCycler in separate capillaries and melting curves were evaluated for each sample. The assay can also be performed using a LightScanner (Idaho Technology) or other instrument for melting curve analysis. The primers are designed to match each SNP, so that the reference or wild-type sequence is an imperfect match and has a lower melting temperature.

AutoGenomics Warfarin XP Assay

The AutoGenomics Warfarin XP assay was performed per manufacturer instructions with a single multiplex PCR on a PE9700 thermal cycler, followed by allele-specific primer extension, hybridization and detection performed

Table 1. SNPs Detected by Each Assay

Assay	<i>CYP2C9</i> SNPs	<i>VKORC1</i> SNPs
Third Wave Invader Plus	*2,*3	–1639G/A
ParagonDx/SmartCycler	*2,*3	1173C/T, 3730G/A
Idaho Technology/LightCycler	*2,*3	–1639G/A
AutoGenomics Infiniti	*2,*3,*4,*5,*6,*11	–1639G/A, 497T/G, 698C/T, 1173C/T, 1542G/C, 2255C/T, 3462C/T, 3730G/A

Note: –1639G>A and 1173C>T are in 100% linkage disequilibrium (see Table 2).

on the automated Infiniti instrument (AutoGenomics). The extended primers, incorporating Cy3-labeled dCTPs, are hybridized to chips using the AutoGenomics ZipCode-anti-ZipCode technology, and fluorescence at each set of the SNP-specific sites is read by the onboard confocal microscope. Onboard software analyzes the raw data, reports fluorescence values for both alleles at each of 14 SNPs (Table 1), and reports SNP determination. AutoGenomics also markets a Food and Drug Administration approved Infiniti Warfarin assay, which detects only *CYP2C9* *2 and *3 and *VKORC1* -1639 (3673), that was not analyzed in this comparison study.

DNA Sequencing

Selected samples from patients on high doses of warfarin were subjected to dideoxy sequencing of the promoter and coding regions of the *VKORC1* gene and capillary electrophoresis on an Applied Biosystems 3130xl genetic analyzer.

Dosing Algorithm Evaluation

Patients treated at the University of North Carolina anticoagulation clinic who were on long-term, stable warfarin therapy were retrospectively analyzed for the accuracy of the dosing algorithms to predict stable warfarin dose. *VKORC1* and *CYP2C9* genotype and clinical information were available from a previous study.⁶ *VKORC1* 1173 (6484) genotype was assumed to be in complete linkage disequilibrium with *VKORC1* -1639 (3673).¹ Additional patient data necessary for each of the dosing algorithms were acquired through chart review, with appropriate informed consent per University of North Carolina Institutional Review Board approval. Sufficient information for all of the algorithms was available on 75 patients (81% Caucasian, 19% African-American; 63% male, 37% female). The required data for each of the 75 patients was entered into each algorithm and the recommended warfarin dosage calculated.

For the Wash U algorithm, data for initial warfarin dose calculation was input on the website <http://www.warfarindosing.org> (last accessed July 21, 2008). For the UCSF, Louisville, and Newcastle algorithms, an Excel spreadsheet was devised to calculate dose using published equations.¹⁴⁻¹⁶ The Newcastle algorithm incorporates age, height, the *VKORC1* -1639 (3673) SNP, and *CYP2C9* *2 and *3. The other algorithms include the same genetic variables plus additional variables. The Louisville algorithm incorporates age, gender, and weight instead of height. The Wash U algorithm incorporates age, body surface area, ethnicity, target international normalized ratio (INR), history of smoking, clinical indication for warfarin, and the presence of other medications that can affect warfarin metabolism (amiodarone). The UCSF algorithm includes the *VKORC1* 2255 (7566) SNP in addition to the genetic variants included in the other algorithms, along with age, height, weight, gender, ethnicity, history of smoking, and the presence of medications that can affect warfarin metabolism (amiodarone or sulfamethoxazole).

Actual dose for each patient was determined as the mean of the last five doses of warfarin on reaching target

prothrombin time INR. The calculated recommended dose from each algorithm was plotted against each patient's actual dose in an XY scatter plot using Microsoft (Redmond, WA) Excel 2000 (version 9.0.3821 SR-1). A best-fit trendline, correlation coefficient (*r*), and coefficient of determination (*r*²) were determined by linear regression using Excel. The Excel linear regression calculations for the best-fit trendline are as follows: $m = \frac{n\sum(xy) - \sum x \sum y}{n\sum(x^2) - (\sum x)^2}$; $b = \frac{\sum y - m\sum x}{n}$; $r = \frac{n\sum(xy) - \sum x \sum y}{\sqrt{[n\sum(x^2) - (\sum x)^2][n\sum(y^2) - (\sum y)^2]}}$, where *n* is the number of data points, *m* is the slope, *b* is the *y*-intercept, and *r* is the correlation coefficient.

Results

Genotyping Method Comparison

The SNPs detected by each assay are listed in Table 1. All of the methods evaluated detect *CYP2C9* *2 and *3 and at least one of the relevant *VKORC1* SNPs, -1639 (3673) G>A or 1173 (6484) C>T. These two *VKORC1* SNPs are in 100% linkage disequilibrium and are both associated with increased sensitivity to warfarin. The ParagonDx and AutoGenomics assays also detect *VKORC1* 3730(9041) G>A, which is present in group B *VKORC1* haplotypes H7 and H8 and has been associated with higher dosage requirements of warfarin.^{1,6} The AutoGenomics Warfarin XP detects six *CYP2C9* variants associated with reduced metabolism and eight *VKORC1* SNPs, which enables determination of nine described *VKORC1* haplotypes (Table 2).

Data compiled for turnaround time (TAT) and hands-on time for each assay are shown in Table 3. The figures are based on actual time during setup and analysis of a run consisting of six samples and three controls. The ParagonDx and Idaho Technology assays had the lowest hands-on time and TAT because data are collected during or immediately after PCR onboard the instrument. The AutoGenomics required less than 1 hour of hands-on time because the allele-specific primer extension, hybridization, and detection are all automated, but had a longer TAT. The TAT increases with the number of samples because of sequential analysis by the Infiniti instrument. The Third Wave assay required the most labor because of the manipulations after PCR required for the Invader assay, but the rapidity of its detection method gave it a significantly shorter TAT than the AutoGenomics assay. Reagent list prices and instrument costs are compared in Table 3.

A summary of genotype results for the 20 previously characterized samples is shown in Table 4. The table includes data for each SNP that is interrogated by more than one of the assays evaluated (*CYP2C9* *2 and *3; *VKORC1* -1639A>G or 1173C>T and *VKORC1* 3730G>A). For comparison, results for the *VKORC1* SNPs -1639A>G and 1173C>T, which are in complete linkage disequilibrium, are reported as *VKORC1* -1639. Some samples were not tested at all SNPs with every assay because of limited availability of reagents. Each assay demonstrated 100% accuracy, with the exception of the AutoGenomics assay, which gave one indeterminate result for *CYP2C9*

Table 2. VKORC1 Haplotypes

Haplotype	−4930 (381)	−4450 (861)	−1639 (3673)	497 (5808)	698 (6009)	1173 (6484)	1542 (6853)	2255 (7566)	3462 (8733)	3730 (9041)
H1	C	C	A	T	C	T	C	T	C	G
H2	C	C	A	G	C	T	C	T	C	G
H3	C	C	G	T	C	C	C	C	C	G
H4	C	C	G	T	C	C	G	T	C	G
H5	T	C	A	G	C	T	C	T	C	G
H6	T	C	G	T	C	C	G	C	C	G
H7	T	C	G	T	C	C	G	C	C	A
H7*	T	C	G	T	C	C	G	C	T	A
H8	T	A	G	T	C	C	G	C	C	A
H9	T	A	G	T	T	C	G	C	C	G

Shown is the VKORC1 sequence at each of the SNPs shown for each haplotype. The VKORC1 H7* haplotype is a subset of haplotype H7 with the 3462(8773) C>T SNP. SNPs in bold are typed by the AutoGenomics Warfarin XP assay. AutoGenomics cannot differentiate H2 versus H5 (can haplotype all others). Haplotypes H1 and H2 (group A) are associated with lower doses of warfarin; haplotypes H7 to H9 (group B) with higher doses.^{1,14}

(95% accuracy). The accuracy of the AutoGenomics assay for genotyping additional SNPs in *VKORC1* was also examined for the 20 samples by correlation with previous results for SNPs 497 (5808) T/G, 698 (6009) C/T, 1542 (6853) C/G, and 2255 (7566) T/C,⁶ and demonstrated 100% accuracy (data not shown).

The advantages and disadvantages of each assay are summarized in Table 5. The technically simplest assays were the Idaho Technology and ParagonDx assays because they use established, one-tube methods. The Third Wave Invader Plus assay required more manipulation in transferring the PCR products into the Invader reaction and then into the fluorometer. The AutoGenomics is a relatively new instrument and uses novel technology, which required specialized training. Capital instrumentation requirements differ for each of the assays. The Third Wave assay requires only a fluorometer, the ParagonDx requires a Cepheid SmartCycler, and the Idaho Technology assay requires a Roche LightCycler 2.0 or another melting curve analysis instrument. The largest capital investment requirement is the Infiniti instrument from AutoGenomics.

Dosing Algorithm Comparison

A comparison of the four algorithms for determining warfarin dosage is shown in Figure 1. The Wash U algorithm requires more clinical information than the other three, which limited the number of patients we were able to analyze in this retrospective study. Only those patients for whom all four algorithms could be run were included in the analysis ($n = 75$). Overall, the subpopulation of patients for which we were able to run the algorithms was

similar demographically and genotypically to the total study population ($n = 117$)⁶ and therefore it is unlikely that the data are biased toward any particular group (see Supplemental Table S1 at <http://jmd.amjpathol.org>). The study population is similar demographically to the total patient population followed at the University of North Carolina. The recommended dosage from the Wash U and UCSF algorithms had the best correlation with stable dosage in the patient population, with correlation coefficients (r) of 0.60 compared with 0.58 for the Louisville algorithm and 0.48 for the Newcastle algorithm. The difference between the Wash U, UCSF, and Louisville algorithms is not significant, whereas the Newcastle algorithm was significantly less predictive of stable dose. The Louisville algorithm calculated a high warfarin dose of greater than 12 mg/day for two patients whose actual warfarin dose was less than 9 mg/day.

Two of the patients had much higher actual warfarin doses than predicted by any of the algorithms (Figure 1, square symbols). Both of these outlier patients were African-American females with an actual stable warfarin dose of >12 mg/day. Removal of these two data points improved the correlation coefficient for each algorithm, particularly the Wash U and UCSF algorithms ($r = 0.67$) (Table 6). All four algorithms performed less well among the African-American population, even after removal of the outlier patients (Table 7). However, the sample size was too small ($n = 14$) to make conclusions about the efficacy of the dosing algorithms in this population.

For the two outlier patients, sequencing of the coding region of *VKORC1* was performed to examine for mutations, and previously performed SNP analysis⁶ was ex-

Table 3. Turnaround Time, Labor, and Cost Comparison

Supplier	TAT (hours)	Labor time (hours)*	Reagent cost per sample (list price) [†]	Instrumentation required	Equipment cost (approximate) [‡]
Third Wave Invader	3.2	1.42	\$55.00	Thermal cycler, 96-well fluorometer	\$12,000
ParagonDx/Cepheid	1.5	0.75	Not available [§]	Cepheid SmartCycler	\$34,000
Idaho Technology	2	0.75	\$12.00	Roche LightCycler	\$40,000
AutoGenomics	10.6	0.83	\$75.00	Thermal cycler, Infiniti analyzer	\$125,000

* Figures are based on a run of six patient samples and three controls; DNA extraction time not included.

[†] Cost does not include DNA extraction costs, polymerase enzyme, or disposables.

[‡] Cost of thermal cycler when required not included.

[§] The ParagonDx/Cepheid assay is not commercially available at this time.

Table 4. *CYP2C9* and *VKORC1* Genotyping Results

Sample	<i>CYP2C9</i>				<i>VKORC1</i>			
	Third Wave	Idaho Tech	ParagonDx	AutoGenomics	Third Wave*	Idaho Tech*	ParagonDx†	AutoGenomics
01	*3/*3	*3/*3	*3/*3	*3/*3	–1639/–1639	–1639/–1639	–1639/–1639	–1639/–1639
02	wt/wt	wt/wt	ND	wt/wt	wt/wt	wt/wt	3730/3730	3730/3730
03	*2/wt	*2/wt	*2/wt	*2/wt	wt/wt	wt/wt	ND	3730/3730
04	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	ND	3730/wt
05	wt/wt	wt/wt	ND	wt/wt	–1639/wt	–1639/wt	–1639/wt	–1639/wt
06	*2/wt	*2/wt	*2/wt	IND	–1639/–1639	–1639/–1639	ND	–1639/–1639
07	*3/wt	*3/wt	*3/wt	*3/wt	–1639/wt	–1639/wt	–1639/wt	–1639/wt
08	wt/wt	wt/wt	ND	wt/wt	–1639/wt	–1639/wt	–1639/3730	–1639/3730
09	*3/wt	*3/wt	*3/wt	*3/wt	–1639/wt	–1639/wt	–1639/3730	–1639/3730
10	*2/wt	*2/wt	*2/wt	*2/wt	–1639/–1639	–1639/–1639	ND	–1639/–1639
11	wt/wt	wt/wt	ND	wt/wt	wt/wt	wt/wt	3730/3730	3730/3730
12	*2/*3	*2/*3	*2/*3	*2/*3	–1639/wt	–1639/wt	–1639/3730	–1639/3730
13	wt/wt	wt/wt	ND	wt/wt	–1639/–1639	–1639/–1639	–1639/–1639	–1639/–1639
14	*2/wt	*2/wt	ND	*2/wt	wt/wt	wt/wt	3730/3730	3730/3730
15	wt/wt	wt/wt	ND	wt/wt	wt/wt	wt/wt	3730/3730	3730/3730
16	wt/wt	wt/wt	ND	wt/wt	–1639/–1639	–1639/–1639	–1639/–1639	–1639/–1639
17	*3/wt	*3/wt	*3/wt	*3/wt	–1639/wt	–1639/wt	ND	–1639/3730
18	wt/wt	wt/wt	ND	wt/wt	wt/wt	wt/wt	3730/wt	3730/wt
19	*2/wt	*2/wt	*2/wt	*2/wt	–1639/wt	–1639/wt	ND	–1639/3730
20	wt/wt	wt/wt	wt/wt	wt/wt	–1639/–1639	–1639/–1639	–1639/–1639	–1639/–1639
Accuracy	100%	100%	100%	95%	100%	100%	100%	100%

–1639 = *VKORC1* SNP –1639 (3673) G>A. 3730 = *VKORC1* SNP 3730 (9041) G>A.

* Method does not genotype *VKORC1* 3730.

† ParagonDx method genotypes *VKORC1* 1173, reported here as –1639 assuming 100% LD for clarity. ND, sample not tested at this locus because of limited reagents available at the time of testing. IND, indeterminate results, sample inadequate to repeat. wt, wild type. All results were confirmed by sequencing or SNP analysis.⁶

amined to determine *VKORC1* haplotypes. The first patient had an actual mean warfarin dose of 17.4 mg/day. This individual was *CYP2C9* *1/*1 (wild type) and had *VKORC1* SNPs 2255 (7566) C/T and 3730 (9041) G/A, indicating that the patient is heterozygous for *VKORC1* haplotype H4 and a group B haplotype (H7 or H8). The *VKORC1* 2255C>T SNP is seen in group A haplotypes, which are associated with increased warfarin sensitivity, as well as in haplotype H4, a less common haplotype for which there is little information on warfarin dose correlation. The 3730 G>A SNP is associated with group B haplotypes, which have been correlated with higher warfarin doses,^{1,5} but it is not included in any of the dosing algorithms. In addition, DNA sequencing demonstrated a novel single nucleotide change in the promoter, *VKORC1* –477 (4835) G/A, which to our knowledge has not been published previously. The impact of this novel change is unknown.

The second outlier patient had an actual mean warfarin dose of 13.8 mg/day. This individual was also *CYP2C9*

*1/*1 and was heterozygous for *VKORC1* –1639 (3673) G/A, 1173 (6484) C/T, and 2255 (7566) C/T and homozygous for 1542 (6853) C/C (*VKORC1* haplotypes H1/H3). Haplotype H1 is a group A haplotype associated with increased sensitivity to warfarin. Haplotype H3 is a less common haplotype with little information on warfarin dose correlation. No *VKORC1* coding region mutations were found by DNA sequencing and chart review revealed nothing remarkable that could account for the high warfarin dose requirement in either patient.

Discussion

Warfarin is one of the most commonly prescribed medications in the U.S. Because of its narrow therapeutic range and the wide variation in warfarin dose requirement in different individuals, there is a significant risk of bleeding or thromboembolic events during warfarin therapy.

Table 5. Advantages and Disadvantages of Each Method

Method	Advantages	Disadvantages
Third Wave Invader	Low capital expense Established methodology Readily scalable to larger run sizes	Post-PCR manipulations Hands-on time
ParagonDx/Cepheid SmartCycler	Short TAT Ease of use Established methodology Low hands-on time	Not commercially available Capital investment Small run size
Idaho Technology LightCycler	Short TAT Ease of use Established methodology Low hands-on time	Capital investment
AutoGenomics Infiniti Warfarin XP	Number of SNPs offered Post-PCR automated Easily adaptable to include additional SNPs	Long TAT Large capital investment Less established methodology

Figure 1. Comparison of four algorithms for determining warfarin dosage. Shown is the actual mean warfarin dose (mg/day) plotted against the warfarin dose predicted by each of the dosing algorithms for patients on long-term, stable warfarin therapy ($n = 75$). Blue diamonds represent each patient analyzed. Red squares represent outlier patients with high warfarin doses not predicted by any of the dosing algorithms. For each graph, solid dark lines represent the best-fit trendline plotted in Excel using linear regression and dashed light lines represent the line of identity. The linear correlation coefficient (r) for each algorithm is shown.

The recent understanding that genetic variability in *CYP2C9* and *VKORC1* can account for some of the variability in individual warfarin dose requirements has prompted the development of commercially available genotyping assays and of dosing algorithms that incorporate genotype information. We compared four different analysis platforms and four dosing algorithms for determining warfarin dosage in a local patient population.

Genotyping Test Platforms

All four platforms performed well and gave accurate genotyping results. The selection of genotyping platform must take into consideration needs, demands, and available resources of each clinical laboratory. Although the Idaho Technology and ParagonDx methods have the shortest TAT and hands-on time of the four, the additional SNPs available on the AutoGenomics Infiniti may be valuable in collecting data in non-Caucasian patients and those with less common genotypes. The Third Wave assay offers comparable TAT and the advantages of low capital equipment investment and scalability to medium test volumes. Although most of the platforms demonstrated 100% accuracy, the AutoGenomics assay gave one indeterminate result for *CYP2C9*. In our experience,

indeterminate results on the AutoGenomics assay are usually resolved with repeat testing. A recent publication compared two of these platforms plus two different *2C9-VKOR* genotyping platforms and also found very good genotyping accuracy by each of the commercial platforms.¹⁷

TAT may be a crucial factor for warfarin genotyping and for pharmacogenomic (PGx) testing in general. Many of the adverse events associated with warfarin occur during the initiation period. Ideally, genetic variability that affects drug response would be known before drug administration. This may not always be feasible for drugs such as warfarin, which may need to be administered quickly, although in some clinical situations a faster-acting anticoagulant such as heparin may be used before warfarin. Some investigators have advocated using a loading dose of warfarin and incorporating genotype into subsequent dose calculations.¹⁸ Nonetheless, although the need for rapid TAT of warfarin genotyping has not been demonstrated, the demand for clinical laboratories to provide useful and timely information that impacts warfarin initiation or dose modification may require assays with low failure rates and rapid TAT. As clinical information grows to accommodate dosing recommendations using the full spectrum of known SNPs, the

Table 6. Evaluation of Warfarin Dosage Algorithms

Algorithm	All patients*		Less two outliers†	
	r, r^2	Trendline equation	r, r^2	Trendline equation
Wash U	0.60, 0.36	$y = 0.9131x + 0.9966$	0.67, 0.45	$y = 0.8346x + 1.1584$
UCSF	0.60, 0.36	$y = 1.0665x + 0.6290$	0.67, 0.45	$y = 0.9725x + 0.8346$
Louisville	0.58, 0.33	$y = 0.6985x + 1.7400$	0.62, 0.38	$y = 0.6088x + 2.0118$
Newcastle	0.48, 0.23	$y = 0.8926x + 1.8266$	0.54, 0.29	$y = 0.8147x + 1.9115$

Comparison of correlation coefficient (r), coefficient of determination (r^2), and trendline equations for each algorithm.

* $n = 75$; 81% Caucasian, 19% African-American; 63% male, 37% female.

† $n = 73$; 84% Caucasian, 16% African-American; 64% male, 36% female.

Table 7. Evaluation of Warfarin Dosage Algorithms in African-American Patients

Algorithm	All African-American patients*		Less two "outliers"†	
	r, r^2	Trendline equation	r, r^2	Trendline equation
Wash U	0.36, 0.13	$y = 1.1131x + 0.8642$	0.52, 0.27	$y = 0.8447x + 1.1604$
UCSF	0.33, 0.11	$y = 1.2723x + 0.1863$	0.62, 0.38	$y = 1.2580x - 1.0661$
Louisville	0.30, 0.09	$y = 0.4872x + 3.7178$	0.56, 0.31	$y = 0.4750x + 2.4520$
Newcastle	0.34, 0.12	$y = 0.9184x + 3.0793$	0.54, 0.29	$y = 0.7451x + 2.5902$

Comparison of correlation coefficient (r), coefficient of determination (r^2), and trendline equations for each algorithm.

* $n = 14$; 64% male, 36% female.

† $n = 12$; 75% male, 25% female.

added genetic information may justify the longer TAT and cost of the AutoGenomics platform or may dictate development of more efficient assays with more complete genomic information.

Dosing Algorithms

Development of testing in a clinical laboratory includes not only analytic validation of testing platform, but also test interpretation and guidance for clinicians. Several groups have devised dosing algorithms through regression analysis of the relative effect of *CYP2C9* and *VKORC1* variants and various demographic and clinical factors on warfarin dose. These studies have indicated that *VKORC1* and *CYP2C9* genotype may account for up to 40% of warfarin dose variance, and genotype in addition to other known factors may account for 50 to 80% of warfarin dose variance seen in patients with effective anticoagulation.^{14–16,19–23} Thus, genetic variability appears at least as important as other factors that are currently considered by clinicians when dosing warfarin.

To incorporate warfarin response genotyping at our institution, we analyzed whether four available dosing algorithms could accurately predict warfarin dose in a local patient population. There was a good linear relationship between actual warfarin dose and predicted dose for each of the algorithms. The UCSF and Wash U algorithms demonstrated the best correlation with actual warfarin dose, with correlation coefficients (r) of 0.60 in the total patient population and 0.67 after the removal of two outlier patients. Both of these algorithms include ethnicity, smoking status, and the presence of a *CYP2C9* inhibitor as factors whereas the other algorithms do not, which may be why they performed somewhat better. The Newcastle algorithm performed the least well and tended to underdose patients, perhaps because it includes height but not body mass index or weight. Wu and colleagues¹⁴ compared several dosing algorithms in a multiethnic American population and found a similar correlation with actual warfarin dose as in our study ($r = 0.64$ to 0.68) with similar worse correlation for the Newcastle algorithm ($r = 0.55$). Although the Wash U algorithm requires that more clinical information be gathered on each patient, it did not perform better than the UCSF algorithm in our population. The web-based format of the Wash U algorithm is convenient and easily accessible, which may make it more easily adaptable by a wide group of clinicians.

After removal of the two outlier data points, 45% of warfarin dose variance (r^2) was explained by the genetic

and clinical factors included in the Wash U and UCSF algorithms and less than 40% by the other two algorithms. This is more than the variance in dose that has been attributed to clinical factors alone, but less than that reported by other groups. It is unclear why these algorithms explain less of the warfarin dose variance in our study population than in the populations studied by the groups that derived the algorithms. There may be factors unique to different geographic or demographic groups that affect the efficacy of dosing algorithms derived from a specific patient population. Wu and colleagues¹⁴ predictably found the best correlation with warfarin dose using the training set of patients that was used to derive their own algorithm. Because ours was a small retrospective analysis, there may have been missing or confounding factors that were not taken into account, or the results may be skewed by individual patients. This may simply indicate the better performance of such an approach in a large group of patients than in an individual patient.

African-American Patients

None of the algorithms predicted warfarin dose very well in the African-American subgroup, although the data were skewed by the two outlier patients on high doses of warfarin. However, even after removal of the two outlier African-American patients, the predicted warfarin doses correlated less well with actual warfarin dose in this subgroup than for the total population. The UCSF algorithm, which was derived in a multiethnic population, performed somewhat better in this subgroup, although the number of patients was too small to make statistically rigorous conclusions. Only the Wash U and UCSF algorithms incorporate ethnicity as a factor. Although part of difference in warfarin response in different ethnic groups may be explained by genetic factors, the spectrum of causative genetic differences is not well understood and there are almost certainly important environmental factors that differ between ethnic groups. Accordingly, a recent study indicated that African-American patients require higher warfarin maintenance doses even after adjusting for known clinical and *CYP2C9-VKORC1* genetic covariants.²⁴

Because many of the studies of the effect of genotype on warfarin dose have been performed primarily in European Caucasians, genetic variants more common in other ethnic groups may be ignored. Thus, *CYP2C9* variants other than *2C9* *2 and *3 that affect warfarin metabolism are not incorporated (eg, *CYP2C9* *4, *5, *6, and *11), nor are *VKORC1* haplotypes other than those com-

mon in Caucasians, such as H3 to H6 and other rarer haplotypes that have been described.¹⁴ Many of these variants are more common in African-Americans. The UCSF multiethnic study, which included Caucasian, Asian, Hispanic, and African-American populations, found a significant effect of the *VKORC1* SNP 2255(7566) C, which is seen in haplotypes H3 and H6 to H9 and was associated with higher doses of warfarin.¹⁴ However haplotypes H3 to H6 were grouped together and were not analyzed individually because of their low frequency. Further studies are needed in African-American and other non-European Caucasian populations to understand the relative effect of genetic and other parameters on warfarin dose requirements.

Warfarin Resistance

The explanation for the high warfarin dose requirement in the two African-American outlier patients is not known. *VKORC1* SNP analysis indicated that each was heterozygous for *VKORC1* H3 or H4, haplotypes reported to be more common in African-Americans and which may require higher doses of warfarin.¹⁴ However, this is unlikely to explain the extremely high doses of warfarin required by these patients. At least six different mutations in the *VKORC1* coding region have been described that are associated with warfarin resistance, possibly because of altered binding of warfarin.^{25–32} Examination of the *VKORC1* coding region for mutations in these patients was negative. The effect of the novel promoter region variant found in one of the patients is unknown, although it is possible that this change could result in higher expression of VKOR and higher dose requirements.

Currently, the only genetic variants that are included in the Food and Drug Administration warfarin package insert and incorporated by most of the dosing algorithms are those associated with increased sensitivity to warfarin (*CYP2C9* *2 and *3, *VKORC1* –1639/3673 G>A). Therefore patients with *VKORC1* variants associated with high dose requirements of warfarin may not be captured by current practice. The –1639(3673) SNP, which is seen in *VKORC1* group A haplotypes (H1 and H2) as well as H5, has consistently been associated with significantly lower dose requirements of warfarin, possibly because of decreased expression of the VKOR protein.¹ It is possible that *VKORC1* –1639 (or a linked SNP) is the only relevant functional SNP and explains all of the variability in warfarin response attributable to *VKORC1* haplotype. However, other possible explanations are that the effect of other variants did not reach statistical significance in some studies because they were rare in the populations studied or because they were not analyzed. Wu and colleagues¹⁴ found that elimination of cases with rare genotypes improved the correlation between actual warfarin dose and dose predicted by various algorithms, indicating that rare variants may have an effect on warfarin dose that is currently not incorporated in such dosing algorithms.

The significance of nongroup A *VKORC1* haplotypes requires further investigation. Group B haplotypes (H7 to

H9) have been correlated with higher dose requirements of warfarin.^{1,14} Haplotypes H3, H4, and H6, which are more common in African-Americans, have also been correlated with higher doses of warfarin,¹⁴ but require further study in a larger group of patients with these haplotypes. In addition, patients with certain *VKORC1* coding region mutations may require very high warfarin doses exceeding 10 mg/day.^{25–32} One of these, the Asp36Tyr mutation, has been reported to be a common variant in Ashkenazi and Ethiopian Jews.^{29,33} We sequenced the *VKORC1* coding region for all patients on high doses of warfarin (>10 mg/day) from the 117 patients in the University of North Carolina study and from a similar study of Brazilian patients. Although none of the nine University of North Carolina patients on >10 mg/day of warfarin had coding region mutations, 2 of 11 Brazilian patients on >10 mg/day of warfarin had a Val66Met mutation (unpublished data), which has previously been reported to be associated with warfarin resistance.^{26,32} Thus, variants in the VKOR coding region associated with extreme warfarin resistance may be common in certain populations, but are not currently included in any of the available genotyping test platforms or dosing algorithms.

Future Directions

The question remains—how will genetic information affecting warfarin response be used clinically? Although there is a well-documented relationship between specific *CYP2C9* and *VKORC1* variants and increased sensitivity to warfarin, the clinical utility of testing for these variants in determining optimal warfarin dose has not been well established. It remains unclear whether dosing algorithms that predict final warfarin dose in patients on stable long-term therapy will be effective in patients initiating warfarin therapy. Several clinical trials have been performed to investigate whether incorporation of genotype in warfarin dosing results in better patient management. Genotype-guided dosage has been associated with fewer dosage changes, faster time to target INR, more time in target INR, and a decrease in out of range INRs in some studies.^{12,13,23,34–38} However, overall the results of these studies have been inconclusive and warfarin genotyping has not yet been incorporated into daily practice. It is unclear whether complicated dosing algorithms will be widely adopted by the bulk of prescribing physicians. Perhaps a more practical approach would be for clinicians to take genotype information into consideration along with other factors when dosing warfarin empirically, for example using an incremental reduction in empirical starting dose based on the number of sensitivity variants present. Those patients that will require the greatest dose modification are those that are homozygous for or have multiple relevant genetic variants, which represent a small proportion of the population.

There is still much to be done in the field of pharmacogenomics of warfarin dosage. Further studies to elucidate the effect of genotypes more common in non-Caucasian ethnic groups and to identify other genes that may have an effect on warfarin response are needed. In ad-

dition, there is a need for additional clinical trials to demonstrate whether there exists clear clinical utility of genotype-guided dosing and if so, the formulation of easily implementable recommendations for dosing modification.

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