The Journal of Molecular Diagnostics, Vol. 22, No. 7, July 2020

ELSEVIER

the Journal of Molecular Diagnostics
jmd.amjpathol.org

SPECIAL ARTICLE

Recommendations for Clinical Warfarin Genotyping Allele Selection

A Report of the Association for Molecular Pathology and the College of American Pathologists

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Accepted for publication
April 1, 2020.

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The goal of the Association for Molecular Pathology (AMP) Clinical Practice Committee's AMP Pharmacogenomics (PGx) Working Group is to define the key attributes of PGx alleles recommended for clinical testing and a minimum set of variants that should be included in clinical PGx genotyping assays. This document series provides recommendations for a minimum panel of variant alleles (tier 1) and an extended panel of variant alleles (tier 2) that will aid clinical laboratories when designing assays for PGx testing. The AMP PGx Working Group considered functional impact of the variants, allele frequencies in multiethnic populations, the availability of reference materials, as well as other technical considerations for PGx testing when developing these recommendations. The ultimate goal is to promote standardization of PGx gene/allele testing across clinical laboratories. These recommendations are not to be interpreted as prescriptive but to provide a reference guide. Of note, a separate article with recommendations for *CYP2C9* allele selection was previously developed by the PGx Working Group that can be applied broadly to *CYP2C9*-related medications. The warfarin allele recommendations in this

Standard of practice is not defined by this article, and there may be alternatives. See *Disclaimers* for further details.

The Pharmacogenomics Working Group of the Clinical Practice Committee, Association for Molecular Pathology (AMP), with organizational representation from the College of American Pathologists (A.M.M.) and the Clinical Pharmacogenetics Implementation Consortium (M.W-C.). The AMP 2018 and 2019 Clinical Practice Committee consisted of Antonia Sepulveda (2018 Chair), Daniel Jones (2019 Chair), Jess Peterson, Josh Deignan, Pinar Bayrak-Toydemir, Jianling Ji, Keyur Patel, Noah A. Brown, Marian Harris, Kandelaria Rumilla, Pranil Chandra, Jonathan Earle, Susan Butler-Wu, Kenneth L. Muldrew, Daniel Cohen, Mark Boguski, Justin Zook, Annette Meredith, Alex Greninger, Megan Wachsmann, and Celeste Eno.

Supported exclusively by the Association for Molecular Pathology.

Disclosures: The Indiana University School of Medicine Pharmacogenomics Laboratory, University of North Carolina Medical Genetics Laboratory, Millennium Health, Mayo Clinic Laboratories, and Sema4 are fee-for-service clinical laboratories that offer clinical pharmacogenetic testing. V.M.P. is supported by the Implementing Genomics in Practice project grants U01 HG007762 and HG010245 and the Indiana University Health—Indiana University School of Medicine Strategic Research Initiative. A.L.D. is employed by Millennium Health, LLC. S.A.S. is employed by Sema4. H.H. is an active employee and a stockholder of Translational Software, a pharmacogenomic interpretative service. M.W.C. is a member of the Clinical Pharmacogenetics Implementation Consortium. A.M.M. is a member of the College of American Pathologists/American College of Medical Genetics and Genomics Biochemical and Molecular Genetics Committee and Pharmacogenetics Workgroup.

https://doi.org/10.1016/j.jmoldx.2020.04.204

report incorporate the previous *CYP2C9* allele recommendations and additional genes and alleles that are specific to warfarin testing. *(J Mol Diagn 2020, 22: 847–859; https://doi.org/10.1016/j.jmoldx.2020.04.204)*

Clinical pharmacogenomics (PGx) testing assays can vary significantly between laboratories. Some assays test for a single pharmacogene, whereas others test for several thousand variants in a variety of pharmacogenes. The star (*) alleles or haplotypes detected and reported for each pharmacogene can also differ between testing laboratories, and the variants used to define those PGx haplotypes may not be consistent. Results from a study by the Centers for Disease Control and Prevention's Genetic Testing Reference Materials Coordination Program revealed the variability of variant alleles included in assays used to test for PGx genes and demonstrated that differences in assay design or variant/allele selection can result in discrepant genotyping results.[1] This variability in PGx alleles tested by different clinical laboratories can also lead to discordant interlaboratory quality assessments [eg, College of American Pathologists (CAP) PGx proficiency testing (PT) surveys].[2] As such, this clinical genotyping variability can result in inconsistencies in reported haplotype and diplotype assignment, which may impact phenotype prediction, test interpretation, and ultimately patient care.

To facilitate standardization of clinical PGx testing, the Association for Molecular Pathology (AMP) PGx Working Group is developing a series of documents that recommend a minimum set of variant alleles to include in clinical PGx assays. The current document communicates a set of alleles to include in clinical warfarin-associated genotyping panels. These recommendations are intended to provide guidance to clinical laboratory professionals and assay manufacturers who develop, validate, and offer clinical PGx assays, with the goal of promoting standardization of PGx testing across clinical laboratories. This series of AMP PGx Working Group documents should be implemented together with other clinical guidelines, such as those issued by the Clinical Pharmacogenetics Implementation Consortium (CPIC), which focus primarily on the interpretation of PGx test results and therapeutic recommendations for specific drug-gene pairs.[3]

The AMP PGx Working Group has previously developed recommendations for clinical *CYP2C9* testing that are intended to be applied to *CYP2C9*-related medications, including warfarin.[4] As there are additional well-characterized genes/alleles contributing to interindividual variation in warfarin sensitivity, the Working Group felt the need to develop a separate document for genes/alleles specifically related to warfarin sensitivity, including the *CYP2C9* alleles. The current document suggests alleles for inclusion in clinical genotyping panels for warfarin sensitivity and defines the key attributes of those alleles. The AMP PGx Working Group has developed a two-tier strategy and selection criteria for recommending PGx variant alleles for clinical testing. Tier 1 PGx variant alleles are a minimum set of alleles recommended for clinical testing, whereas tier 2 variant alleles are additional alleles that do not meet tier 1 criteria but that may be considered for clinical testing (see *Materials and Methods* for details). A description of the rationale for these clinical PGx genotyping recommendations and the development of this two-tier classification strategy has been previously described in the *CYP2C19* and the *CYP2C9* recommendation documents by this PGx Working Group.[4,5] Of note, common benign variants in high linkage disequilibrium (LD) with established functional variant(s) are not currently being considered for inclusion as tier 1 or tier 2 variant alleles in routine clinical PGx genotyping panels.

Drug: Warfarin

Warfarin (brand names of Coumadin, Jantoven, and others), a coumarin anticoagulant, is one of the most widely prescribed drugs[6] and is administrated orally as a racemic mixture of *S*-warfarin and *R*-warfarin. The effects of warfarin and other coumarin anticoagulants are derived from inhibiting the activation of the vitamin K–dependent clotting factors. Warfarin specifically inhibits vitamin K epoxide reductase, an enzyme responsible for conversion of vitamin K epoxide to the reduced form of vitamin K, which is the rate-limiting step in vitamin K recycling. The reduced form of vitamin K serves as a necessary cofactor for carboxylation and activation of coagulation factors II, VII, IX, and X.[7] Warfarin has a narrow therapeutic index, and as such, a small variation in plasma concentrations can lead to concentration-dependent adverse drug reactions, such as bleeding or lack of efficacy. Clinically, each individual's warfarin dose is tailored according to the international normalized ratio (INR), with a target INR of 2 to 3 for most indications. Genetic variants, together with other variables, such as age, body size, dietary vitamin K intake, comorbidities, and concomitant medications, contribute to warfarin sensitivity and the optimal dose required to achieve the desired level of anticoagulation.

Genes: *CYP2C9*, *VKORC1*, and *CYP4F2*

CYP2C9

The cytochrome P450 2C9 (*CYP2C9*) is a member of the CYP2C subfamily of the cytochrome P450 enzymes and is the major metabolizing enzyme for *S*-warfarin and several other widely prescribed medications. A significant association between variant *CYP2C9* star (*) alleles and warfarin dose requirements has been well established, and *CYP2C9* testing is routinely included in PGx testing for warfarin

sensitivity. The AMP PGx Working Group has previously developed recommendations for clinical *CYP2C9* testing that can be applied to *CYP2C9*-related medications, including warfarin.[4] In brief, on the basis of allele function and frequency and availability of reference materials, the Working Group recommended *CYP2C9*2*, **3*, **5*, **6*, **8*, and **11* to be included in the tier 1 variant allele list; and *CYP2C9*12*, **13*, and **15* to be included in the tier 2 variant allele list.[5]

VKORC1

The vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene is located on chromosome 16p11.2 and encodes the enzyme target of warfarin. Numerous studies have demonstrated that genetic variants in *VKORC1* are significantly associated with warfarin sensitivity or resistance. The common *VKORC1* promoter variant allele c.-1639G>A (NM_024006.5:c.-1639G>A; *rs9923231*) has been widely studied and strongly associated with warfarin sensitivity and predicting warfarin dose requirements.[8–13] There are other common *VKORC1* variants associated with warfarin sensitivity; however, these variants and haplotypes were not found to improve warfarin dose prediction beyond c.-1639G>A.[11,13] In contrast, *VKORC1* missense variants have been implicated in warfarin resistance and confer higher warfarin dose requirements to achieve adequate anticoagulation.[14,15]

VKORC1 is currently included in the Food and Drug Administration (FDA) Table of Pharmacogenetic Biomarkers for warfarin (*https://www.fda.gov/Drugs/ScienceResearch/ucm572698.htm*, last accessed July 11, 2019). Most clinical laboratories that offer PGx testing include *VKORC1* c.-1639G>A and/or the intronic NG_011564.1:g.6399C>T (ie, 1173C>T; *rs9934438*) variant, which is in high LD with the *VKORC1* c.-1639G>A variant.

CYP4F2

The cytochrome P450 4F2 (*CYP4F2*) enzyme is a member of the CYP4F subfamily of the cytochrome P450 enzymes that is primarily expressed in the liver and kidneys. Its substrates include fatty acids, eicosanoids, vitamin E, and vitamin K. This enzyme is encoded by the *CYP4F2* gene on chromosome 19p13.12. CYP4F2 acts in counterpart to VKORC1 as it limits the accumulation of vitamin K by converting it to hydroxyl-vitamin K1.[16] A single variant in *CYP4F2*, the *CYP4F2*3* allele (NM_001082.4:c.1297G>A; p.Val433Met; *rs2108622*), is associated with a modest increase in warfarin dosing in white and Asian populations, but not among those with African ancestry.[16,17]

CYP4F2 is not currently included as a PGx biomarker in the FDA-approved labeling information for warfarin. However, some clinical laboratories may test for the *CYP4F2*3* allele in addition to *CYP2C9* and *VKORC1* variants, and the 2017 CPIC warfarin dosing guideline includes dosing recommendations based on *CYP4F2* genotype when results are available.[14,18]

Clinical Testing

According to the NIH Genetic Testing Registry (*https://www.ncbi.nlm.nih.gov/gtr*, last accessed July 11, 2019), warfarin sensitivity testing is offered as either single-gene tests or in panels of *CYP2C9* and *VKORC1* with or without additional genes, such as *CYP4F2*. Warfarin testing is also commonly included in clinical PGx panel tests that include genes associated with response to many medications. Not only is the inclusion of specific genes/variants variable across different laboratory tests, but the methods of genotyping can also vary, ranging from targeted genotyping of a few variants to sequencing of the entire coding region or selected exons. A variety of techniques may be employed, including PCR with hydrolysis probes, microarray, PCR with allele-specific hybridization, bidirectional Sanger sequencing, next-generation sequencing, or massive parallel sequencing with or without deletion/duplication analysis. Although an increasing number of laboratories use next-generation sequencing as a platform for detecting PGx variants, targeted variant analysis is still the preferred approach, because it can help to avoid the challenges of interpreting and reporting variants of uncertain clinical significance in the context of PGx indication. Several important pharmacogenes or loci, including *CYP2D6* locus on chromosome 22 and the human leukocyte antigen alleles, cannot be reliably sequenced by next-generation sequencing, although bioinformatics tools are being developed to assist with the diplotype and haplotype assignment. In addition, warfarin sensitivity–related PGx variants are often included in clinical exome sequencing reports from laboratories that report PGx variants as secondary findings. However, without specifying what PGx variants/genes are included in the exome sequencing capture, it is challenging to determine if the patient is negative for certain noncoding variants (ie, *VKORC1* c.-1639G>A) or whether nonreporting is due to lack of detection by the platform. Regardless of a platform a clinical laboratory chooses to use, it is important to define a minimum recommended list of PGx variant alleles in an effort to promote standardization in assay design and interpretation of PGx testing across clinical laboratories. Rare *VKORC1* variants associated with warfarin resistance are not included in most commercial targeted genotyping platforms. Moreover, the current CPIC warfarin dosing guideline does not provide recommendations for warfarin resistance variants. However, if sequencing approaches, such as Sanger or next-generation sequencing/massive parallel sequencing, are utilized for clinical PGx testing, additional rare variants may be detected. It is at the discretion of the testing laboratory whether to evaluate the significance of any additional or novel variant(s) identified and whether to include these variants in the clinical report.

Existing Guidelines

Clinical PGx guidelines for warfarin sensitivity testing are available from other professional societies, including CPIC,[14] American College of Medical Genetics and Genomics,[19] and the Canadian Pharmacogenomics Network for Drug Safety.[20] The Dutch Pharmacogenetics Working Group, funded by the Royal Dutch Pharmacists Association,[21] has guidelines for acenocoumarol and phenprocoumon, anticoagulants that are similar to warfarin. The original CPIC warfarin dosing guideline[14] included *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* c.-1639G>A in warfarin dosing recommendations. In the 2017 updated CPIC guideline, use of *CYP2C9*5*, *CYP2C9*6*, *CYP2C9*8*, *CYP2C9*11*, and *CYP4F2*3* were included as optional recommendations for patients of non-African ancestry for dosing adjustment in addition to the dosing adjustment based on published PGx algorithms with *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* c.-1639G>A.[14] However, for patients of African ancestry in whom the *CYP2C9*5*, **6*, **8*, and **11* are most often detected, the CPIC guidelines state that genotype-based warfarin dosing should not be used to guide warfarin dosing unless these additional *CYP2C9* alleles are tested. The guidelines have a moderate recommendation to reduce warfarin dose when one of these alleles is detected, but there is no recommendation to include *CYP4F2* genotype for individuals of African ancestry. If a patient is African American and the recently reported *CYP2C* gene cluster variant *rs12777823* is detected, there is a moderate recommendation for additional warfarin dose reduction on the basis of the available evidence for the effect of this variant on warfarin dose requirements and clearance in African Americans.[22] The Canadian Pharmacogenomics Network for Drug Safety currently only recommends *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* c.-1639G>A genotype-based PGx dosing algorithms and does not provide recommendations for *CYP2C9*5*, *CYP2C9*6*, *CYP2C9*8*, *CYP2C9*11*, or *CYP4F2*3* alleles.[20]

The 2008 American College of Medical Genetics and Genomics Policy Statement on PGx testing of *CYP2C9* and *VKORC1* alleles for warfarin did not include a recommendation for or against routine *CYP2C9* and *VKORC1* genotyping, because of the lack of strong evidence available at that time to support the association with clinical outcomes.[19] However, it stated that *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* c.-1639G>A genotypes can be used as part of the workup for an unusually low maintenance dose of warfarin or an unusually high INR during standard dosing.

Materials and Methods

The AMP PGx Working Group, including subject matter expert representatives from the clinical PGx testing community, CPIC, and CAP, evaluated genes that have previously been reported to impact warfarin sensitivity and/or response (see *Results*). Genes that are known to be clinically tested to predict warfarin response, those included in the existing dosing guidelines, and genes described in the literature as potentially having an impact on warfarin response were considered. Alleles/variants within the selected genes were then categorized on the basis of the three criteria: i) the allele/variant is well characterized and known to be functionally significant, leading to an alteration in a drug response phenotype, ii) presence at an appreciable allele frequency in at least one population, and iii) reference materials (RMs) are publicly available. Each of these three criteria received equal weight during the group's deliberations. Variants/alleles meeting all three criteria were included in tier 1, whereas those that meet at least one but not all three were considered for inclusion in tier 2.

Allele function information was derived from literature searches as well as the CPIC/PharmGKB Allele Function Tables found by gene (*https://www.pharmgkb.org/page/pgxGeneRef*, last accessed July 11, 2019). Population frequency information was cited from the 1000 Genomes Project (*http://www.internationalgenome.org/1000-genomes-browsers*, last accessed July 11, 2019), Genome Aggregation Database (*https://gnomad.broadinstitute.org*, last accessed July 11, 2019), and the CPIC/PharmGKB Frequency Tables found by gene at the aforementioned URL. Data from the Get-RM project[1] were queried to identify RMs. Information regarding specific cell lines/RMs that were identified for *VKORC1* and *CYP4F2* is included in Table 1, whereas information regarding RMs for *CYP2C9* is available in a prior publication.[4]

To identify alleles that may be commonly tested, a list of genes/variants included on commercially available platforms was also generated using data from manufacturer websites (Table 2). Similar data for *CYP2C9* were previously published.[4] Although the PGx Working Group attempted to include as many platforms as possible, this may not represent a complete list and inclusion does not indicate endorsement of any particular product, service, or vendor. In addition to commercially available platforms, laboratories may offer additional alleles as part of a laboratory-developed test, which are not captured in Table 2.

Results

The AMP PGx Working Group reviewed additional genes associated with warfarin activity (eg, *CALU* and *GGCX*) and focused the recommendations on genes that were supported by clinical guidelines (eg, CPIC and Dutch Pharmacogenetics Working Group) and/or included in the FDA drug labels on the basis of the strength of their clinical evidence. Variants in genes not supported by guidelines were not considered for tier 1 or tier 2 placement. The alleles or variants recommended for inclusion in tiers 1 and 2 are shown in Tables 3 and 4, respectively, along with previous recommendations for *CYP2C9* allele selection from the AMP PGx Working Group.[4]

Tier 1 Variant Allele: *VKORC1* c.-1639G>A

The *VKORC1* c.-1639G>A allele alters a promoter transcription factor binding site and results in lower gene expression and reduced VKORC1 protein expression.[10,23] The *VKORC1* c.-1639G>A variant has an allele frequency ranging from 41% to 47% in the white (European + North American) and Middle Eastern populations, whereas it is much higher (approximately 88%) in East Asian populations but lower in populations of African ancestry (approximately 13%) and South/Central Asian populations (approximately 15%) (*https://www.pharmgkb.org/chemical/PA451906/guidelineAnnotation/PA166104949*, last accessed July 11, 2019). Other common *VKORC1* variants and haplotypes tested were not found to improve warfarin dose predictions beyond the c.-1639G>A variant.[11,13] However, another *VKORC1* variant (commonly referred to as 1173C>T; NG_011564.1:g.6399C>T; *rs9934438*) can also be informative as it is in high LD with c.-1639G>A in most populations.[13] Although such variant(s) can be used/tested as part of the clinical PGx panel as tag single-nucleotide polymorphisms for the functional variant, they are not considered or listed as either tier 1 or tier 2 alleles because their linked functional variants have been included in tier 1 or 2.

Tier 2 Variant Alleles

*CYP4F2*3*

The alleles or variants recommended for inclusion in tier 2 are shown in Table 4. The *CYP4F2*3* allele is defined by a missense variant in exon 11 (NM_001082.4:c.1297G>A; p.Val433Met; *rs2108622*). The effect of this variant on CYP4F2 function is not fully understood.[24–26] It is associated with reduced enzyme activity, but it is not known how the variant causes loss of enzyme function. The reduced enzyme activity may be due to reduced protein levels, either because of decreased protein translation or increased protein degradation.[25] Its overall minor allele frequency (MAF) is approximately 27% (range, 10% to 40%; *https://gnomad.broadinstitute.org/variant/19-15990431-C-T*, last accessed July 11, 2019) (Table 4). Among different ethnic groups, the allele frequency ranges from 30% to 43% in white (European + North American), Middle Eastern, and South/Central Asian populations, but is lower in individuals of East Asian (approximately 22%) and African (approximately 8%) ancestry. (*https://www.pharmgkb.org/chemical/PA451906/guidelineAnnotation/PA166104949*, last accessed July 11, 2019). This allele is associated with a modest increase in warfarin dosing requirements and can be beneficial in improving warfarin dosing requirements in some ethnic groups, such as whites and Asians, but not in Africans, possibly because of its low frequency in this population.[16,17] Because of the unclear functional impact of the variant, including its unexplained ancestry-specific nature, the Working Group agreed that it did not fulfill the tier 1 requirement of a well-characterized functional allele (ie, the allele/variant is well characterized and known to be functionally significant, leading to an alteration in a drug response phenotype).

Table 1 Current Publicly Available Reference Materials for *VKORC1* and *CYP4F2*

Allele	Coriell number (diplotype)
VKORC1 rs9923231	NA12236 (*A/A*)
VKORC1 rs9923231	NA11839 (*G/A*)
*CYP4F2*3*	NA11839 (**1/*3*)
*CYP4F2*3*	NA17679 [(**2*)/**3*]
*CYP4F2*3*	NA07029 (**3/*3*)

This is not a comprehensive list. Inclusion herein does not represent an endorsement of any product or service by the Association for Molecular Pathology. For a complete list, see the Centers for Disease Control and Prevention's Genetic Testing Reference Materials Coordination Program website (*https://wwwn.cdc.gov/clia/Resources/GETRM/default.aspx*, last accessed July 11, 2019).[1] Alleles in parentheses were those observed but not independently confirmed.[1]

VKORC1 c.196G>A (p.Val66Met) and c.106G>A (p.Asp36Tyr)

Also included in tier 2 are selected *VKORC1* coding variants that have been associated with warfarin resistance and/or higher therapeutic warfarin dose requirements: p.Val66Met (NM_024006.5:c.196G>A; *rs72547529*) and p.Asp36Tyr (NM_024006.5,c.106G>A; *rs61742245*).[15,27–32] These missense variants are seen in >1 in 1000 individuals in at least one human subpopulation in the Genome Aggregation Database (*https://gnomad.broadinstitute.org*, last accessed July 11, 2019). The p.Val66Met variant is observed in approximately 0.3% of individuals of African descent, whereas the p.Asp36Tyr is present in approximately 4% of Ashkenazi Jewish individuals, and it has been reported in 15% of the Ethiopian population and in 2.5% to 7% of other Northeast African populations.[33–36] There is phylogenetic evidence that the *VKORC1* p.Asp36Tyr variant may have arisen on a common ancestral haplotype in the Northern African population.[35] Because RMs are not available for either variant, they are currently classified as tier 2 alleles. This categorization is subject to change should reference materials become available.

CYP2C Cluster *rs12777823*

A genome-wide association study in African Americans identified a novel association between the *rs12777823* variant, located on chromosome 10 in the *CYP2C* cluster adjacent to *CYP2C18*, and warfarin response.[22] The variant occurs commonly in African Americans, with an MAF of 25% (multiethnic allele frequency 0% to 30%; *https://gnomad.broadinstitute.org/variant/10-96405502-G-A*, last accessed July 11, 2019). Although also common in other populations, the variant was not associated with warfarin dose requirements in white European, Japanese, or Egyptian patients, suggesting that it may not have functional impact but rather may be in LD with one or more functional variants in individuals of West African

Table 2 Genotyping Platforms

Variants/platforms	HGVS genomic nomenclature†	Affymetrix Pharmaco Scan (RUO)‡	Agena Bio sciences iPLEX ADME (RUO)§	Auto Genomics INFINITI (CE marked)¶	BioFire Defense (analyte-specific reagents)‖	GenMark eSensor (FDA cleared)**	TrimGen (FDA cleared)††	LifeTech TaqMan OpenArray (V and RUO)‡
VKORC1 rs9923231	NM_024006.5: c.-1639G>A	X	X	X	X	X	X	V
VKORC1 rs9934438 (1173C>T/G)	NM_024006.5: c.174-136C>T	X	X	X				V
VKORC1 rs7294 (3730G>A)	NM_024006.5:c.*134G>A	X	X	X				V
VKORC1 rs8050894 (6853G>C)	NM_024006.5:c.283+124G>A/C	X	X	X				V
VKORC1 rs61162043 (-8191)	(GRCh37.p13 chr 16) NC_000016.9:g.31114234A>G		X					
VKORC1 rs17884388	(GRCh37.p13 chr 16) NC_000016.9:g.31111064A>G	X						V
VKORC1 rs17878544	NM_024006.5:c.-1877A>G	X						V
VKORC1 rs104894539	NM_024006.5:c.85G>T, p.Val29Leu	X	X					V
VKORC1 rs61742245	NM_024006.5: c.106G>T, p.Asp36Tyr	X	X					V
VKORC1 rs104894540	NM_024006.5:c.134T>C, p.Val45Ala	X	X					V
VKORC1 rs104894541	NM_024006.5:c.172A>G, p.Arg58Gly	X	X					V
VKORC1 rs13337470	NM_024006.5:c.173+486C>A	X						V
VKORC1 rs13336384	NM_024006.5:c.174-429C>T	X						V
VKORC1 rs72547529	NM_024006.5: c.196G>A, p.Val66Met	X	X					V
VKORC1 rs17886199	NM_024006.5:c.283+186T>C	X	X					V
VKORC1 rs17884850	NM_024006.5:c.283+231G>A	X						V
VKORC1 rs17884982	NM_024006.5:c.284-882A>G/T	X						V
VKORC1 rs72547528	NM_024006.5:c.292C>T/G, p.Arg98Trp/Gly	X						V
VKORC1 rs7200749	NM_024006.5:c.358C>T, p.Leu120Leu	X	X					V
VKORC1 rs11540137	NM_024006.5:c.*131C>A	X						V
VKORC1 rs104894542	NM_024006.5:c.383T>G, p.Leu128Arg	X						
VKORC1 rs17880887 (861C>A)	(GRCh37.p13 chr 16) NC_000016.9:g.31110501G>T		X					V
VKORC1 rs17708472	NM_024006.5:c.173+525C>T	X	X					V
*CYP4F2*2 rs3093105*	NM_001082.4:c.34T>G, p.Trp12Gly	X						V
*CYP4F2*3 rs2108622*	NM_001082.4: c.1297G>A, p.Val433Met	X	X					V
CYP4F2 rs2906891	NM_001082.4:c.36G>C, p.Trp12Cys	X						V
CYP4F2 rs2906890	NM_001082.4:c.38C>G, p.Pro13Arg	X						V
CYP4F2 rs3093106	NM_001082.4(CYP4F2):c.165A>G, p.Pro55Pro	X						V
CYP4F2 rs8100960	NM_001082.4:c.279A>C, p.Gly93Gly	X						V
CYP4F2 rs8110714	NM_001082.4:c.336C>A/T p.Asn112Lys/Asn	X						V
CYP4F2 rs3093136	NM_001082.4:c.348C>G/T, p.Ala116Ala	X						V
CYP4F2 rs3093153	NM_001082.4(CYP4F2):c.554G>T, p.Gly185Val	X						V
CYP4F2 rs4605294	NM_001082.4(CYP4F2):c.832C>T/G, p.Leu278Phe/Val	X						V
CYP4F2 rs2074900	NM_001082.4:c.1029C>T, p.His343His	X						V
2C cluster *rs12777823*	(GRCh37.p13 chr 10) NC_000010.10: g.96405502G>A	X						V
GGCX rs11676382	NM_000821.6:c.2084+45G>C		X					V

(*table continues*)

Table 2 (*continued*)

Variants/platforms	HGVS genomic nomenclature[†]	Affymetrix Pharmaco Scan (RUO)[‡]	Agena Bio sciences iPLEX ADME (RUO)[§]	Auto Genomics INFINITI (CE marked)[¶]	BioFire Defense (analyte-specific reagents)[‖]	GenMark eSensor (FDA cleared)**	TrimGen (FDA cleared)[††]	LifeTech TaqMan OpenArray (V and RUO)[‡]
GGCX rs12714145	NM_000821.6:c.214+597G>A	X						V
GGCX rs2592551	NM_000821.6:c.1218C>T, p.Arg406Arg	X						V
GGCX rs699664	NM_000821.6:c.974G>A, p.Arg325Gln	X						V

Variant name in parenthesis refers to legacy nomenclature.

[†]Available from *https://www.ncbi.nlm.nih.gov/snp* (last accessed March 11, 2020). Commercially available platforms as of April 2, 2019, and does not represent a comprehensive list. Inclusion herein does not represent an endorsement of any product or service by the Association for Molecular Pathology.

[‡]Thermo Fisher Scientific (Waltham, MA).

[§]Agena Bioscience (San Diego, CA).

[¶]AutoGenomics (Carlsbad, CA).

[‖]BioFire Defense, LLC (Murray, UT).

**GenMark Diagnostics (Carlsbad, CA).

[††]TrimGen Genetic Diagnostics (Sparks, MD).

CE, Conformité Européene; FDA, Food and Drug Administration; HGVS, Human Genome Variation Society; RUO, research use only; V, variable; X, present in platform

ancestry. The variant is located in an intragenic region and is not likely to be the underlying cause of the observed effect on warfarin dose, but may tag the causative variant.

Quality Assessments

PT is available commercially for some but not all the PGx alleles contained in this document (eg, CAP PGx surveys). CAP PT data were queried to determine which of the tier 1 and tier 2 alleles are currently tested by laboratories and to better understand the potential utility of this document. On the basis of data from the CAP 2017-B PGx survey, 138 laboratories (60%) responded that they perform *VKORC1* testing [c.-1639G>A (*rs9923231*) and/or c.1173C>T (*rs9934438*)]. Six laboratories (4%) do not test the c.-1639G>A variant, whereas 111 (80%) do not test the c.1173C>T variant; 21 laboratories (15%) include both variants. No data are available with regard to the number of laboratories testing *VKORC1* warfarin resistance alleles. In addition, the North American Specialized Coagulation Laboratory Association (*https://www.nascola.com/AccessibleServices/Testing*, last accessed July 11, 2019) also provides PT for *VKORC1* c.-1639G>A and c.1173C>T.

A total of 154 laboratories responded that they test *CYP2C9*.[37] Among the *CYP2C9* tier 1 variants, **2* was tested by 149 laboratories (96.75%), **3* by 153 laboratories (100%), **5* by 108 laboratories (70.13%), **6* by 101 laboratories (65.58%), **8* by 66 laboratories (42.86%), and **11* by 101 laboratories (65.58%). The *CYP2C9* tier 2 alleles are not included in CAP PT surveys. *CYP4F2* is also not currently included in the surveys.

Discussion

Warfarin has been widely used for the past six decades and remains a commonly prescribed oral anticoagulant.[38] The drug is indicated for prophylaxis and treatment of thromboembolism in numerous conditions, and interindividual variability in therapeutic dose mandates frequent INR monitoring after warfarin initiation until target anticoagulation is achieved. Since 2010, clinicians have been able to obtain *CYP2C9* and *VKORC1* genotypes and use either the FDA prescribing label or PGx dosing algorithms to define warfarin dose requirements for their patients. Although experts have contributed to establishing high-quality genotype-based recommendations for warfarin[14] and the accessibility of clinical PGx testing continues to increase, the diversity of available testing platforms and variants interrogated can lead to inconsistencies in results among laboratories. Content differences between testing panels and laboratories may result in patients receiving discordant genotyping results and dosing recommendations. Moreover, although the initial CPIC guideline recommendations included variants that are more common among whites and Asians,[39] the updated 2017 guideline incorporated additional variants that are predictors of warfarin dose requirements in patients of African descent.[14] To implement recommendations from the recent CPIC warfarin guideline, both the availability of self-reported ancestry and interrogation for specific alleles are therefore essential.

Members of the AMP PGx Working Group are among the early adopters and have accumulated substantial knowledge and expertise about PGx testing in clinical settings. This document offers a two-tier categorization of variants as an aid for designing genotyping assays that are relevant to optimization of warfarin dosing. By engaging a multidisciplinary team, the Working Group aimed to define a tier 1 minimum target list of variants to be interrogated by laboratories and to identify those tier 2 variants with gaps of knowledge requiring additional evidence or RMs before incorporation into routine clinical PGx testing. The Working

Table 3 Tier 1 Variant Alleles

Gene	Allele	Allele functional status[†]	Defining functional variant	HGVS genomic nomenclature	HGVS cDNA nomenclature	HGVS protein nomenclature	Reference material available	Multiethnic allele frequency, %
CYP2C9[‡]	**2*	Decreased function	*rs1799853*	NG_008385.1: g.8633C>T	NM_000771.3: c.430C>T	p.Arg144Cys	Yes	0–12
CYP2C9[‡]	**3*	Decreased function	*rs1057910*	NG_008385.1: g.47639A>C	NM_000771.3: c.1075A>C	p.Ile359Leu	Yes	1–11
CYP2C9[‡]	**5*	Possibly decreased function	*rs28371686*	NG_008385.1: g.47644C>G	NM_000771.3: c.1080C>A	p.Asp360Glu	Yes	0–1
CYP2C9[‡]	**6*	No function	*rs9332131*	NG_008385.1: g.15626delA	NM_000771.3: c.818delA	p.Lys273fs	Yes	0–1
CYP2C9[‡]	**8*	Possibly decreased function	*rs7900194*	NG_008385.1: g.8652G>A	NM_000771.3: c.449G>A	p.Arg150His	Yes	0–5
CYP2C9[‡]	**11*	Possibly decreased function	*rs28371685*	NG_008385.1: g.47567C>T	NM_000771.3: c.1003C>T	p.Arg335Trp	Yes	0–2
VKORC1	c.-1639G>A	Decreased gene expression	*rs9923231*	NG_011564.1: g.3588G>A	NM_024006.5: c.-1639G>A	N/A	Yes	10–88

[†]Citations for assignment of function can be found at *https://www.pharmvar.org* (last accessed July 11, 2019).
[‡]Included for completeness,[4] HGVS nomenclature (*https://www.ncbi.nlm.nih.gov/snp*, last accessed July 11, 2019).
HGVS, Human Genome Variation Society.

Group recognizes that the benefit of genotype-guided dosing for warfarin to reduce underdosing or overdosing episodes in patients from diverse ethnicities will not likely be realized unless testing panels account for appropriate clinical variants that are relevant for the ethnic groups to whom the test is offered.

CYP2C9

S-warfarin is three to five times more potent than *R*-warfarin and is metabolized to inactive metabolites by CYP2C9. To predict an accurate CYP2C9 metabolizer phenotype in a multi-ethnic population, with patients of African and non-African descent, the Working Group recommends the following reduced and nonfunctional alleles in tier 1: **2*, **3*, **5*, **6*, **8*, and **11*.[4] Data consistently demonstrate reduced warfarin dose requirements in patients who carry any of these six alleles, and their detection is predictive of reduced metabolism of *S*-warfarin. This list is aligned with the *CYP2C9* alleles that are included in the current CPIC warfarin guideline, and their inclusion in PGx warfarin dosing algorithms is easily achievable. Although clinicians are invited to use the FDA prescribing label, the label does not include dose recommendations for *CYP2C9 *5*, **6*, **8*, and **11*. Failure to account for these variants, particularly in persons of African ancestry, may lead to significant overprediction of warfarin dose requirements.[40] As such, the Working Group recommends that laboratories include all of the tier 1 alleles to serve patients with diverse ethnicities.

For laboratories that use testing technologies that allow them to interrogate for a broader range of *CYP2C9* variants, the tier 2 alleles can be considered. These alleles have been limited to those that are found to confer either reduced enzymatic function (**12* and **13*) or loss of enzymatic function (**15*). These are categorized as tier 2 alleles because they have low MAFs (<0.3%) in major ethnic groups (Table 4). In addition, *CYP2C9*13* and *CYP2C9*15* currently lack available RMs, although several candidate RMs are being developed by the Genetic Testing Reference Materials Coordination Program.

VKORC1

The Working Group recommends the common *VKORC1* c.-1639G>A promoter variant, which is significantly associated with warfarin sensitivity, as a tier 1 allele. The MAF for this variant varies among different ethnic groups (Table 3), and largely explains the differences in average dose requirements between whites, African Americans, and Asian Americans.[13]

For most ethnicities, although various studies have reported significant geographic differentiation in the observed allele frequencies for c.-1639G>A, the inclusion of this single variant is sufficient to accurately predict VKORC1 expression levels and subsequent associated warfarin sensitivity phenotype. The presence of the low-dose–associated c.-1639A allele, either as a heterozygous or a homozygous genotype, can discriminate patients belonging to high-, intermediate-, or low-warfarin dose categories.[39]

The AMP PGx Working Group recognized that in some specific populations, the existence of *VKORC1* resistance-conferring variants can be significant and can lead to underdosing. Those included in tier 2 are *VKORC1* p.Val66Met (NM_024006.5: c.196G>A) and *VKORC1* p.Asp36Tyr (NM_024006.5: c.106G>T). Additional rare missense coding variants in *VKORC1* associated with resistance to warfarin have been described.[33,41–44] However, these additional coding *VKORC1* variants were not included in tier 2 because of their rare frequencies in the

Table 4 Tier 2 Variant Alleles

Gene	Allele	Allele functional status[†]	Defining functional variant	HGVS genomic nomenclature	HGVS cDNA nomenclature	HGVS protein nomenclature	Reference material available	Multiethnic allele frequency, %
CYP2C9[‡]	**12*	Possibly decreased function	*rs9332239*	NG_008385.1: g.55363C>T	NM_000771.3: c.1465C>T	p.Pro489Ser	Yes	0–0.3
CYP2C9[‡]	**13*	Possibly decreased function	*rs72558187*	NG_008385.1: g.8301T>C	NM_000771.3: c.269T>C	p.Leu90Pro	No[§]	0–0.2
CYP2C9[‡]	**15*	No function	*rs72558190*	NG_008385.1: g.14125C>A	NM_000771.3: c.485C>A	p.Ser162Ter	No	0–0.01
CYP4F2	**3*	Possibly decreased function	*rs2108622*	NG_007971.2: g.23454G>A	NM_001082.4: c.1297G>A	p.Val433Met	Yes	10–40
VKORC1		Warfarin resistant	*rs72547529*	NG_011564.1: g.6557G>A	NM_024006.5: c.196G>A	p.Val66Met	No[§]	0–0.25
VKORC1		Warfarin resistant	*rs61742245*	NG_011564.1: g.5332G>T	NM_024006.5: c.106G>T	p.Asp36Tyr	No[§]	0–3.8
2C cluster		Unknown; variant in linkage disequilibrium with warfarin effect in individuals of West African ancestry	*rs12777823*	NC_000010.10: g.96405502G>A			No[§]	0–30

[†]Citations for assignment of function can be found at *https://www.pharmvar.org* (last accessed February 20, 2019).
[‡]Included for completeness,[4] HGVS nomenclature (*https://www.ncbi.nlm.nih.gov/snp*, last accessed July 11, 2019).
[§]Genetic Testing Reference Materials Coordination Program reference material verification study in process.
HGVS, Human Genome Variation Society.

general population (<0.1%) and the lack of available RMs. Although sequencing assays would detect these variants in large populations, the AMP PGx Working Group does not recommend them for inclusion in routine clinical PGx genotyping platforms at this time.

CYP4F2

A single *CYP4F2* variant, *CYP4F2*3*, was recommended by the Working Group for inclusion in tier 2 at this time. This variant's function is not well defined as the variant's effect on enzyme function is not known. In addition to the well-studied *CYP2C9* and *VKORC1* variants, *CYP4F2* genotypic variations account for a small but significant proportion of the variability in warfarin dose in whites and Asians[17,45] but not in African Americans or Egyptians.[45,46] More specifically, the *CYP4F2*3* allele was associated with warfarin dose in three independent cohorts of white patients, with carriers requiring increased doses because of decreased function of the CYP4F2 enzyme.[17] Because of the differences in the frequency of *CYP4F2*3* among major ethnic groups, the potential clinical benefit of this variant appears to vary by ancestry. Accordingly, from a population perspective, the expected contribution of this variant to warfarin dosing in patients with African ancestry is likely to be less than in whites and Asians. *CYP4F2*3* was categorized as tier 2 because the functional status is not well defined and appears to vary by ancestry.

CYP2C Cluster Variant

The PGx Working Group examined the clinical relevance of the variant *rs12777823*, which has not been fully characterized, located within the *CYP2C* gene cluster upstream of *CYP2C18* on chromosome 10q23. Although its location indicates that it may affect *CYP2C9* transcription, its clinical significance in the general population is not straightforward. Presence of this variant has been associated with reduced warfarin dose requirements in African Americans (MAF, 25%) with predominantly West African ancestry, but not in European Americans (MAF, 14%)[22] or other African populations.[22] Thus, it is possible that this variant may be in LD with another functional variant (or variants) in African Americans but not in white Americans or Africans. According to the latest CPIC guideline, the inclusion of genotype results for this emerging variant can be considered for testing African Americans, the only group in whom it has been associated with a noticeable clinical effect on warfarin dose.[14] The evidence reviewed for the CPIC guideline was limited and included data from two cohorts.[22] Further studies showed that addition of this variant improves the dosing algorithm published by the International Warfarin Pharmacogenetics Consortium in African Americans.[12] The intergenic location of this variant complicates the characterization of its functional effects. Although pharmacokinetic analysis showed reduced *S*-warfarin clearance in individuals who have the *rs12777823 A* allele,[22] additional studies are

warranted to evaluate the exact biological function(s) it regulates. Moreover, it was reported that the *rs12777823 A* allele occurred disproportionately more often along with a *CYP2C9*5*, **6*, **8*, or **11* allele, but many African Americans who require lower warfarin doses have this variant in the absence of the *CYP2C9*5*, **6*, **8*, or **11* allele.[40]

In summary, the PGx Working Group concluded that inclusion of the *rs12777823* variant in tier 1 was not warranted at this time, primarily because the mechanism underlying its association with warfarin dose requirements in African Americans is unknown. The Working Group recognizes its role in improving dose predictions in African Americans and classified it as a tier 2 variant. The ethnic-dependent clinical effect observed with the *rs12777823* variant is likely to reflect a complex haplotype structure of the *CYP2C* locus, which requires further investigation. If interrogated, laboratories may consider reporting the *rs12777823* genotype results for every individual tested, but it is recommended that they specifically indicate that its clinical effect (lower dose requirement) is applicable only to African American individuals. Most clinicians may not be familiar with this variant, and its interpretation could further get complicated by the fact that commonly used warfarin genotype-based dosing algorithms do not include this variant. A new genotype-based dosing algorithm that incorporates *rs12777823* along with several specific *CYP2C9* alleles with higher frequency in African descent populations has been proposed recently and may serve as a useful tool in African Americans.[47]

Other Genes/Variants

The PGx Working Group also reviewed *GGCX* and *CALU* genes as possible candidates for clinical testing recommendations. Given the role of γ-glutamyl carboxylase (GGCX) and calumenin (CALU) in the activation of vitamin K−dependent clotting factors, these genes have been interrogated for their effects on warfarin dose requirements. The GGCX enzyme carboxylates protein-bound glutamate residues to catalyze the biosynthesis of vitamin K−dependent clotting factors.[48] CALU functions as a chaperone of the γ-carboxylation system.[49] Rare *GGCX* variants lead to coagulation factor deficiency,[48] whereas more common *GGCX* variants, including *rs699664*, *rs12714145*, and *rs11676382*, have been shown to impact warfarin dose requirements in several populations, but the data are inconsistent.[50,51] The intronic *CALU rs339097* (NM_001199671.1:c.606+133A>G) variant is common in populations of African descent (MAF, 11% to 14%) but rare in European populations (MAF, <1%).[52] The minor *rs339097 G* allele has been associated with higher warfarin dose requirements in African Americans and Egyptians, but not in those of European ancestry,[51] likely because of its low frequency in this population. *CALU* variants may be included as a tier 1 or 2 variant in the future if shown to significantly contribute to dose requirements after consideration of more recently discovered variants (eg, *rs12777823*).[14] Because these genes are not included in the most recent CPIC warfarin dosing recommendations because of insufficient evidence, they are not currently included in the AMP PGx Working Group's recommendations.

Additional Considerations

Warfarin Resistance and *VKORC1*

Over 26 rare *VKORC1* missense variants have been reported in association with warfarin resistance, some identified as recently as 2018.[33,41,42] These coding variants have been identified among individuals with warfarin dose requirements of >10 mg/day, ranging from two to seven times the normal dose requirements, or even complete warfarin resistance.[41] Mechanistic and computational modeling studies indicate that they inhibit or prevent binding of warfarin to the VKORC1 enzyme.[41,53,54] Many of these variants are extremely rare and therefore have a paucity of publications reproducing their warfarin resistance across research cohorts. However, the two *VKORC1* resistance variants included in tier 2 (p.Val66Met and p.Asp36Tyr) are recurrent in specific subpopulations and have several published studies supporting their association with higher warfarin dose requirements.[15,27−35]

Variants Associated with Bleeding

A recent genome-wide association study with warfarin-treated African American patients identified the *rs78132896* variant on chromosome 6 as associated with major bleeding occurring at an INR <4.[55] This variant is located upstream of the *EPHA7* gene, which is involved in hematopoiesis, and together with the enhancer region *rs16871327* variant, was found to increase *EPHA7* expression. The *rs78132896* occurs almost exclusively among individuals of African descent (MAF, 7%)[56] and may represent a marker for bleeding risk with warfarin in this population. As additional evidence accumulates, this variant may eventually be included in either tier 1 or tier 2 recommended alleles for routine clinical warfarin sensitivity genotyping.

Limitations

This document is limited to recommendations of alleles to include in clinical laboratory testing for warfarin genotyping. This document does not include, for example, mapping of genotypes to phenotype, clinical interpretation of genotypes, or recommendations for changes to medication therapy based on genotype, as these were considered to be out of scope for this document and/or available from other sources (eg, PharmGKB, CPIC, warfarin prescribing information, and warfarin dosing algorithms). Clinical laboratories should follow best practices for assay validation and adhere to the applicable regulatory requirements for their location.

Conclusions

This document provides recommendations for PGx alleles included in clinical genotyping tests for prediction of warfarin sensitivity. These recommendations are intended to facilitate the design and implementation of genetic testing by clinical laboratories. In addition, these recommendations should help to standardize testing and genotyping concordance between laboratories, which is the basis for haplotype/diplotype assignment, and downstream reporting/interpretations.

Disclaimers

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