CME/SAM

An Improved Algorithm for Activated Protein C Resistance and Factor V Leiden Screening

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ABSTRACT

Objectives: *To evaluate the performance of a Russell viper venom–based activated protein C resistance (APCR) screening test relative to DNA analysis for the factor V Leiden mutation.*

Methods: *We evaluated the concordance between Pefakit APCR screening results and DNA analysis for 435 patients homozygous (n = 11), heterozygous (n = 310), or wild-type (n =114) for the G1691A allele.*

Results: *Using receiver operating characteristic analysis, we found that a cutoff of 1.89 for the APCR ratio yields a sensitivity and specificity of 99.1%. In patients with discrepant genotype-phenotype correlation, their APCR may provide a more clinically relevant result.*

Conclusions: *We compared several strategies for employing reflex testing and found that performing initial APCR screening followed by confirmatory molecular analysis on a subset of cases in the borderline regions between the diagnostic groups can reduce unnecessary testing by approximately 80% without compromising diagnostic accuracy.*

Upon completion of this activity you will be able to:

- analyze how activated protein C resistance (APCR) and molecular genotyping tests work and discuss why these methods are advantageous relative to older methodologies for factor V Leiden screening and confirmation.
- discuss the most common genetic risk factors for venous thrombosis.
- describe the utility of evaluating a patient's transplant and transfusion history when examining results from APCR screening and molecular genotyping assays.

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Questions appear on p 441. Exam is located at www.ascp.org/ajcpcme.

Venous thromboembolism is a common cardiovascular disease that is a major cause of morbidity and mortality, causing between 100,000 and 300,000 deaths in the United States each year.[1-3] Clots are formed that disrupt local blood flow in the deep veins of the leg or arm, and patients can develop chronic venous insufficiency and life-threatening pulmonary emboli.[3,4] Venous thromboembolism is a multifactorial disease influenced by genetics and environmental factors. Common environmental risk factors for venous thrombosis include surgery, immobilization, pregnancy and puerperium, malignancy, obesity, antiphospholipid syndrome, and oral contraceptive use.[3,5] Mutations in the factor V or factor II genes can be inherited and may alter protein interactions or transcript stability to exert a procoagulant effect. Less frequently, the levels or activity of anticoagulant proteins,

including antithrombin, protein C, and protein S, can also cause inherited thrombophilias.[6]

The factor V Leiden (FVL) mutation is a single-nucleotide substitution at position 1691 in the coding region of the gene (*F5* c._G1691A). The mutation results in a single amino acid change at one of the sites where activated protein C (APC) cleaves factor V. This single-residue change from arginine to glutamine at position 506 (F5 p._R506Q) renders factor V resistant to inactivation by APC, which has a procoagulant effect.[7,8] Patients with an FVL mutation have an increased risk of venous thrombosis, pulmonary emboli, pregnancy loss in the second or third trimester, venous leg ulcers, and other thrombotic complications.[9]

The prevalence of heterozygotes for the FVL mutation differs by ethnic groups and has been estimated at 5.3% in whites, 2.2% in Hispanic Americans, 1.3% in Native Americans, 1.2% in African Americans, and 0.5% in Asian Americans.[10] Heterozygotes are approximately 7 times more likely to develop a deep venous thrombosis relative to a person without the FVL mutation, and relative risk is increased by ~80-fold in patients who carry 2 copies of this mutant allele.[11,12]

The presence of the FVL mutation can be assessed by a DNA-based molecular diagnostic assay or by performing functional assays that measure the resistance of factor V to activated protein C degradation.[7,8] DNA analysis is considered the "gold standard" method because functional APC resistance (APCR) can be affected by a number of exogenous variables, including a mutation in a different site of factor V that affects its activity, low levels of common pathway factors in the coagulation cascade, low levels or inhibitors of protein C, or medications such as factor Xa inhibitors or direct thrombin inhibitors.[13-16] The degree to which these variables influence APCR depends on the assay method.

The Pefakit (Pentapharm, Basel, Switzerland) assay measures clot formation in the presence and absence of exogenous APC. The addition of exogenous APC substantially prolongs the clotting time in plasma from patients with wild-type factor V but does not have as great an effect in patients with FVL because the R506Q mutation renders the FV protein resistant to APC proteolysis. Therefore, the ratio of the clotting time in the presence and absence of APC is higher in wild-type patients than in individuals with the FVL mutation. The Pefakit assay is advantageous relative to earlier-generation screening methods because the test is performed with APC in the absence of free calcium and phospholipid, thereby reducing interferences due to protein C, protein S, or lupus anticoagulant in the patient's plasma.[13] In addition, dilution of the patient's sample with factor V–deficient plasma in the initial part of the Pefakit assay decreases the effect of other factor deficiencies on APCR.[13] These advances are significant from a clinical testing standpoint because earlier-generation APCR screening tests would have a greater number of abnormal results in the absence of the FVL mutation.

We previously found that the Pefakit assay was more accurate and cost-effective in distinguishing heterozygotes from wild-type patients relative to the first-generation APCR assay used in our hospital's clinical laboratory.[17] The design of this study was to evaluate the Pefakit Russell viper venom (RVV) functional test relative to DNA sequence analysis for a large retrospective cohort of patients to examine the overall assay performance in distinguishing wild-type, heterozygote, and homozygote FVL patients. Due to the sensitivity and specificity of 100% reported in several smaller studies,[17,18] we wanted to evaluate the Pefakit assay performance in a large cohort of patients to decide whether this test could eliminate the need for DNA testing or if screening cutoffs could be adjusted to use laboratory resources more efficiently.

Materials and Methods

Sample Collection

Venous blood was collected in standard citrated tubes (Becton Dickinson, Franklin Lakes, NJ) and transported to the clinical laboratories. Samples were centrifuged at 1,300*g* for 15 minutes at room temperature. The pellets were discarded and plasma supernatants were placed on ice for immediate use or frozen in aliquots at –70°C for storage.

Data Collection and Analysis

Samples were collected from patients at Brigham and Women's Hospital (BWH, Boston, MA) and its affiliated clinics between March 19, 2008, and November 1, 2012. There were no specific inclusion or exclusion criteria, and all patients with complete data for both DNA analysis and Pefakit values were used in this study. Economic analysis included reagent costs, consumables, and technologist time but not capital equipment. Medical record review was performed to evaluate relevant medical and transfusion history, as well as other coagulation parameters.

Ethics Statement

This project was approved by the Institutional Review Board at BWH under study protocol 2012-P-002486/1.

Pefakit APCR Assay

The Pefakit APCR assay (Pentapharm) was performed according to the instructions of the manufacturer. In total, 30 μL of patient plasma was combined with 20 μL of pooled normal human plasma and the RVV reagent in the absence or presence of APC. After incubation for 3 minutes, both samples were incubated with a prothrombin activator reagent. The clotting time in the presence and absence of

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APC was measured using an STA Compact (Diagnostica Stago, Parsippany, NJ).

DNA Analysis Using the Invader Assay

DNA sequencing for the FVL mutation was performed using the Invader assay (Hologic, Madison, WI) as previously described.[19,20] Prior studies have shown that the Invader assay is a reliable alternative method to traditional polymerase chain reaction genotyping.[19,21]

Statistical Analysis

The D'Agostino and Pearson normality test was used to evaluate the distributions of APCR values in the wild-type, heterozygote, and homozygote groups. Because these populations did not pass this normality test, the nonparametric Kruskal-Wallis test and Dunn multiple-comparisons posttest were used to compare these 3 groups. Receiver operating characteristic (ROC) analysis was performed to evaluate the optimal APCR cutoff to maximize sensitivity and specificity for detecting the G1691A allele. Statistical and graphical analysis was performed using GraphPad Prism (version 5.0; GraphPad Software, La Jolla, CA) and Origin (version 8.0; Origin Software, Northampton, MA) software.

Results

Demographic Data

The demographic data are provided in **Table 1**. We assessed 435 cases with Pefakit APCR values and DNA analysis performed between 2008 and 2012. We found that 114 individuals carried the wild-type allele, and 310 patients were heterozygous and 11 homozygous for FVL.

We would expect to find an overrepresentation of patients positive for the G1691A allele in this cohort for 2 reasons. First, referring physicians ordering this assay would have some clinical suspicion of an underlying coagulopathy in their patients, and FVL is one of the most common thrombophilic risk factors. Second, when Pefakit testing was initiated in our clinical laboratories in March 2008, an APCR value of 2.5 was used as the initial cutoff for DNA testing; therefore, most samples in this study were positive for FVL. We also observed that approximately two-thirds of our study population was female. This sex ratio is attributable to thrombotic risk factors that are specific to women, including pregnancy, puerperium, oral contraceptive use, and postmenopausal hormone replacement. In addition, we observed that the mean age of homozygous patients was nearly 10 years younger than patients in either of the other 2 groups, which is consistent with previous reports in the literature.[12]

Comparison of APCR Values and FVL Mutation Genotype

We wanted to evaluate the concordance between APCR results and genotype groups for patients homozygous (n = 11), heterozygous (n = 310), or wild type (n = 114) for the G1691A allele **Figure 1**. We found that these 3 populations were significantly different when compared by the Kruskal-Wallis test ($P < .001$) and Dunn multiple-comparison test ($P < .05$). While the populations are significantly different from each other, we did observe some overlap in a few APCR values when evaluating the range for each genotype group.

Table 1
Demographic Data for Patient Samples Used in This Study

Characteristic	Factor V Leiden Wild-Type Allele	Factor V Leiden G1691A Allele	Total No. (%)
No. of cases	114	321	435
Sex, No.			
Male	31	111	142 (32.6)
Female	83	210	293 (67.4)
Age, mean (range), y	47.1 (0-87)	46.0 (0-84)	
Ethnic background, No.			
White	253	69	322 (74.0)
African American	22	4	26 (6.0)
Hispanic American	9	4	13 (3.0)
Asian American	1	2	3 (0.7)
Other/unknown	13	58	71 (16.3)
Referring service, No.			
Hematology	5	12	17 (3.9)
Cardiology	7	37	44 (10.1)
Oncology	9	24	33 (7.6)
Obstetrics and gynecology	22	35	57 (13.1)
Primary care	0	21	21 (4.8)
Inpatient (service not specified)	51	105	156 (35.9)
Office visit (service not specified)	17	65	82 (18.9)
Internal medicine	0	8	8 (1.8)
Emergency department	1	3	4 (0.9)
Other	3	10	13 (3.0)

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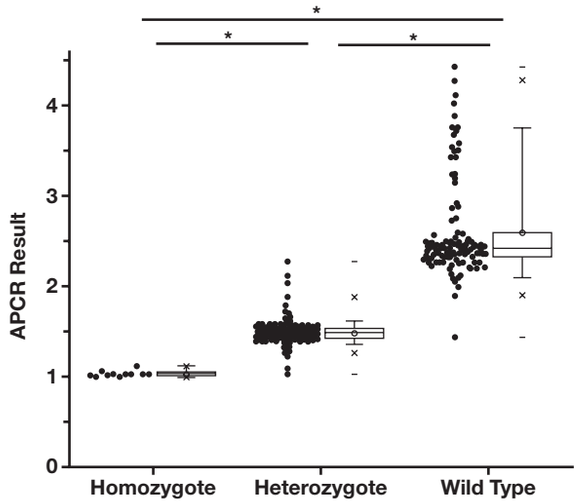

Figure 1 Comparison of activated protein C resistance (APCR) values and factor V Leiden mutation genotype. The APCR values for patients homozygous (n = 11), heterozygous (n = 310), or wild type (n = 114) for the G1691A allele are compared. The box range indicates 25th to 75th percentiles, and whisker plots indicate 5th and 95th percentile data for the 3 populations. *The 3 populations are significantly different compared with the Kruskal-Wallis test ($H = 260$, $df = 2$, $P < .001$) and Dunn multiple-comparison test ($P < .05$).

Specifically, all homozygotes had APCR values between 1.00 and 1.12, heterozygotes ranged from 1.03 to 2.28, and patients with the wild-type allele had values ranging from 1.44 to 4.43. The overlap in these ranges was due to a small number of cases with discrepant genotypic and phenotypic assays; therefore, in addition to the absolute ranges, we also examined percentiles for wild-type, heterozygote, and homozygote patients. Examining the 5th and 95th percentiles indicated that most patients had nonoverlapping APCR ranges between 1.00 and 1.12 for homozygotes, 1.36 and 1.62 for heterozygotes, and 2.10 and 3.79 for individuals with 2 copies of the wild-type allele **Table 2**.

Sensitivity and Specificity of Pefakit APCR for Distinguishing Patients With the FVL Allele

Since most patients with the FVL allele are heterozygotes, we evaluated the sensitivity and specificity of the Pefakit APCR assay by combining patients with either 1 or 2 copies of the G1691A allele into one group (n = 321) and patients having 2 copies of the wild-type allele (n = 114) in the second group **Figure 2**. Using an ROC curve, we found that the area under the curve was 0.993 (95% confidence interval [CI], 0.980-1.005). We also found that an APCR ratio cutoff of 1.89 corresponds to a sensitivity and specificity of 99.1% using the Pefakit assay. We compared this with the sensitivity and specificity of an APCR cutoff of 2.5, which was the value initially used by our clincial laboratories after the assay was adopted in 2008 **Table 3**.

Proposed Screening Algorithm

Instead of employing an absolute APCR cutoff screen, an alternative screening method using selective confirmatory testing on cases at the borderline is proposed. Using APCR values alone to group wild-type, homozygous, and heterozygous patients would have incorrectly diagnosed 6 individuals in this cohort. Two patients genotyping as heterozygotes had APCR values within the homozygous range. One patient had a wild-type genotype with an APCR screen within the heterozygous range, and 3 patients with heterozygote genotypes had APCR values within the wild-type range. We found that 2 of these genotype-phenotype discrepancies were patients with a past medical history of bone marrow transplant. The remaining 4 patients were close to the cutoffs between diagnosis groups and would have been correctly identified using an algorithm that selectively genotypes patients at the borderline regions in between diagnosis groups. Because 1 case with a genotype-phenotype discrepancy was near the homozygote-heterozygote border and 3 cases were near the heterozygote–wild-type border, we propose an alternative screening method using confirmatory testing selectively on cases that are outside of the 95% CIs of the heterozygote group **Figure 3**.

Table 2
Descriptive Statistics for APCR Functional Screening Assay

Statistic	Wild-Type Factor V Leiden	Heterozygote Factor V Leiden	Homozygote Factor V Leiden
Mean (SD)	2.60 (0.53)	1.49 (0.11)	1.03 (0.03)
SEM	0.05	0.01	0.01
95% CI	2.10-3.79	1.36-1.62	1.00-1.12
Range	1.44-4.43	1.03-2.28	1.00-1.12
No. of samples	114	310	11
Samples with indeterminate APCR screens	14	1	1

APCR, activated protein C resistance; CI, confidence interval.

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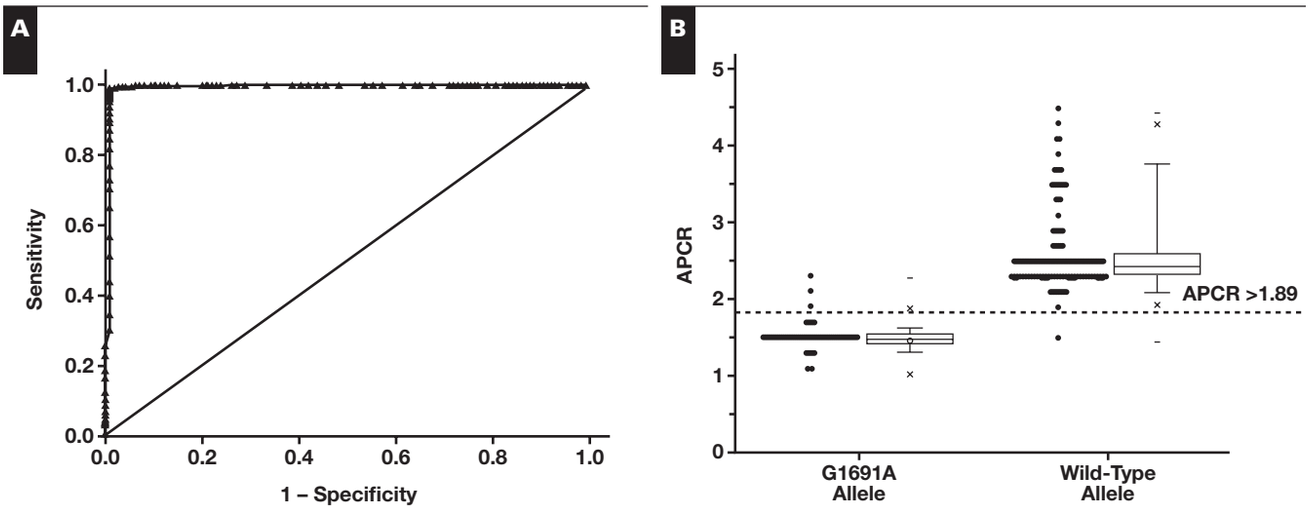

Figure 2 Sensitivity and specificity of Pefakit (Pentapharm, Basel, Switzerland) activated protein C resistance (APCR) for distinguishing patients with the factor V Leiden allele. Plasma APCR cutoffs were evaluated using a receiver operating characteristic (ROC) curve for patients with the wild-type (n = 114) and G1691A (n = 321) allele. **A**, Area under the curve was 0.993 (95% confidence interval, 0.980-1.005). **B**, APCR values for patients with the wild-type (n = 114) or G1691A (n = 321) allele used to compute the ROC curve are plotted with box ranges indicating 25th to 75th percentiles and whisker plots representing 5th and 95th percentile data. An APCR cutoff value of 1.89 corresponds to a sensitivity and specificity of 99.1% using the Pefakit assay.

Cost Analysis

We compared costs for reflex molecular testing following the APCR screen using several different cutoffs and also estimated expenditures based on the proposed algorithm of focusing molecular testing on cases with borderline APCR values **Table 4**. We observed that the absolute APCR cutoffs at 2.5 (the APCR cutoff initially used at BWH after adopting the Pefakit screen) or 1.89 (the suggested APCR value based on our ROC analysis) resulted in cost estimates signficantly higher than the proposed screening algorithm depicted in Figure 3. We also evaluated the relative costs of eliminating the Pefakit screening assay and performing the molecular assay on all samples and observed that performing the screening algorithm with reflex molecular testing for borderline cases is favored because it is the most cost-effective method and does not compromise diagnostic accuracy.

Table 3
Comparison of Sensitivity and Specificity Using Molecular Testing as a Gold Standard Assay

Characteristic	Reflex DNA Testing With Screening APCR <2.5	Reflex DNA Testing With Screening APCR <1.89[a]
Positive APCR assay, No.		
Presence of G1691A allele	321	318
Absence of G1691A allele	77	1
Negative APCR assay, No.		
Presence of G1691A allele	0	3
Absence of G1691A allele	37	113
Sensitivity	1.00	0.99
Specificity	0.32	0.99
Positive predictive value	0.81	1.00
Negative predictive value	1.00	0.97

APCR, activated protein C resistance.
[a] Analysis was performed using an APCR cutoff of 1.89 as determined by area under the curve analysis in Figure 2.

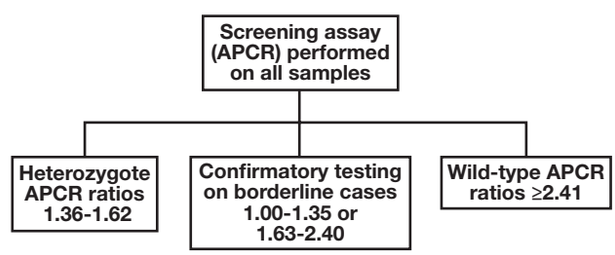

Figure 3 Proposed screening algorithm. Plasma samples with activated protein C resistance (APCR) results at the border region between heterozygotes and homozygotes as well as normal and heterozygote groups were the most likely to exhibit discrepancies between APCR values and molecular testing. Instead of employing an absolute APCR cutoff screen, an alternative screening method using selective confirmatory testing only on cases at the borderline is proposed.

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Table 4
Cost Analysis for Reflex Testing Using 2 Absolute APCR Screening Cutoffs vs Selective Reflex Testing of Borderline Assays[a]

Characteristic	Reflex DNA Testing With Screening APCR <2.5	Reflex DNA Testing With Screening APCR <1.89[b]	Reflex DNA Testing With Borderline APCR[c]	Molecular Testing Without APCR Screening
Pefakit APCR assay	2,994.64	2,994.64	2,994.64	0.00
Factor V Leiden molecular testing				
Positive results	20,473.38	20,282.04	2,423.64	20,600.94
Negative results	2,909.83	37.79	1,851.71	4,837.12
Indeterminate APCR values				
Positive results	127.56	127.56	127.56	0.00
Negative results	529.06	529.06	529.06	0.00
Total costs	27,034.47	23,971.09	7,926.61	25,438.06

APCR, activated protein C resistance.
[a] Values are presented as costs in US dollars. Pefakit (Pentapharm, Basel, Switzerland) screening costs are estimated at $6.64 per assay, including the cost of kits, plasma controls, disposable reagents, and labor. Molecular testing costs are estimated at $37.79 for a factor V Leiden–negative assay and $63.78 for a positive assay because all positives are confirmed by running the molecular assay twice prior to reporting results. Molecular diagnostic cost estimates include reagents, disposables, technologist time, and employee benefits for performing DNA extraction followed by molecular testing.
[b] Analysis was performed using an APCR cutoff of 1.89 as determined by the receiver operating characteristic curve in Figure 2.
[c] Algorithm for reflex testing depicted in Figure 3. Molecular testing was performed on borderline APCR results from 1.00 to 1.35 (to distinguish homozygotes and heterozygotes) or between 1.63 and 2.40 (to distinguish heterozygotes and wild types).

Discussion

In this study, we compared several thresholds in the analysis of an APCR screening method. We found that lowering the APCR cutoff from 2.5 to 1.89 reduces the number of tests sent by 20% (from 398 to 319 cases) but would have incorrectly identified 3 heterozygotes as wild type. Alternatively, sending the borderline cases for confirmation decreased the number of tests by approximately 80% (from 398 to 87 cases), without a significant impact on diagnostic accuracy. Selectively performing molecular testing on cases in the borderline regions has an excellent correlation with DNA testing and is a more cost-efficient approach for performing screening and confirmatory assays.

In our analysis of 435 cases, we identified 6 patients with APCR values that were not well matched to their genotype. Interestingly, 2 patients had a past medical history of chronic lymphocytic leukemia and allogeneic bone marrow transplant prior to FVL testing. We believe that this genotype-phenotype discrepancy arises because the patient's genotype may reflect his or her donor's FVL status (since the DNA analysis is performed on nucleated white blood cells that are marrow derived), whereas the APCR assay reflects the patient's endogenous factor V genotype (because this protein is produced in the liver). This phenomenon has been reported several times in the literature, both for patients who have a bone marrow transplantation and develop a different factor V genotype[22,23] as well as for patients who undergo liver transplantation and acquire the phenotypic FVL status of their donor.[24-26]

A third individual with a heterozygote genotype was a critically ill patient transferred from an outside hospital with recent abdominal surgery and venous thrombosis of the superior mesenteric vein with extension into the portal vein. This patient's APCR screen was 2.28, and it is plausible that recent transfusion in combination with critical illness may have affected the screening assay result. Clinical factors that might account for borderline APCR values were not obvious in the 3 remaining cases. These cases would have been correctly diagnosed using the algorithm in which borderline APCR assays are sent for molecular genotyping.

The screening algorithm currently used in the BWH clinical laboratory assayed the 6 discrepant cases by APCR and used genotype analysis to provide the final diagnosis. Because we were unable to document the pretransplant FVL status of these patients, these cases contributed to the broad range of APCR values for the 3 genotype groups. The proposed screening algorithm in which confirmatory testing is performed only on borderline cases would have reflexed 5 of the 6 aberrant cases for molecular testing, and 1 patient with prior history of a bone marrow transplant would not have been genotyped. This individual had an APCR value of 1.44, indicative of a heterozygote (which may reflect the patient's FVL genotype), with a concomitant genotype assay of wild type (which may reflect the donor's FVL genotype). Since the patient's APCR result should be more reflective of his or her coagulopathic risk and carrier status, the phenotypic assay may provide a more clinically relevant result. Because of the close association between BWH and the Dana-Farber Cancer Institute, we have a substantial patient population with a prior history of allogeneic transplants. In patients with a prior history of bone marrow transplantation, it may be useful to perform confirmatory testing from a second site, such as sampling DNA from a skin biopsy specimen or buccal swab rather than using peripheral blood leukocytes for genetic analysis.[23]

Although documenting a patient's FVL carrier status by genotypic analysis provides a sense of diagnostic certainty, the relationship between genotypic and phenotypic APCR testing

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is complex. Multiple studies have shown that resistance to APC correlates with venous thromboembolism and recurrent pregnancy loss even in the absence of the FVL mutation.[27-31] While APCR is attributed to the FVL mutation in most patients, other factors have been implicated in approximately 5% of cases.[31] The mechanism for these phenomena may be related to an elevation of factor VIII activity levels, acquired or genetic abnormalities affecting protein C or protein S activity, or rare mutations in factor V or factor VIII.[31,32] Another recent study found that patients with multiple myeloma have a statistically significant decrease in the APCR relative to healthy individuals in the absence of the FVL mutation.[33] Our results indicate that the percentage of cases that are APCR positive and factor V negative may be less than 5% when newer-generation APC tests such as Pefakit are employed as the APCR screening assay.

While the performance of the Pefakit APCR assay yielded high sensitivity and specificity in patients on traditional anticoagulant medication such as heparin and coumadin, the effect of new anticoagulant medications on this assay will have to be carefully evaluated in future studies. There have been several reports of aberrant assays run in the presence of factor Xa inhibitors and direct thrombin inhibitors, which are medications relevant to patients undergoing a workup for suspected coagulopathy. Rivaroxaban blocks the active site of factor Xa independently of thrombin and is used as an anticoagulant for deep venous thrombosis prophylaxis, whereas dabigatran inhibits thrombin-mediated platelet aggregation and conversion of fibrinogen into fibrin during clot formation.[34,35]

In a recent study by Hillarp et al,[14] 10 plasma samples were spiked with rivaroxaban in a concentration range from 0 to 1,000 μg/L, and these samples were analyzed using a variety of common coagulation assays. The Pefakit assay exhibited an increase in APCR values with the addition of increasing amounts of exogenous rivaroxaban, but the magnitude of the change was negligible and would not have been sufficient to cause misdiagosis of patients with FVL. A similarly designed study performed with dabigatran indicated a potentially significant increase in APCR values using the Pefakit assay.[16] However, these were small studies performed under nonphysiologic conditions, with rivaroxaban or dabigatran dissolved in DMSO and spiked into plasma samples. Therefore, additional analysis of assay interference should be performed using a larger group of samples from patients who are taking these medications. Until these studies are performed, caution should be exercised when interpreting APCR assays in patients taking these medications.[14,16]

Conclusions

At this point, we recommend combining functional APCR assays with confirmatory DNA sequence analysis in patients with borderline APCR results. The patient's transplant and transfusion history, family history, medication list, and other coagulation testing parameters should also be thoroughly examined. The sampling site used for confirmatory testing should be carefully considered in patients with a history of bone marrow or liver transplantation so that the patient's FVL genotype is likely to reflect his or her APCR phenotype. Elevated factor VIII activity levels, abnormalities affecting protein C or protein S, or rare mutations in factor V or factor VIII should also be considered in patients with a decreased APCR in the absence of the FVL mutation. We believe that performing initial APCR screening followed by confirmatory molecular analysis on cases in the borderline regions between the diagnostic groups in conjunction with careful scrutiny of the patient's clinical history can decrease costs by eliminating unnecessary testing without compromising diagnostic accuracy.

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