Rivaroxaban Causes Missed Diagnosis of Protein S Deficiency but Not of Activated Protein C Resistance (Factor V Leiden)

Elena Maryamchik, MD; Matthew W. Rosenbaum, MD; Elizabeth M. Van Cott, MD

• ***Context.***—**Rivaroxaban causes a false increase in activated protein C resistance (APCR) ratios and protein S activity.**

Objective.—**To investigate whether this increase masks a diagnosis of factor V Leiden (FVL) or protein S deficiency in a "real-world" population of patients undergoing rivaroxaban treatment and hypercoagulation testing.**

Design.—**During a 2.5-year period, we compared 4 groups of patients (n = 60): FVL heterozygous (FVL-HET)/taking rivaroxaban, wild-type/taking rivaroxaban, FVL-HET/no rivaroxaban, and normal APCR/no rivaroxaban. Patients taking rivaroxaban were tested for protein S functional activity and free antigen (n = 32).**

Results.—**The FVL-HET patients taking rivaroxaban had lower APCR ratios than wild-type patients ($P < .001$). For FVL-HET patients taking rivaroxaban, mean APCR was 1.75 ± 0.12, versus 1.64 ± 0.3 in FVL-HET patients not taking rivaroxaban ($P = .005$). Activated protein C resistance in FVL-HET patients fell more than 3 SDs below the cutoff of 2.2 at which the laboratory reflexes FVL DNA testing. No cases of FVL were missed despite rivaroxaban. In contrast, rivaroxaban falsely elevated functional protein S activity, regardless of the presence or absence of FVL ($P < .001$). A total of 4 of 32 patients (12.5%) had low free protein S antigen (range, 58%–67%), whereas their functional protein S activity appeared normal (range 75%–130%). Rivaroxaban would have caused a missed diagnosis of all cases of protein S deficiency during the study if testing relied on the protein S activity assay alone.**

Conclusions.—**Despite rivaroxaban treatment, APCR testing can distinguish FVL-HET from normal patients, rendering indiscriminate FVL DNA testing of all patients on rivaroxaban unnecessary. Free protein S should be tested in patients taking rivaroxaban to exclude hereditary protein S deficiency.**

(*Arch Pathol Lab Med.* 2018;142:70–74; doi: 10.5858/arpa.2016-0616-OA)

Rivaroxaban is a direct, antithrombin-independent factor Xa inhibitor, which inhibits not only free factor Xa, but also clot-bound factor Xa and the prothrombinase complex. It is used for treatment and prevention of venous thromboembolism, as well as for prophylaxis of stroke in patients with atrial fibrillation.[1] Rivaroxaban has become a commonly prescribed anticoagulant, to the extent that almost half of the special coagulation laboratories in the United States now offer an anti-Xa test to measure rivaroxaban activity levels.[2] Many patients taking rivaroxaban undergo hypercoagulation testing to rule out common inherited or acquired causes of hypercoagulability, including assays for activated protein C resistance (APCR) to detect factor V Leiden (FVL), and tests for protein S deficiency. However, a previous study reported that spiking normal plasma with rivaroxaban caused an artifactual increase in the APCR ratio.[3] In another study, rivaroxaban was found to falsely elevate protein S activity.[4]

Factor V Leiden is the most common inherited risk factor for venous thromboembolism. Heterozygosity for FVL confers a 3- to 7-fold increase in the risk of thromboembolic events, whereas homozygosity is associated with an 80-fold increase.[5,6] FVL affects about 5% of the white population and is responsible for more than 95% of cases of APCR.[5,7,8] Activated protein C is an intrinsic plasma anticoagulant that cleaves factor Va at several conserved arginine residues. The cleaved factor Va, in turn, acts as a cofactor for activated protein C in degrading factor VIIIa.[5,9,10] The molecular basis for FVL is a point mutation in the factor V gene at G1691A, resulting in an arginine to glycine substitution at amino acid 506, which makes activated factor V resistant to cleavage by activated protein C.[5,9,10]

The most commonly used APCR test is a clot-based assay that measures the activated partial thromboplastin time (aPTT) before and after activated protein C is added to the sample. It is expressed as a ratio of these 2 values. Originally, it was conducted on undiluted patient plasma, which made it susceptible to the effects of anticoagulants, factor deficiencies, factor elevations, and acute thrombosis.[5,8,11] However, an improved, "modified" version soon became available, in which a patient's plasma was diluted 5-

Accepted for publication March 21, 2017.

Published as an Early Online Release September 18, 2017.

From the Department of Pathology, Massachusetts General Hospital, Boston.

The authors have no relevant financial interest in the products or companies described in this article.

Presented in part at the United States & Canadian Academy of Pathology annual meeting; March 6, 2017; San Antonio, Texas.

Reprints: Elizabeth M. Van Cott, MD, Department of Pathology, Gray/Jackson 235, Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114.

fold into factor V–deficient plasma containing a heparin neutralizer. This increased the selectivity of the test for FVL by normalizing the concentrations of other plasma proteins involved in the formation and regulation of thrombin.[12,13] It also rendered the test suitable for use in patients on heparin and vitamin K antagonists (eg, warfarin), as well as in patients with acute thromboembolic events.[6,8,11,14] The sensitivity and specificity of the APCR test in those patients approached 100%.[8,12,13,15] However, data concerning its sensitivity in patients taking Xa inhibitors, such as rivaroxaban, are still scarce. With the increasing use of these medications, a new question has arisen about the value of the modified APCR assay for FVL mutation screening in patients on rivaroxaban.

In this study, we sought to determine the magnitude of rivaroxaban interference in APCR and protein S activity testing in an actual patient population by analyzing hypercoagulation panels received at our high-volume special coagulation laboratory. To our knowledge, this is the first study investigating the effect of rivaroxaban on APCR and protein S activity in real patients undergoing rivaroxaban treatment rather than in spiked normal samples, when using the most commonly used testing platforms: Coatest V (Chromogenix, West Chester, Ohio) for APCR, and the Star Evolution analyzer (Stago, Parsippany, New Jersey) for protein S.

MATERIALS AND METHODS

Patients

We prospectively reviewed all APCR test results performed by the special coagulation laboratory at Massachusetts General Hospital between March 12, 2014, and September 2, 2016. Results from consecutive patients testing heterozygous for FVL while taking rivaroxaban were recorded in group 1. For each patient in group 1, 3 matching patients were randomly selected among the patients tested on the same day for each of the 3 following control groups (groups 2–4). Group 2 consisted of patients who were also heterozygous for FVL but were not taking rivaroxaban. Group 3 included patients taking rivaroxaban who tested normal ("wild type") for FVL by DNA testing. Group 4 comprised patients who were not taking rivaroxaban and who had a normal APCR ratio. If control patients tested on the same date as a group 1 patient was not available, control patients were selected from the nearest dates possible that used the same equipment and reagent lot as in group 1, thus reducing bias that could potentially arise from operational differences.

We also identified all patients taking rivaroxaban who underwent protein S testing during the same period. All patients taking rivaroxaban were tested for protein S with both an activity assay and a free-antigen assay.

Laboratory Methods

The patients were evaluated using the standard FVL testing protocol adopted in our institution. It included an aPTT-based APCR assay with dilution in factor V–deficient plasma (Coatest APC Resistance V assay, Chromogenix), on a Star Evolution analyzer, and an FVL DNA assay (Invader assay, Hologic, Boston, Massachusetts). Activated protein C resistance ratios of 2.0 and below were considered abnormal, and to ensure detection of FVL, values of 2.2 or lower underwent further workup by DNA testing. Activated protein C resistance and DNA analysis was performed for all patients taking rivaroxaban.

Protein S functional activity was measured using the STACLOT Protein S assay (Stago) on a Star Evolution analyzer, and free protein S antigen was measured using the Asserachrom Free Protein S assay (Stago). Free protein S antigen levels served as a control to compare to the functional activity of protein S in patients on rivaroxaban, because rivaroxaban does not affect free protein S antigen results. All patients tested for protein S were concomitantly tested for FVL as described above.

Rivaroxaban concentration for each specimen was determined using an anti-Xa assay (Stachrom, Stago). The results were provided in U/mL using a low–molecular weight heparin calibrator (Aniara, West Chester, Ohio), and converted into rivaroxaban concentration using rivaroxaban calibration curves.

Statistical Analysis

The geometric mean APCR ratios of the 4 groups were analyzed using analysis of variance and 2-tailed Student *t* test assuming equal variances. The mean protein S activity and free protein S antigen were analyzed using 2-tailed Student *t* test assuming equal variances. *P* values $<.05$ were considered statistically significant. Excel software by Microsoft (Redmond, Washington) was used to perform the calculations.

RESULTS

There were 4 groups of 15 patients identified for the APCR analysis (n = 60). The patients included 31 male and 29 female patients between the ages of 20 and 90 years. The geometric means (±SDs) of the APCR ratios in the 4 groups were: group 1 (patients heterozygous for FVL taking rivaroxaban), 1.75 ± 0.12; group 2 (patients heterozygous for FVL not taking rivaroxaban), 1.64 ± 0.09; group 3 (wild-type patients taking rivaroxaban), 2.63 ± 0.23; and group 4 (patients with normal APCR not taking rivaroxaban), 2.49 ± 0.18. Comparing the groups taking and not taking rivaroxaban, the absolute increase in the mean was 0.11 for patients heterozygous for FVL, and 0.14 for the wild-type patients (Figure 1). Individual APCR ratios were all lower than 2.0 for all FVL heterozygous patients, with or without rivaroxaban.

The FVL heterozygous patients taking rivaroxaban had a higher APCR ratio than heterozygous patients not taking rivaroxaban ($P = .005$). However, these heterozygous patients taking rivaroxaban had a significantly smaller APCR ratio than patients with normal APCR ratios not taking rivaroxaban ($P < .001$). Although there was a nonsignificant trend for the wild-type patients taking rivaroxaban to have a higher APCR ratio than patients with normal APCR not taking rivaroxaban, a 2-sided *t* test did not show a statistically significant difference ($P = .07$). The results are presented in Table 1.

For the protein S study, 32 patients taking rivaroxaban were identified during the abovementioned time period. Of these patients, 8 (25%) were found to concomitantly have FVL, and the remaining 24 (75%) had wild-type factor V. The mean protein S functional activity in patients on rivaroxaban was 124.7% ± 27.9%, and mean free protein S antigen was 83.8% ± 14.8% (Figure 2). The difference between the 2 means was statistically significant ($P < .001$), presumably because rivaroxaban falsely increases protein S activity but does not affect protein S free antigen. A total of 4 of the 32 patients (12.5%) had low protein S free antigen results (range, 58%–67%). Of these 4 patients, 1 patient (25%) also had FVL, and the remaining 3 patients (75%) had wild-type factor V. Protein S activity was falsely normal in all 4 cases (range, 75%–130%; $P < .05$). Therefore, unlike APCR, rivaroxaban would have caused a missed diagnosis of all 4 cases of protein S deficiency if using a protein S activity assay alone. The results are presented in Table 2.

Plasma rivaroxaban concentrations of patients in this study ranged from slightly subtherapeutic to high-therapeutic levels.[16] In the APCR study, the rivaroxaban dosage

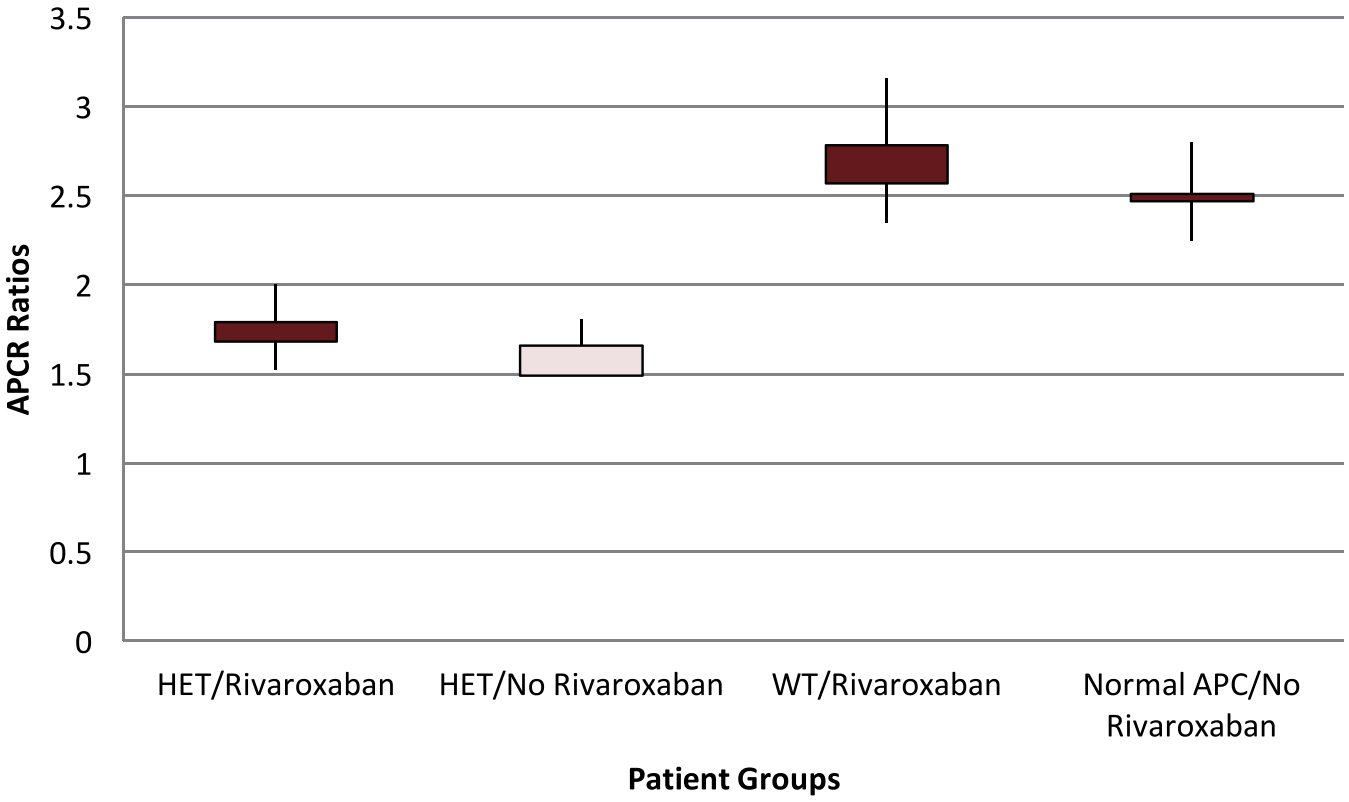

Figure 1. *Activated protein C resistance (APCR) ratios by group. Horizontal axis depicts 4 distinct groups of patients: HET/rivaroxaban, patients heterozygous (HET) for factor V Leiden (FVL) taking rivaroxaban; HET/no rivaroxaban, HET-FVL patients not taking rivaroxaban; WT/rivaroxaban, patients with wild-type (WT) factor V taking rivaroxaban; and normal APCR/no rivaroxaban, patients with normal APCR not taking rivaroxaban. Vertical axis shows magnitudes and ranges of APCR for each of these 4 groups.*

was 20 mg/d in 17 patients (8 FVL heterozygous, 9 wild type), and 15 mg twice daily in 13 patients (7 FVL heterozygous, 6 wild type). In the protein S study, 16 of 32 patients (50%) took 20 mg of rivaroxaban daily (7 of 15 patients with FVL [47%], and 9 of 15 patients with wild type factor V [60%]). Of the remaining 16 patients, 14 (44%) were in their rivaroxaban induction period and took 15 mg twice a day. The information on the daily dosage of the remaining 2 patients (6%) is not available. Both of those patients had wild-type factor V and a therapeutic rivaroxaban concentration.

DISCUSSION

The results of this study indicate that taking rivaroxaban slightly increases the APCR ratio in patients heterozygous for the FVL mutation. However, the magnitude of this increase did not reach the normal APCR range, and the assay was still able to distinguish the heterozygous patients from normal. No patients were misclassified despite rivaroxaban. The mean increase in the APCR ratio was 0.14 for the wild-type patients, and 0.11 for the heterozygous patients. Figure 1 demonstrates a clear separation between the wild-type and the heterozygous patients taking rivaroxaban. One explanation for this phenomenon could be that diluting the patient's plasma 1:5 was sufficient to reduce the rivaroxaban concentration to a level that caused only a minor interference.

These results are reassuring, and suggest that if specimens are submitted for APCR testing while patients are taking rivaroxaban, the laboratory will provide the correct answer even if the clinician does not inform the laboratory about rivaroxaban, or if DNA testing is not available. The results also suggest that laboratories could proceed with this APCR test despite knowing that rivaroxaban is present. However, as an added precaution, we suggest checking an anti-Xa level to rule out a supratherapeutic rivaroxaban concentration, because levels above 349 ng/mL (anti-Xa 1.51 U/mL by a low–molecular weight heparin curve) were not present in this study. Fortunately, because supratherapeutic specimens were not encountered during the 2.5-year study period, the results suggest that supratherapeutic specimens are not common among patients undergoing hypercoagulability testing.

Table 1. Activated Protein C Resistance (APCR) Results With and Without Rivaroxaban in Patients With and Without Heterozygous Factor V Leiden

	Heterozygous/ Rivaroxaban	Heterozygous/ No Rivaroxaban	Wild Type/ Rivaroxaban	Normal APCR/ No Rivaroxaban
APCR, mean (SD), N = 60	1.75 (0.12)	1.64 (0.09)	2.63 (0.23)	2.49 (0.18)
Rivaroxaban concentration, ng/mL, mean (SD; range)	143 (95; 45–349)	Not applicable	147 (79; 68–311)	Not applicable

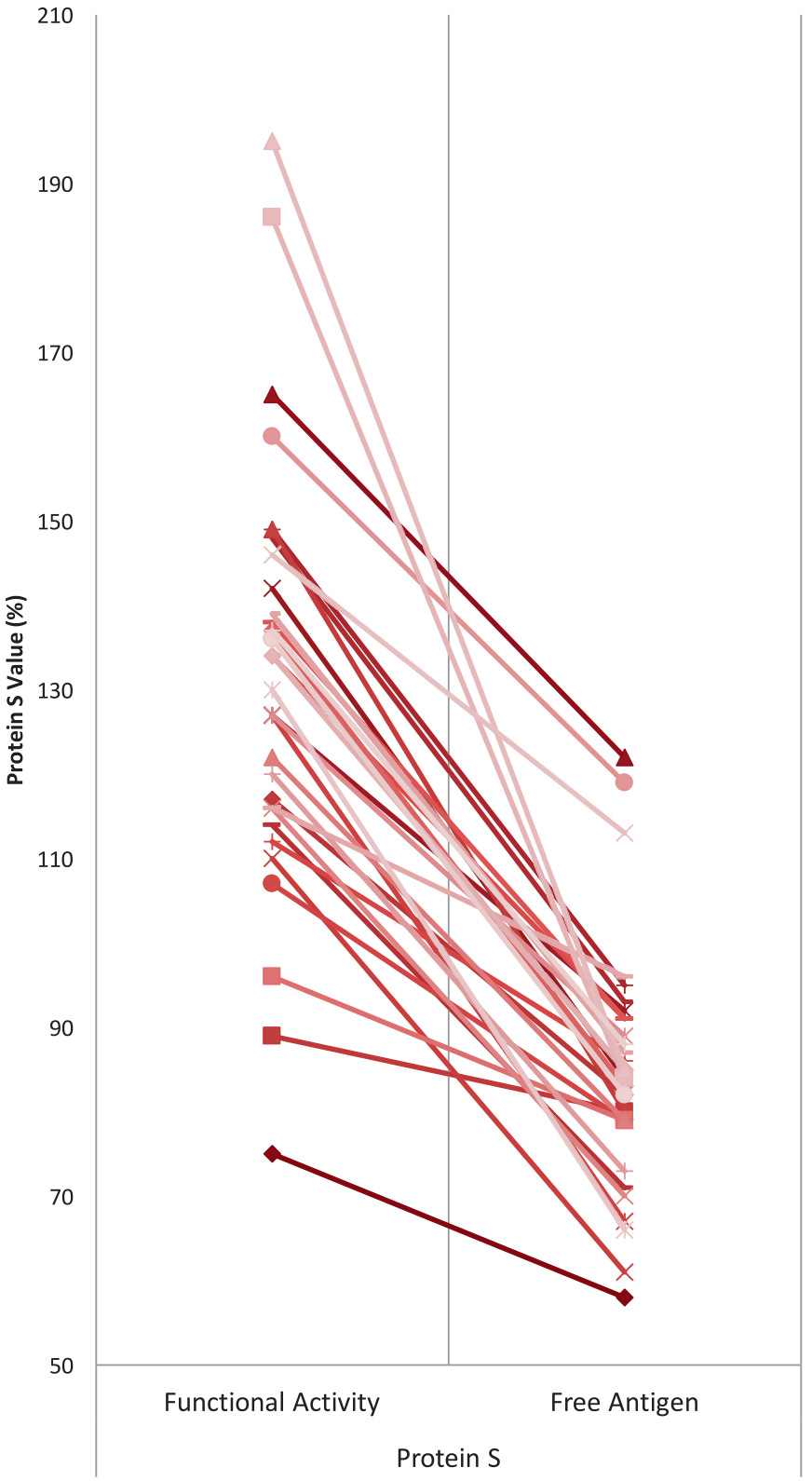

Figure 2. *Protein S functional activity versus free protein S in patients taking rivaroxaban. Horizontal axis depicts 2 distinct tests: protein S functional activity, and protein S free antigen. Vertical axis shows the values of these 2 tests for each of the study participants.*

Table 2. Protein S Analysis of Patients Taking Rivaroxaban

	Rivaroxaban Concentration, ng/mL, Mean (SD; Range)	Protein S Functional Activity, %, Mean (SD)	Protein S Free Antigen, %, Mean (SD)	*P* Value
All patients (n = 32)	145 (97; 23–349)	124 (27.9)	83.8 (14.8)	<.001
Patients with low protein S (n = 4)	154 (101; 23–266)	108 (25.3)	62.9 (4.24)	.047

Rivaroxaban concentrations detected in this study (Table 1) are congruent with the concentrations typically seen in patients taking rivaroxaban. In 1 study, with a 10-mg daily dosage, the steady-state peak was 91 to 196 ng/mL, and the trough was 1 to 38 ng/mL; with a 20-mg daily dosage, the peak was 160 to 360 ng/mL and the trough was 4 to 96 ng/mL (95% confidence interval).[16]

We did not encounter any patients homozygous for FVL and taking rivaroxaban during the investigation period. However, patients homozygous for FVL exhibit an even greater degree of APCR than heterozygous patients. Therefore, their APCR ratios are even lower than those of heterozygous patients, and fall well below the normal cutoff point.

In a similar study previously conducted by our group, argatroban (a direct thrombin inhibitor) was able to mask the diagnosis of FVL by raising the APCR ratio into the normal range,[17] and dabigatran (direct thrombin inhibitor) substantially raised the APCR ratio.[18] In contrast to these direct thrombin inhibitors, rivaroxaban (an antithrombin-independent factor Xa inhibitor) raised the APCR ratio only slightly, and not into the normal range in any of our cases, highlighting that these different anticoagulants can have different effects on APCR elevation.

Simultaneous testing of functional protein S activity and free protein S antigen in patients on rivaroxaban showed that functional protein S activity was on average 1.5-fold higher than protein S free antigen. This elevation was observed regardless of the presence or absence of FVL, and could be attributed to rivaroxaban. Furthermore, low free protein S antigen occurred in 4 of 32 patients (12.5%) in this study, and the protein S activity assay missed all cases of low protein S during rivaroxaban treatment. Therefore, free protein S antigen is the preferred method in all patients taking rivaroxaban in order to exclude a hereditary protein S deficiency. However, clinicians should keep in mind that in order to detect a rare qualitative type II protein S deficiency (normal levels of protein S free antigen, but decreased protein S function), the protein S activity testing would need to be performed after a patient discontinues rivaroxaban.

The false protein S elevation is likely due to the fact that the protein S activity assay is aPTT based, and protein S prolongs the aPTT by serving as a cofactor for activated protein C–mediated cleavage of factors V and VIII. The degree of aPTT prolongation is proportional to the amount of protein S in the specimen. Rivaroxaban prolongs the aPTT in the assay, causing an overestimation of the amount of protein S present.

Factor V Leiden can cause falsely low protein S activity in some assays. Those assays rely on the patient as the only source of factor V. When the patient has FVL, the patient's factor V resists degradation by the activated protein C/protein S complex, making it seem like there is less protein S present, whereas in reality this apparent decrease in protein S function is caused by the resistance of abnormal factor V to degradation by the activated protein C/protein S complex. The present study avoids such consequences by using a protein S activity assay that supplies exogenous normal factor V, thus minimizing this interference.

CONCLUSIONS

The data obtained in this study suggest that the Coatest APCR V assay can be used to test patients anticoagulated with rivaroxaban for the FVL mutation. Because FVL specimens are generally reliably detected at an APCR cutoff of 2.0 or lower, in an effort to ensure 100% sensitivity in detecting FVL, our special coagulation laboratory currently uses a protocol in which all specimens with APCR ratios of 2.2 or lower undergo DNA testing. In the present study, this protocol detected all patients heterozygous for FVL, suggesting that DNA testing of patients with APCRs above 2.2 is unnecessary regardless of rivaroxaban use. As an added precaution, an anti-Xa assay could be performed to confirm that supratherapeutic rivaroxaban levels are not present, because this study included high-therapeutic specimens up to 349 ng/mL rivaroxaban (anti-Xa 1.51 U/mL by a low–molecular weight heparin curve), but supratherapeutic specimens were not encountered (Note added in proof: While the manuscript was in press, we encountered 2 patients heterozygous for factor V Leiden with supratherapeutic rivaroxaban concentrations of 590 ng/mL and 401 ng/mL [anti-Xa 2.48 μ/mL and 1.72 μ/mL by a LMWH curve, respectively]. The APCR assay remained accurate with ratio results of 1.89 and 1.85, respectively. Thus, the APCR assay appears accurate even with supratherapeutic rivaroxaban). Protein S free antigen is preferred instead of protein S activity for patients taking rivaroxaban, with the caveat that protein S activity testing would need to be performed after rivaroxaban has been discontinued in order to detect a rare qualitative type II protein S deficiency.

References

1. Douxfils J, Mullier F, Loosen C, et al. Assessment of the impact of rivaroxaban on coagulation assays: laboratory recommendations for the monitoring of rivaroxaban and review of the literature. *Thromb Res*. 2012;130(6):956–966.
2. Zantek ND, Hayward CP, Simcox TG, et al. An assessment of the state of current practice in coagulation laboratories. *Am J Clin Pathol*. 2016;146(3):378–383.
3. Hillarp A, Baghaei F, Fagerberg Blixter I, et al. Effects of the oral, direct factor Xa inhibitor rivaroxaban on commonly used coagulation assays. *J Thromb Haemost*. 2011;9(1):133–139.
4. Mani H, Hesse C, Stratmann G, et al. Ex vivo effects of low-dose rivaroxaban on specific coagulation assays and coagulation factor activities in patients under real life conditions. *Thromb Haemost*. 2013;109(1):127–136.
5. Van Cott EM, Khor B, Zehnder JL. Factor V Leiden. *Am J Hematol*. 2016;91(1):46–49.
6. Hall C, Andersson NE, Andras M, et al. Evaluation of a modified APTT-based method for determination of APC resistance in plasma from patients on heparin or oral anticoagulant therapy. *Thromb Res*. 1998;89(5):203–209.
7. van Oerle R, van Pampus L, Tans G, et al. The clinical application of a new specific functional assay to detect the factor V(Leiden) mutation associated with activated protein C resistance. *Am J Clin Pathol*. 1997;107(5):521–526.
8. Svensson PJ, Zoller B, Dahlback B. Evaluation of original and modified APC-resistance tests in unselected outpatients with clinically suspected thrombosis and in healthy controls. *Thromb Haemost*. 1997;77(2):332–335.
9. Khor B, Van Cott EM. Laboratory evaluation of hypercoagulability. *Clin Lab Med*. 2009;29(2):339–366.
10. Segers K, Dahlback B, Nicolaes GA. Coagulation factor V and thrombophilia: background and mechanisms. *Thromb Haemost*. 2007;98(3):530–542.
11. Freyburger G, Javorschi S, Labrouche S, et al. Proposal for objective evaluation of the performance of various functional APC-resistance tests in genotyped patients. *Thromb Haemost*. 1997;78(5):1360–1365.
12. Jorquera JI, Montoro JM, Fernandez MA, et al. Modified test for activated protein C resistance. *Lancet*. 1994;344(8930):1162–1163.
13. Trossaert M, Conard J, Horellou MH, et al. Modified APC resistance assay for patients on oral anticoagulants. *Lancet*. 1994;344(8938):1709.
14. Johnson NV, Khor B, Van Cott EM. Advances in laboratory testing for thrombophilia. *Am J Hematol*. 2012;87(suppl 1):S108–S112.
15. Kadauke S, Khor B, Van Cott EM. Activated protein C resistance testing for factor V Leiden. *Am J Hematol*. 2014;89(12):1147–1150.
16. Mueck W, Borris LC, Dahl OE, et al. Population pharmacokinetics and pharmacodynamics of once- and twice-daily rivaroxaban for the prevention of venous thromboembolism in patients undergoing total hip replacement. *Thromb Haemost*. 2008;100(3):453–461.
17. Shaikh S, Van Cott EM. The effect of argatroban on activated protein C resistance. *Am J Clin Pathol*. 2009;131(6):828–833.
18. Kim YA, Gosselin R, Van Cott EM. The effects of dabigatran on lupus anticoagulant, diluted plasma thrombin time, and other specialized coagulation assays. *Int J Lab Hematol*. 2015;37(4):e81–e84.