

Digging into drug's effect on aPTT-based assays

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December 2020—As ongoing studies reveal the merits of emicizumab for hemophilia A patients—fewer bleeding episodes, longer duration between treatments—laboratories need to be alert to the drug's effect on coagulation testing.

"If you take anything away from this presentation," said Andrew J. Goodwin, MD, speaking in October in a CAP20 session, "aPTT assays in patients on emicizumab are not going to be accurate. And the effect of the drug on our aPTT-based assays can last up to six months following discontinuation of that medication."

"It presents an interference, which is a real challenge," said Dr. Goodwin, clinical pathology division chief and CLIA director, University of Vermont Medical Center. His co-presenter was David Unold, MD, associate medical director, transfusion services, University of California Davis Medical Center.

Dr. Unold reported the results of recent HAVEN studies of emicizumab (Hemlibra, Genentech), a bispecific factor IXa and factor X-directed antibody that mimics the activity of factor VIII. Emicizumab is unlike factor VIII replacement therapies because "it actually binds both the precursor—the zymogen—as well as the active forms of factors IX and X," he said. It binds only human forms of factor IX and X, which is significant for laboratory testing.



Dr. Unold

"The nice thing about it, even though the schedule can vary based on the different concentrations available, is you can give the drug as few times as every four weeks," Dr. Unold said. The half-life of the drug is 30 days (Müller J, et al. *Thromb Haemost.* 2019;119[9]:1384-1393).

The most recent trial of emicizumab—HAVEN 4—studied hemophilia A patients age 12 and older, with and without inhibitors. "Essentially, they wanted to look at this increased duration between injections," Dr. Unold said. The trial found an annualized rate of treated bleeds of 2.4, which while slightly higher than the rates of 1.5 and 1.3 found in the earlier HAVEN 3 trial, still demonstrated benefits with a longer duration between injections. "And no thrombotic events or antidrug antibodies with neutralizing potential were identified," he said (Pipe SW, et al. *Lancet Haematol.* 2019;6[6]:e295-e305).

Congenital hemophilia A, a genetic deficiency of the procoagulant factor VIII, is a disease negatively impacting secondary hemostasis, and in this disorder the PT would be normal, the aPTT would be prolonged, and the 50:50 mixing studies (incubated or nonincubated) "would correct into the normal range or to within whatever criteria are often used by the laboratory," Dr. Goodwin said. And the fibrinogen and PFA-100 results would be normal.

"In a patient with lupus coagulant—an acquired thrombotic disorder—depending on the reagents, the aPTT can also be prolonged," Dr. Goodwin said. "But the difference is that the 50:50 mix fails to correct. So the 50:50 mix has some very good discriminating information with the results that can be used if you're a reference laboratory or a laboratory that doesn't have any history or information. A 50:50 mix is always a very good next step when you have prolonged screening coagulation tests."

The fibrinogen and PFA-100 would be expected to be normal for a lupus coagulant patient, he added.

In patients with primary hemostatic disorders, such as von Willebrand disease, the PT and the aPTT can be normal. The aPTT can also be prolonged depending on the severity of the disease and the type of von Willebrand the patient has, Dr. Goodwin said.

Since the PT, aPTT, 50:50 mix, and fibrinogen test results can look similar to those of a patient with hemophilia, “and certainly in a patient with von Willebrand disease, we would have to do von Willebrand studies as well.”

In patients with fibron clot cross-linking or fibrinolytic defects that lead to a hemostatic disorder, the screening tests have limited sensitivity, Dr. Goodwin said. “They are not designed to pick up a patient, for example, with factor XIII deficiency. So in a patient who has delayed or significant bleeding following surgery—usually a few days later, when their screening coagulation tests are all normal—it’s time to start thinking about fibrinolytic defects.”

For specialized coagulation testing for hemophilia, “there are two main flavors of factor VIII activity monitoring”—aPTT-based clotting and chromogenic substrate assays—and there is variability in the values based on the methodology and the activator, Dr. Goodwin said.

“You can have normal aPTT results in a patient with hemophilia, particularly mild hemophilia. And in a male patient with a presentation or bleeding disorder that fits to a secondary hemostatic disorder and has concern for hemophilia A, if the clot-based activity is normal, it is recommended that you consider the phenomenon called discrepant hemophilia and follow up testing with a chromogenic FVIII activity assay.”

The chromogenic substrate assays are “more sensitive to certain mutations that are encountered, and that helps us in patients who present again with a bleeding disorder for whom we have a strong suspicion but a normal clot-based factor VIII activity,” he said. The chromogenic assay will have increased sensitivity and can show a lower factor VIII activity compared with the clot-based assay.

A paper published earlier this year showed that in some scenarios “the difference between the clot-based activity and the chromogenic activity could be on the order of two to four to even six times’ difference,” Dr. Goodwin said, with clot-based activity results being higher compared with chromogenic activity results (Al-Huniti A, et al. *Am J Clin Pathol*. 2020;154[1]:78-87).

Emicizumab: impact on coagulation laboratory tests

Test	Effect
Activated partial thromboplastin time (aPTT)	Decrease aPTT (potential for false normal)
aPTT-based factor assays (VIII, IX, XI, XII)	Falsely increase factor activity levels
Activated clotting time	Falsely decreases clotting time
Prothrombin time (PT)*	Not affected
PT-based factor assays (VII, X, V, II)*	Not affected
Clotting-based Bethesda assay for inhibitors	Miss/underestimate inhibition
Fibrinogen	Not affected
Thrombin time	Not affected
Anti-Xa activity (heparin assays)	Not affected
VWF activity and antigen	Not affected
Factor XIII antigen	Not affected
Thromboelastography (TEG)	Normal R value (still useful for monitoring platelet function, as maximum amplitude will not be affected)

*Confirm with local reagents

aPTT-based assays are not accurate in a person taking emicizumab for up to six months after discontinuation of medication.

Adamkewicz JJ, Chen DC, Paz-Priel I. Effects and interferences of emicizumab, a humanised bispecific antibody mimicking activated factor VIII cofactor function, on coagulation assays. *Thromb Haemost*. 2019;119:1084-1093.

Müller J, Pekrul I, Pötzsch B, Berning B, Oldenburg J, Spannagl M. Laboratory monitoring in emicizumab-treated persons with hemophilia A. *Thromb Haemost*. 2019;119:1384-1393.

Jenkins PV, Bowyer A, Burgess C, et al. Laboratory coagulation tests and emicizumab treatment a United Kingdom Haemophilia Centre Doctors' Organisation guideline. *Haemophilia*. 2020;26:151-155.

The impact of extended half-life factor replacement products on coagulation testing is noteworthy, Dr. Goodwin said.

"What's important to understand when you look at the papers, often they're going to present percent recovery of factor VIII in a patient receiving an extended half-life product. And the critical component you need to pay attention to is what's the reagent, the assay type, and the activator being used. The activators behave differently in these extended half-life products" (Van den Bossche D, et al. *Int J Lab Hematol*. 2018;40[suppl 1]:21-29).

For example, Van den Bossche and coauthors found that an aPTT-SP reagent—a one-stage clotting assay with a silica activator—underestimated the percentage of factor VIII expected recovery of Afstyla (CSL Behring)—a single-chain, B-domain truncated, extended half-life medication—by about half (52 percent) compared with the chromogenic assay result. Using the correction factor of two, as indicated in the product insert, resulted in a percent factor recovery value of 104. "This is not a value of percent activity," Dr. Goodwin noted.

"As a laboratory, we need to communicate with our clinicians and understand what particular medication a patient is being treated with for their hemophilia A so we are certain that the proper conversion factors are being used in a patient who's having a clot-based assay," Dr. Goodwin said.

In the Van den Bossche study, the chromogenic substrate assay showed better "percentage of FVIII:C expected recovery (%)" at normal levels, making it the manufacturer-preferred assay type for this medication.

However, when looking at low levels of factor VIII, for which the definitions can vary, "some of these assays perform quite well," Dr. Goodwin said. The same aPTT-SP assay did recover about 100 percent of low levels of factor VIII (without using the correction), while the chromogenic assay overestimated the percent recovery at about 150 percent.

Dr. Goodwin explained that the impact of emicizumab, the first nonfactor replacement medication, on common coagulation assays is evident in the aPTT-based factor activity assay. He pointed to three recently published reviews (Adamkewicz JI, et al. *Thromb Haemost*. 2019;119[7]:1084-1093; Müller J, et al. *Thromb Haemost*. 2019;119[9]:1384-1393; and Jenkins PV, et al. *Haemophilia*. 2020;26[1]:151-155).

In a chromogenic FVIII activity assay using human-derived reagents, the effect of emicizumab is apparent and results in an overestimation of the patient's FVIII activity. Thus, laboratories need to recognize this point and use a chromogenic FVIII activity assay with bovine-derived reagents (using bovine FIXa and FX components), which are insensitive to the presence of emicizumab.

Some clotting and chromogenic assays, such as fibrinogen, thrombin time, PT-based APC resistance, anti-Xa, protein C activity (chromogenic method), antithrombin activity, and plasminogen activity, are not affected by emicizumab. "Certainly the ELISA-based assays, the latex immunoturbidimetric assays that we use for von Willebrand's and other things, are not going to be impacted by emicizumab, and obviously genetic tests would not either," Dr. Goodwin said.

He explained why emicizumab affects the aPTT clot-based assays in particular. "Let's say we're monitoring a patient factor VIII activity level. We'll take the sample, we'll add factor VIII deficient plasma, and we'll add our activator [with phospholipid and calcium sources]. We will run a PTT, record that clot time, and we will understand that the PTT is inversely proportional to the concentration of the patient's factor VIII." If the aPTT is prolonged, factor VIII is low. If it's short, factor VIII activity is higher.

Since emicizumab causes shortening of the aPTT, “it’s no wonder that you’re going to have an overestimation of the patient’s factor VIII activity.”

Chromogenic factor VIII activity assays that use a human reagent will be sensitive to the presence of emicizumab “and in the U.S. particularly, it can act as a qualitative assessment for emicizumab activity,” he said—is emicizumab present or absent? Chromogenic factor VIII activity assays using bovine reagents provide a quantitative assessment of factor VIII activity in the patient sample due to either factor replacement therapy or the patient’s own endogenous factor VIII. “This is the one you would use if you were doing a Bethesda assay to look for potential inhibitors in the patient.”

The chromogenic assay works by adding activated factor IX, activated thrombin, and factor X inactivated, in excess, to the patient sample with phospholipid and calcium. “The patient, dependent on how much factor VIII they have, is going to bind with factor IX and activate factor X, so this assay in the first stage is going to generate factor Xa.”

The limiting factor is how much factor VIII the patient has, he added.

“We then take that patient’s plasma that now has activated X and we add a chromogenic substrate. Factor X, in its activated form, will cleave the chromogenic substrate and produce a chromogenic signal, which can then be measured at 405 nanometers. In this scenario, the amount of signal that is measured at 405 nanometers is proportional to the amount of factor VIII the patient had in their sample back in the first stage.”

Whether the reagent is human or bovine is the crucial point to remember, Dr. Goodwin said. The former is sensitive to the presence of emicizumab, the latter is not. Thus, in using a chromogenic assay in a dose-dependent manner, the human reagent will show increasing factor VIII activity based on the concentration of emicizumab. With a bovine reagent, “the only factor VIII activity that is going to be detected is what the patient is able to contribute to the assay system” (Adamkewicz JL, et al. *Thromb Haemost.* 2019;119[7]:1084-1093).

Drs. Unold and Goodwin completed the CAP20 presentation with hypothetical case-based scenarios to help solidify the information presented. For case No. 1, the participants were asked to choose which findings were most consistent for a patient with hemophilia A:

- 14-year-old woman with heavy menses and frequent, life-long epistaxis with normal PT, prolonged aPTT, and thrombocytopenia.
- 17-year-old man with easy bruising, frequent joint dislocations, a painful right knee joint, and visible vasculature with normal PT, aPTT, and VWF assays.
- 45-year-old man with oozing from his surgical site beginning 24 hours following a colon resection, with normal PT and aPTT, and increased lysis on a viscoelastic assay.
- two-year-old boy with multiple bruises on both his extensor and non-extensor surfaces, plus a swollen knee two days after falling out of bed, with normal PT and a prolonged aPTT, which fully corrects after immediate and incubated 50:50 aPTT mixing studies.
- 34-year-old woman with four miscarriages and a current edematous right lower extremity, with normal PT, prolonged aPTT, and a 50:50 aPTT immediate mixing study that fails to correct.

The correct answer is the fourth, the two-year-old boy. Dr. Unold explained that the scenario for the 14-year-old female patient would be more consistent with von Willebrand disease since hemophilia A affects primarily males, though females can be carriers. The patient's prolonged aPTT could have been due to decreased factor VIII as it relates to von Willebrand disease.

The 34-year-old female patient's history of miscarriages prompted thoughts of thrombophilia and potential antiphospholipid antibodies, and her edematous lower extremity likely represents a DVT. "The failure to mix would also indicate potential antiphospholipid antibodies, which are inhibitors essentially, rather than a factor deficiency," he said.

The normal aPTTs of the two older male patients reduced the likelihood of hemophilia A diagnoses for them. Of the 17-year-old male, Dr. Unold said, "Certainly this clinical presentation, with easy bruising and joint dislocations, you would see in a patient with connective tissue disorder, perhaps Ehlers-Danlos." The 45-year-old man who had oozing from the surgical site and increased lysis on a viscoelastic assay (TEG or ROTEM, for example) "makes us think of fibrinolysis, which may occur sometimes in the postoperative period in some patients, perhaps due to decreased plasminogen activator inhibitor." But it wouldn't be consistent with hemophilia A, Dr. Unold said.

He called the two-year-old the "prototypical patient with hemophilia A."



Dr. Goodwin

"You have a young patient who's presenting with a new bleeding or bruising. He has a swollen knee—joints are a typical target of bleeding in patients with hemophilia. And the correction of the mixing studies lets us know that we're dealing with a factor deficiency, and in this case factor VIII."

The patient is found to have the intron 22 inversion on the *F8* gene, Dr. Goodwin said. He is started on standard therapy but fails to show appropriate therapy response three years later. A Bethesda assay shows an inhibitor to factor VIII, and the patient begins treatment with emicizumab. The *F8* intron 22 inversion "often presents with a severe type of disease" and has about a 30 percent chance to develop an alloimmune inhibitor, he added.

Dr. Goodwin continues with the same patient who is now a 10-year-old who falls on a rock while hiking out of state and visits the emergency department for treatment of a significant hematoma that develops over four hours. Dr. Goodwin asked session viewers to select the correct impact of emicizumab on a series of coagulation tests ordered by the emergency physician:

- aPTT: falsely overestimate.
- aPTT clot-based factor VIII activity: falsely increased activity.
- PT: falsely underestimate.
- Thrombin time: falsely overestimate.
- Fibrinogen Clauss method: falsely underestimate.

The PTT will not falsely overestimate; it will falsely underestimate—the clot time will be shorter, he said. "Therefore, you're going to have a PTT clot-based assay that will lead to falsely increased activity because of the shortened PTT. The PT is generally not impacted, nor are the thrombin time and fibrinogen Clauss method."

In this case, the aPTT clot-based factor VIII activity assay result would show falsely increased activity because of

the shortened PTT.

In another scenario, a 14-year-old male is admitted to a hematology consult service for a swollen left knee joint, determined to be hemarthrosis. Laboratory testing is performed with the following results: prolonged aPTT at 49 seconds, normal PT at 11.2 seconds, and factor VIII activity (human-reagent chromogenic assay) is critically low at less than one percent. A Bethesda assay (chromogenic, bovine) is performed with a result of 5.2 Bethesda units (reference interval: zero to factor VIII).

Based on the results, Dr. Unold asked, which of the following is correct:

- This patient has not developed an antibody to emicizumab as literature lacks documented cases of neutralizing antibodies.
- The chromogenic FVIII activity (human) is insensitive to the presence of emicizumab.
- The detected Bethesda units are inaccurate.
- Both the aPTT and PT results are accurate.
- The FVIII PTT clot-based activity assay is best to assess the patient's factor VIII activity.

The answer: Both the aPTT and the PT results are accurate.

"Knowing that the human-based chromogenic activity would be affected by emicizumab, there was less than one percent factor VIII in this hemophiliac," Dr. Unold said. "We would expect with emicizumab on board that we would actually have had a falsely increased factor VIII activity because this test is affected. But in this case, because there was less than one percent, we were thinking the emicizumab was either not on board or not affecting the assay."

Two reasons for that result could be noncompliance with the medication or development in the patient of neutralizing antibodies against emicizumab.

"Knowing that the drug was not affecting the test led us to presume then that the aPTT was actually accurate and not falsely elevated by the drug," Dr. Unold said. And PT is usually not affected by emicizumab, though this varies depending on the PT performed. "Detected Bethesda units would be accurate in this case, certainly because a bovine assay would not be expected to be affected by the drug, much less the fact that the drug did not seem to be either present or affecting this human-based test."

There are instances in the literature—the HAVEN trials—showing that neutralizing antibodies can be developed to emicizumab, he said. "And remember that a PTT clot-based assay is not the best way to assess a patient's factor VIII activity if they're on emicizumab, because these clot-based assays will be affected by the presence of the drug."□

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