JOHNS HOPKINS HOSPITAL

HEMOSTASIS MANUAL

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JOHNS HOPKINS
MEDICINE
THE JOHNS HOPKINS HOSPITAL

HEMOSTASIS MANUAL

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Baltimore, Maryland

CONTENTS

LIST OF FIGURES AND TABLES

Figures

Tables

SELECTED ABBREVIATIONS

ACA	anticardiolipin antibody
APC	activated protein C
APLA	antiphospholipid antibodies
APS	antiphospholipid antibody syndrome
AT	antithrombin; antithrombin III
AvWS	acquired von Willebrand syndrome
CLSI	Clinical Laboratory Standards Institute (formerly NCCLS)
DIC	disseminated intravascular coagulation
DOAC	direct oral anticoagulant
dRVVT	dilute Russell viper venom time
DTI	direct thrombin inhibitor
ELISA	enzyme-linked immunosorbent assay
FDP	fibrin degradation products (aka fibrin split products)
FEU	fibrinogen equivalent units
FVL	factor V Leiden
HIT	heparin-induced thrombocytopenia
HMWK	high-molecular-weight kininogen
HMWM	high-molecular-weight multimer
HSP	Henoch-Schönlein purpura
INR	international normalized ratio
ISI	international sensitivity index
ISTH	International Society on Thrombosis and Haemostasis
LMWH	low molecular weight heparin
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
NORD	National Organization for Rare Disorders
PAI-1	plasminogen activator inhibitor-1
PF4	platelet factor 4
PT	prothrombin time
PTT	partial thromboplastin time
RT	reptilase time
RVVT	Russell viper venom time
SLE	systemic lupus erythematosus
SSC	Scientific and Standardization Subcommittee (of the ISTH)
TEG®	thromboelastograph
TFPI	tissue factor pathway inhibitor
tPA	tissue plasminogen activator; tissue-type plasminogen activator
TT	thrombin time; thrombin clotting time; TCT
TTP	thrombotic thrombocytopenic purpura
UFH	unfractionated heparin
VTE	venous thromboembolism
vWD	von Willebrand disease
vWF	von Willebrand factor

INTRODUCTION

Hemostasis and Laboratory Testing

A Brief Overview of Hemostasis

Maintenance of normal hemostasis depends upon the balance between the procoagulant proteins (e.g., factor IX, etc.), the endogenous anticoagulant proteins (e.g., protein C, protein S, etc.), and the proteins of the fibrinolytic system (e.g., tissue plasminogen activator, plasminogen).

Figure 1 shows the classical concept of the coagulation cascade. This depiction is useful clinically as it provides a good illustration of what the prothrombin time (PT) and partial thromboplastin time (PTT) measure. A list of hemostatic factors and molecules is provided in Appendix I.

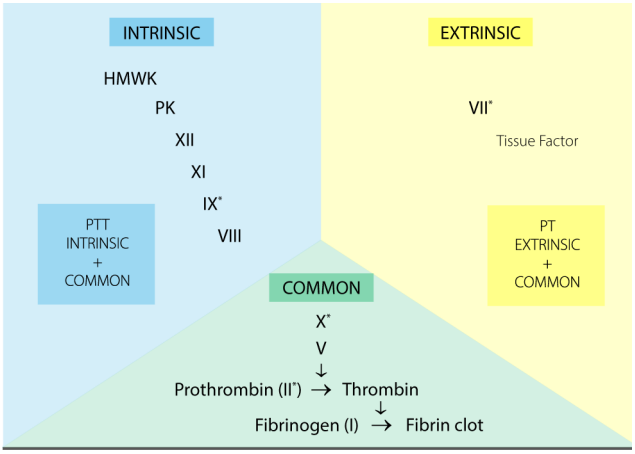

Figure 1. The classical coagulation "cascade." This series of reactions is a convenient way to think about various in vitro clotting assays. The partial thromboplastin time (PTT) measures intrinsic factors and the prothrombin time (PT) measures the extrinsic factors, with both measuring the factors of the common pathway. Since the endpoint of the test is a clot, abnormally low fibrinogen levels will prolong both the PT and the PTT. Proteins C and S and antithrombin III (not shown here) serve as inhibitors to exuberant thrombin formation. HMWK, high-molecular-weight kininogen; PK, prekallikrein. * requires vitamin K for its synthesis.

In addition, note several points regarding the biosynthesis of proteins involved in hemostasis:

- Fibrinogen, prothrombin, factors V, VII, IX, X, XII, XIII, and probably XI, as well as prekallikrein, high-molecular-weight kininogen (HMWK), proteins C and S, and antithrombin are synthesized in the liver.
- Factors II, VII, IX, and X, as well as proteins C and S require the presence of vitamin K for their synthesis.

- The majority of the clotting factors are serine proteases, and the only important non-enzyme factor is factor VIII.

Before you get overwhelmed, just remember that the coagulation cascade generates thrombin. Thrombin converts water-soluble fibrinogen to water-insoluble fibrin. Too little thrombin generation leads to bleeding, and a relative abundance of thrombin can lead to thrombosis. How simple!

Clinical Considerations

History-taking

It cannot be overemphasized that coagulation tests can be interpreted accurately only in the context of the patient history. The minimal information required is:

- The nature of the problem (e.g., hemorrhage or thrombosis).
- A complete list of medications, particularly antithrombotic medications—such as heparin, warfarin, direct oral anticoagulants (DOACs) and direct thrombin inhibitors (DTIs), and antiplatelet drugs—and hormonal preparations.
- The patient's age and gender.
- The family history of bleeding or thrombosis.
- If bleeding is the clinical issue, details about the type of bleeding.
- If thrombosis is the clinical issue, the location of the thrombosis—arterial, venous, or both—is important. It is also important to determine whether the thrombosis is idiopathic or associated with a transient risk factor.

Table 1, adapted from guidelines released by the International Society on Thrombosis and Haemostasis (ISTH), provides a useful systematic approach to assessing whether a bleeding disorder is present. Use this tool in conjunction with the clinical history; Appendices II and III provide suggested topics to cover.

Initial Workup of a Bleeding Patient

When confronted with a bleeding patient without a known coagulopathy, the initial tests should include a complete blood count, peripheral smear, PT, and PTT. Figure 2

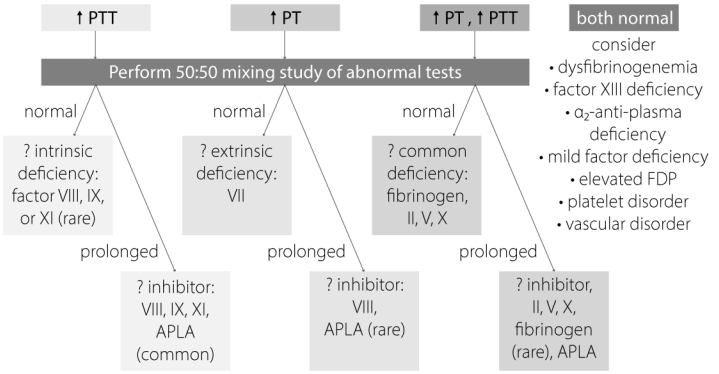

Figure 2. Initial laboratory evaluation of a bleeding patient.
APLA, antiphospholipid antibodies; FDP, fibrin degradation products.

BLEED TYPE	SCORE			
	1	2	3	4
epistaxis	> 5/yr or > 10 min	consulted physician	packing, cautery, or antifibrinolytic	transfusion[1]
cutaneous	≥ 5 bruises in exposed areas	consulted physician	extensive bruising	spontaneous hematoma with transfusion
minor wounds	> 5/yr or > 10 min	consulted physician	surgical hemostasis	transfusion[1] or desmopressin
oral cavity	present	consulted physician	surgical hemostasis or antifibrinolytic	transfusion[1] or desmopressin
gastro-intestinal	unprovoked	consulted physician	surgical hemostasis or antifibrinolytic	transfusion[1] or desmopressin
hematuria	macroscopic	consulted physician	surgical hemostasis or iron therapy	transfusion[1] or desmopressin
tooth extraction	≤ 25% of all procedures, no therapy	> 25% of all procedures, no therapy	resuturing or packing	transfusion[1] or desmopressin
surgery	≤ 25% of all procedures, no therapy	> 25% of all procedures, no therapy	surgical hemostasis or antifibrinolytic	transfusion[1] or desmopressin
menorrhagia	consult only, > 2 pads/hr, clot & flood, or PBAC > 100	> 2 sick days/yr, iron, hormones, or antifibrinolytic	since menarche and > 12 mo or antifibrinolytic plus hormones	transfusion[1], desmopressin, emergency visit, or surgical cure
post-partum	consult only, syntocin, or lochia > 2 weeks	iron or antifibrinolytic	transfusion[1], desmopressin, EUA, or packing	surgical cure
muscle hematoma	post-trauma, no therapy	spontaneous, no therapy	replacement therapy or desmopressin	surgery or blood transfusion
hemarthrosis	post-trauma, no therapy	spontaneous, no therapy	replacement therapy or desmopressin	surgery or blood transfusion
CNS	-	-	subdural	intracerebral
other	present	consulted physician	surgical hemostasis or antifibrinolytic	transfusion[1] or desmopressin

Table 1. Assessment of a patient's bleeding history.
Adapted from Rodeghiero F, et al. ISTH/SSC bleeding assessment tool. J Thromb Haemost. 2010;8(9):2063-5. See this work for important details and interpretative guidance. PBAC, pictorial blood loss assessment chart; EUA, exam under anesthesia. [1]blood transfusion or replacement therapy

Condition	Suggested tests
Defect in primary hemostasis	platelet count; peripheral blood smear; PFA-100 assay; vWF antigen; ristocetin cofactor assay; factor VIII activity; platelet aggregation study
Defect in secondary hemostasis	PT; PTT; thrombin time; fibrinogen level; add factor assays if the screening test results are suggestive
Delayed bleeding	factor XIII screen (add factor XIII activity if suspicion is high); α2-antiplasmin; PAI-1
Venous thromboembolism	PTT; APC resistance assay (order factor V Leiden sequencing if positive); prothrombin G20210A gene mutation analysis; dRVVT with confirmation; anticardiolipin and β2-glycoprotein 1 antibody testing; protein C, protein S, and antithrombin activity
Arterial thromboembolism	PTT; thrombin time; fibrinogen level; reptilase time; dRVVT with confirmation; anticardiolipin and β2-glycoprotein 1 antibody testing; homocysteine

Table 2. Workup of bleeding and thrombotic disorders. A defect in primary hemostasis manifests as mucocutaneous bleeding, which indicates a disorder of platelet-plug formation. A defect in secondary hemostasis is marked by the type of bleeding seen in coagulation factor deficiencies: hemarthroses, deep-tissue hematomas. PFA-100, platelet function analyzer; vWF, von Willebrand factor; PT, prothrombin time; PTT, partial thromboplastin time; PAI-1, plasminogen activator inhibitor 1; APC, activated protein C; dRVVT, dilute Russell viper venom time.

shows an algorithm for interpreting the PT and PTT in this workup. Suggestions for initial tests based on specific bleeding characteristics are listed in Table 2. See Chapters 1 through 3 for descriptions of these tests. Disorders of the fibrinolytic system are described in Chapter 5.

Venous	Venous and Arterial
Inherited	Inherited
antithrombin deficiency	hyperhomocysteinemia
protein C deficiency	dysfibrinogenemia
protein S deficiency	homocystinuria
factor V Leiden	dysfibrinogenemia
prothrombin G20210A	Acquired
elevated factor VIII	malignancy
Acquired	APS
previous thrombosis	hormone therapy
age	hematopoietic disorders
immobility	HIT
surgery	DIC
pregnancy	
obesity	
infection	
APC resistance	
smoking	

Table 3. Risk factors for thrombosis. Adapted from Konkle BA. Bleeding and thrombosis. In: Harrison's Principles of Internal Medicine, 20e. APC, activated protein C; APS, antiphospholipid antibody syndrome; HIT, heparin-induced thrombocytopenia; DIC, disseminated intravascular coagulation

Routine screening for a bleeding tendency before surgery is not recommended. Recall that a prior incident is the strongest predictor of bleeding risk (or thrombosis). If the patient has had one or more major hemostatic challenges, such as surgery or trauma, without excessive bleeding, a clinically important hereditary bleeding disorder is unlikely to be present. Conversely, if a personal or family history suggests a bleeding disorder, you cannot exclude this possibility based on routine screening tests; refer the patient to a hematologist.

Approach to Thrombosis

A hypercoagulable state can be due to an inherited thrombophil-

ia or an acquired condition. The most common inherited cause is factor V Leiden, which has been implicated in up to 50% of cases of familial thrombophilia. Risk factors for thrombosis are summarized in Table 3.

Current practice recommends that clinicians pursue a workup for an inherited thrombophilia or acquired hypercoagulable state only if such information will change clinical management. In general, patient characteristics (age, medications, systemic diseases), confer a greater risk for thrombosis than genetic mutations. For example, the extent and nature of a surgery are the factors that most strongly influence the probability of developing post-operative venous thromboembolism. The presence or absence of a familial predisposition to thrombosis rarely influences the decision to prescribe prophylaxis. Therefore, preoperative testing for such abnormalities is not necessary.

Consider thrombophilia testing for patients with recurrent thromboembolism, a first event at age 50 or younger, a strong family history of thromboembolism, or thromboembolism in an unusual site—for example, the hepatic, mesenteric, or cerebral vasculature. The location of the thromboses (i.e., arterial or venous) offers a reasonable guide for laboratory testing, although there is a good deal of disease overlap between the two locations. Suggested initial tests are described in Table 2.

Descriptions of tests for diagnosing thrombophilias are covered in Chapter 4, including a discussion of antiphospholipid antibody syndrome and tests used for its diagnosis. Dysfibrinogenemia, which can lead to abnormal bleeding or clotting, is addressed in Chapter 5.

Medications

The therapeutic anticoagulants in use today may or may not affect screening coagulation tests. We provide specific comments regarding these agents in the description of individual tests. The effect of anticoagulants on a selection of tests is presented in Appendix IV.

Keep in mind that any medication or supplement that modifies the concentration of a clotting factor may affect hemostasis—in particular, drugs that affect vitamin K metabolism or alter a natural prothrombotic or antithrombotic mechanism. The best example here is the common medication aspirin, which affects platelets.

Sample Collection

Sodium citrate is the principal anticoagulant used for specimens submitted for coagulation testing. It inhibits coagulation by complexing free Ca^{2+} ions, which are essential for several chemical reactions in the coagulation cascade. For this reason, virtually all coagulation tests are initiated by adding calcium to the

Analytical Errors in Coagulation

(Or, Kickler's DOs and DON'Ts)

The quality of a coagulation result is crucial, and there are many variables with which to contend—reagents, instruments, different test kits, lack of standardization of calibration curves. Preanalytical variables, in fact, account for 60% to 70% of all errors in testing. Table 4 lists some DOs and DON'Ts regarding common errors. Following these rules can help our coagulation laboratory troubleshoot efficiently and, ultimately, deliver high-quality results, These issues are common to all coagulation tests, including those described in this manual. Note that some of these came to me from colleagues at the erstwhile NewYork Hospital. ♦

DO be ruthless about your specimen. Adhere to the 9:1 blood-to-anticoagulant ratio and keep the CLSI guideline of a ± 10% fill rate.	**DON'T** think you are helping by ignoring this guideline. An underdrawn tube (thus, excess anticoagulant) can prolong an otherwise normal result.
DO remember that PTTs must be performed within a four-hour window, whereas PTs are stable for 24 hours.	**DON'T** add a PTT to a sample older than four hours. It is tempting to report a normal result, but recall that when plasma contacts platelets, PF4 is released and neutralizes heparin. If the patient is on a low dose of heparin, the result may falsely appear in the normal range.
DO confirm any PTs or PTTs that result in "no clot" using an alternative method or by checking the cuvette for a clot.	**DON'T** just assume these are prolonged—they may have clotted. This can occur with small clots or when the patient is receiving recombinant factor VIIa.
DO find out if patients are on heparin, warfarin, direct thrombin inhibitors, or direct oral anticoagulants.	**DON'T** think only unfractionated heparin or warfarin can prolong results. Some LMWHs prolong PTT, and DTIs (hirudin, argatroban) can prolong screening tests and clot-based tests.
DO make sure that your normal range reflects your patient population. Use a minimum of 20 people, with equal numbers of males and females.	**DON'T** use ER or pre-op patients, who may have elevated acute phase reactants, such as fibrinogen and factor VIII. Such elevations can shorten your normal range.
DO realize the importance of the normal range. It sets the INR, normal vs. abnormal, and the therapeutic range of heparin (1.5 to 2.5 times the mean of the normal range).	**DON'T** prepare your range all in one day. Rather, run it over a longer period of time so that you can capture interday variation.
DO know your reagents; they can be your worst nightmare.	**DON'T** assume a normal PT and PTT equals a normal patient, unless you know your reagents.
DO know how sensitive your reagents are for factors, even if you perform only PTs and PTTs.	**DON'T** run a reagent lot without knowing how many seconds represent a factor deficiency. For example, if the PTT upper limit is 37 seconds but reflects only 19% factor IX, the patient may bleed during surgery.
DO know the actual therapeutic range for unfractionated heparin.	**DON'T** think that just using 1.5 to 2.5 times the mean of the PTT normal range is good enough.
DO obtain a heparin therapeutic range by collecting specimens from 30 patients on all dosages. Patients must have a normal PT. For each specimen, run a PTT with the reagent lot as well as a heparin level. Plot a graph of the PTT against the heparin level; the seconds that correspond to 0.3 to 0.7 iU/ml of heparin represent the therapeutic range.	**DON'T** just spike normal plasma with known concentrations of heparin and run a PTT. Doing so masks the biological variation seen in patients on heparin.

DO make sure that you are working with platelet-poor plasma (PPP)—that is, plasma with a platelet count of less than 10,000.	**DON'T** assume centrifuging makes specimens platelet-poor. Check your centrifuges. Be wary of the StatSpin. Specimens spin fast, but if the specimen sits, platelets diffuse back into the plasma.
DO remember that platelets are phospholipids, as are coagulation reagents.	**DON'T** forget that the presence of platelets will shorten clotting times.
DO be careful when you are separating and freezing specimens.	**DON'T** think that more plasma is always better. If you pick up platelets from the buffy coat you will shorten clotting times. If you freeze and thaw specimens, the platelets will burst and interfere with the phospholipid in the reagent, again shortening clotting times.
DO use a PT reagent with an ISI < 1.5. Reagents with high ISIs are less sensitive to warfarin.	**DON'T** use insensitive reagents with high ISIs. Remember that in the formula for the INR, the ISI is the exponent, so the higher the ISI, the greater the error in calculating the INR.
DO know that the INR is valid only on patients who are on a stable dose of warfarin. It takes about two weeks for a patient to become stable.	**DON'T** use the INR for diagnosing other possible coagulopathies.
DO obtain new normal ranges if you switch from glass to plastic tubes.	**DON'T** think it has to be done only when you change a reagent lot. According to CLSI guidelines, it must be done annually or when you change instruments, lots, or collection systems.
DO use only 3.2% sodium citrate. The specimens are more stable, results are more accurate, and the anticoagulant is closer to plasma osmolality.	**DON'T** use 3.8% sodium citrate. Your testing will contain more variability, and 3.2% is an important part of standardizing methods.
DO avoid traumatic venipunctures; it can release tissue factor and initiate coagulation.	**DON'T** assume that a bad draw with normal results is OK; times may be artificially shortened.
DO remember that a hematocrit > 55% will prolong coagulation results.	**DON'T** forget that a hematocrit of < 20% may not contain sufficient anticoagulant and lead to a clotted specimen.
DO remember that every accession number and every medical record number is attached to a patient.	**DON'T** think that just giving a number for a result is enough. Understand the importance of giving quality results and the role it plays in patient care.

Table 4. Kickler's DOs and DON'Ts.

sample ("recalcification"). Improper collection can affect test results significantly. Citrated vacutainers ("blue tops") contain 0.5 mL of anticoagulant (3.2% sodium citrate), and the vacuum is set to draw in precisely 4.5 mL of blood. Anything that disturbs this ratio can alter test results. The two most common situations in which this occurs are

- when the vacuum is not used (e.g., manually filling the from a separate syringe, with or without removal of the blue top) and
- when the plasma volume is abnormal (as with erythrocytosis or severe anemia).

Platelets are not included in most coagulation tests—in fact, it is essential that they be removed, since they accelerate several key enzymatic reactions. Serum also lacks fibrinogen and factors V, VIII, and XIII, all of which remain in the clot. Thus, serum does not reflect the in vivo state of circulating blood.

Specimens for Special Coagulation tests should never be drawn from a central line or heparin lock without extensive flushing (at least 20 mL of blood). A clean venipuncture at a separate site is greatly preferred. Blood should be taken from the opposite arm when patients are receiving therapeutic heparin infusions.

Hemolyzed samples cannot be tested. Free hemoglobin activates platelets and in vitro clotting. Lipemic samples, samples from patients who are receiving intravenous fat emulsions or who have very high bilirubin levels (> 20 mg/dL), and samples with low fibrinogen concentrations may give erratic results with our routine lab procedures. Occasionally, some values will need to be checked by more than one method.

Only one test requires sample collection and processing on ice: homocysteine. **Do not refrigerate any blood sample sent to us for coagulation testing other than homocysteine**.

See Appendix V for a list of coagulation tests orderable through Epic.

CHAPTER 1

Screening Tests

Prothrombin time and INR
Partial thromboplastin time
Heparin neutralization
Mixing studies
Fibrinogen
Thrombin time
Reptilase time
D-dimer
Cryofibrinogen

Prothrombin Time (PT) and INR

Also known as Quick Time, after Armand Quick, MD, PhD

This assay is used to evaluate the extrinsic and common pathways of the coagulation cascade (Figure 1).

Indications

- Monitoring warfarin (Coumadin) therapy
- Assessment of clotting-factor deficiency in a vitamin K–deficient state
- Diagnosis of congenital and acquired deficiencies of coagulation proteins in the extrinsic (factor VII) and common (factors II, V, X and fibrinogen) pathways
- Diagnosis of inhibitors of factors II, V, VII, or X
- Assessment of liver function

Test Principle

A mixture of thromboplastin (which contains phospholipid and tissue factor/factor III) and calcium chloride is added to citrated patient plasma. Using photo-optical detection, the time to clot formation is determined. The clotting time of fresh normal plasma ranges from approximately 9 to 11 seconds.

In Europe, human placenta is used as a source of commercial thromboplastin, and in the past, we used to grind up rabbit brains to make it. Today, labs in the United States generally use recombinant human thromboplastin—in particular, Innovin, which is supplemented with a high concentration of phospholipid. Recombinant thromboplastins are often called high-sensitivity thromboplastins because they are very sensitive to reductions in vitamin K–dependent coagulation factors (II, VII, IX, and X). As a consequence, these thromboplastins are similar in sensitivity to the WHO reference standard and thus have a low ISI, about 0.98 to 1.1.

Innovin, which is used in our lab, is insensitive to therapeutic concentrations of heparin (0.3 to 0.7 IU/mL), so PT results do not change significantly once heparin is discontinued in patients receiving both heparin and warfarin. In addition, Innovin is generally not affected by most antiphospholipid antibodies.

A Word About the INR

The international normalized ratio (INR) is used to monitor warfarin therapy. It is a contrived measurement that reflects the sensitivity of thromboplastin to changes in factor VII alone. It should not be used to monitor newer anticoagulants, such as DOACs or DTIs.

The WHO and the ISTH recommend that PT tests for patients on warfarin also include the INR. The INR is independent of the reagent and method used: It represents the prothrombin ratio that would have been obtained if the WHO reference thromboplastin reagent had been used to perform the PT. The calculation is:

$$\text{INR} = \left(\frac{\text{Patient PT}}{\text{geometric mean of PT of normal pooled plasma}} \right)^{\text{ISI}}$$

The ISI for a given thromboplastin preparation is a measure of its responsiveness to reductions in vitamin K–dependent coagulation factors. Commercial rabbit brain thromboplastins had ISIs ranging from 2.0 to 2.6. With the advent of recombinant thromboplastins, such as the one we use in the coagulation laboratory, ISIs down to 1.0 have become available.

We have used Innovin from Siemens for PT testing since around 2000. It is insensitive to heparin and to most antiphospholipid antibodies. Each year, the lot changes, and the ISI may undergo small adjustments. We have noticed very little effect upon test results or the normal range, so our check-in of this reagent is relatively easy. We do pay strict attention to calibration of the ISI for our instruments and to the correct entry of the ISI into our computer system. Now that there are FDA-approved INR calibrators, these risks are reduced.

Our critical action value for the INR is 5.0. We also monitor delta changes for warfarin patients. The biggest discrepancies arise because the person drawing blood collected into EDTA. Just because the tube has a blue cap on it does not mean the collection was not originally in EDTA. ♦

Possible Results & Interpretation

The PT depends on the concentration of factors II, V, VII, and X, and fibrinogen. It is most sensitive to small changes in factor levels when the levels are quite low and least sensitive when levels are near normal. The PT is not influenced by the concentrations of VIII, IX, XI, XII, XIII, prekallikrein, or HMWK.

The most common cause of a prolonged PT is an acquired condition—e.g., warfarin therapy, vitamin K deficiency, DIC, liver disease. In each case, the factor VII concentration is low. Congenital causes of a prolonged PT are uncommon but include deficiencies in the abovementioned coagulation components (II, V, VII, X, and fibrinogen).

A prolonged PT in conjunction with a normal PTT suggests a deficiency of VII. Prolongation of both the PT and PTT is generally due to deficiencies of multiple factors. This can occur in severe liver disease, severe vitamin K deficiency, DIC, and anticoagulant therapy. Also remember that you need at least 75 mg/dL of fibrinogen to form a clot; therefore, if the fibrinogen level is markedly reduced, both the PT and PTT will be prolonged.

Some antiphospholipid antibodies are associated with antibodies to prothrombin (factor II), leading to reduced prothrombin and significant prolongation of the PT (20 to 25 seconds). In these patients, adding normal plasma in a mixing study will not correct the PT: These patients bleed from severe hypoprothrombinemia. Even though interference appears to

be less frequent with Innovin, the thromboplastin we use, you should not use the INR to monitor warfarin in these patients but rely on the chromogenic factor X assay instead.

A shortened PT may represent the lower end of the normal range; increased tissue factor in the sample from traumatic venipuncture; excessive plasma to anticoagulant ratio (e.g., collection error, severe anemia), or a recent infusion of recombinant VIIa.

PT/INR at the Point of Care

While many hospitals—including ours—use point of care (POC) devices to monitor warfarin, I am not personally enthusiastic about this approach. Too many preanalytical variables can affect the results, and staff not trained in the laboratory sciences are unlikely to have the background or experience to adequately troubleshoot problems or address quality assurance issues. Fortunately, our POC PT/INR meters have at least started to use high-sensitivity thromboplastins, including Innovin. ♦

Factors Affecting the PT

Erroneous results can be caused by improper collection, storage, or processing. In particular, patients with a hematocrit > 55% require an adjustment in the collection amount in order to ensure the correct ratio between anticoagulant and blood. Other conditions (malnutrition, congestive heart failure, hepatic disease, hyperthyroidism, fever, steatorrhea, renal failure, antibiotic therapy, prolonged use of narcotics, etc.) can deplete the concentration of one or more clotting factors or interfere with their activity. Other drugs and conditions (hypothyroidism, hyperlipidemia, edema, diarrhea, total parenteral nutrition, hereditary resistance to warfarin, etc.) may decrease the PT as well as reduce the effects of warfarin.

Partial Thromboplastin Time (PTT)

This test is used to evaluate the intrinsic pathway of the coagulation cascade (factors VIII, IX, XI, and XII, prekallikrein, and HMWK. The test used to be performed without activation; now, all PTT tests are performed with activation. Hence, there is no need to use the term "aPTT." Only people who cannot face new realities still use "aPTT" and perhaps should hang it up.

Indications

- Screening for hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), von Willebrand disease, and congenital or acquired deficiency states of the rest of the intrinsic pathway (XI, XII, prekallikrein, and HMWK)
- Monitoring unfractionated heparin therapy
- Screening for inhibitors of factor VIII, IX, XI, or XII
- Screening for the presence of antiphospholipid antibodies; requires a low phospholipid reagent

Test Principle

The PTT test is performed by recalcifying plasma in the presence of platelet membrane–like phospholipids and an activator of the contact factors of the intrinsic coagulation pathway (actin or kaolin). Actin and kaolin activate factor XII.

This mixture triggers activation of the intrinsic pathway and, subsequently, the

common pathway of coagulation. Therefore, the PTT is sensitive to deficiencies of all coagulation factors except factors VII and XIII. Using the present reagent lot (as of July 14, 2020), the normal PTT clotting time is 23.1 to 30.9 seconds. The PTT generally becomes abnormal when one of the PTT clotting factors is decreased to less than 25% to 35% of normal. Consequently, a patient with a normal PTT can still have mild factor deficiencies. Our coagulation lab uses a low phospholipid PTT reagent that is sensitive to the presence of some antiphospholipid antibodies; thus, the PTT may be prolonged if an antiphospholipid antibody is present.

Possible Results & Interpretation

A prolonged PTT indicates a deficiency of factor I (fibrinogen), II, V, VIII, IX, X, XI, or XII, prekallikrein, or HMWK, or an inhibitor of one or more of these components. Common causes of a prolonged PTT include heparin therapy, heparin contamination, antiphospholipid antibodies, DIC, and congenital or acquired deficiency of VIII or IX. Less common causes of a prolonged PTT include deficiency of XI, another specific intrinsic pathway factor deficiency, or an inhibitor.

An abnormally prolonged PTT with a normal PT indicates a deficiency of factor VIII (hemophilia A), IX (hemophilia B), XI, or XII; von Willebrand disease; or the presence of heparin. Prolongation of both the PT and PTT is generally due to multiple factor deficiencies, which can be caused by severe liver disease, hypo-or dysfibrinogenemia, DIC, warfarin, and severe vitamin K deficiency.

A shortened PTT may represent the lower end of the normal range or result from an excessive plasma to anticoagulant ratio (e.g., with incorrect sample collection, severe anemia, or a traumatic venipuncture). Rarely, a short PTT indicates a hypercoagulable state—but only if repeatedly short!

Factors Affecting the PTT

Erroneous results can result from improper sample collection and processing. A prolonged PTT can be seen when blood is drawn from intravenous catheters that have been flushed with heparin or blue tops are underfilled.

Heparin Neutralization Test (Hepzyme®)

This assay tests for the presence of heparin (both UFH and LMWH). We use recombinant enzyme to remove heparin.

Indications

- To rule out heparin contamination as the cause of an abnormal PTT and/or PT
- To compare the PTT results for heparinized and unheparinized samples when monitoring heparin therapy
- To evaluate patients on combined heparin and warfarin therapy

Test Principle

The presence of heparin in a specimen may interfere with the interpretation of various coagulation tests. Heparinase (Hepzyme) cleaves heparin at multiple sites per molecule, including the antithrombin binding site, producing oligosaccharides that have an average molecular weight of 1,000 daltons and have no antithrombotic activity. Hepzyme can neutralize up to 2 IU of unfractionated heparin or LMWH in 1 mL of citrated plasma. This approach is preferred over adding thrombin, since the thrombin time is not specific for the effects of heparin.

Possible Results and Interpretation

Results are reported as positive, negative, or inconclusive. The difference (in seconds) before and after treatment with Hepzyme is used to identify the presence of heparin in a sample. PTT results should be compared with the laboratory's normal range or ± 15% of the patient's baseline PTT, if available.

Factors Affecting the Heparin Neutralization Test

As performed, the assay will neutralize up to 2 IU/mL of heparin. Higher concentrations of heparin may not be fully neutralized. Note that therapeutic levels of unfractionated heparin are 0.3 to 0.7 IU/mL, and therapeutic levels of LMWH are 0.3 to 1.1 IU/mL.

Mixing Studies

These tests are used to determine if a prolonged PT, PTT, or dilute Russell viper venom time (dRVVT) is due to a factor deficiency or an inhibitor.

Indications

- Evaluation of a prolonged PT, PTT, or dRVVT

Test Principle

If a screening coagulation test is prolonged, it is essential for the clinician to know whether a factor deficiency or an inhibitor is causing the abnormal result, as these conditions are often managed differently. Mixing studies are based on two principles:

- An inhibitor, if present, is in excess and inhibits both normal and patient plasma.
- A 50% reduction of any factor is enough to yield a normal test result. In fact, 30% to 40% of the normal level is generally sufficient to give a normal PT, PTT, and dRVVT.

In a 1:1 mix, equal volumes of patient and normal plasma are mixed; in a 4:1 mix, 3 parts patient plasma are mixed with 1 part normal plasma. After mixing, the test is repeated.

In rare cases, a weak or low-titer inhibitor is detected by the 4:1 mix only. Inhibitors such as antiphospholipid antibodies tend to result in prolongation of the PTT immediately after mixing, whereas inhibitors against specific factors require incubation at 37°C for 1 to 2 hours to fully express their inhibitory properties.

Types of Inhibitors

Inhibitors of specific factors

- Neutralizing antibodies against VIII, IX, XI, XII, thrombin, fibrinogen
- Non-neutralizing antibodies against II, VIII, IX, X (i.e., amyloid-associated)

Global inhibitors

- heparin
- glycosaminoglycans
- direct thrombin inhibitors (e.g., bivalirudin, argatroban), Xa inhibitors (e.g., apixaban)

Non-specific inhibitors

- antiphospholipid antibodies (including lupus anticoagulant)
- fibrin degradation products
- paraproteins

Possible Results & Interpretation

Correction into the normal range after mixing indicates a factor deficiency. Lack of correction suggests the presence of an inhibitor. Lack of correction in only the

4:1 mix suggests a low-titer inhibitor, although it is important to note that at 4:1, a mild factor deficiency may also not correct. A paradoxical increase in the PTT after mixing can be caused by a lupus anticoagulant–type antiphospholipid antibody. This uncommon occurrence is due to the "lupus cofactor" phenomenon, in which the normal plasma potentiates that antibody.

Depending upon the results, the clinician may order additional studies to identify the specific factor (e.g., factor levels) or inhibitor (e.g., Bethesda assay) or to confirm the presence of an antiphospholipid antibody (e.g., dRVVT, anticardiolipin immunoassay).

Factors Affecting Mixing Studies

Heparin contamination must be excluded (using the heparinase neutralization test, p. 20) in any specimen with a suspected inhibitor. This is performed reflexively in specimens submitted for a mixing study that show a prolonged PT, PTT, or dRVVT. If heparin is present, the mixing study will not be performed due to the unpredictable effects of heparin on correction.

Warfarin in the sample will lead to screening tests that correct after mixing, mimicking a factor deficiency. Direct oral anticoagulants (both thrombin and Xa inhibitors) also lead to factitious results.

Fibrinogen

This test measures the concentration of functional fibrinogen.

Indications

- Evaluation of coagulation disorders associated with a prolonged PT, PTT, or thrombin time (TT)
- Evaluation of DIC
- Evaluation of liver disease
- Monitoring of patients undergoing thrombolytic therapy

Test Principle

We use the Clauss method for measuring fibrinogen. This is a functional assay based on the fact that at high thrombin concentrations (≥ 100 NIH units/mL), the thrombin time of dilute plasma is inversely proportional to the plasma fibrinogen concentration (i.e., the higher the fibrinogen, the shorter the TT).

In normal blood coagulation, fibrinogen is converted to fibrin by the enzyme thrombin (factor IIa). This conversion of fibrinogen to fibrin is a two-step process. The first step is thrombin-mediated proteolysis of fibrinopeptides A and B from fibrinogen, leaving a form of fibrinogen termed a "fibrin monomer." In the second step, fibrin monomers polymerize to form a fibrin polymer. The formation of this fibrin polymer is recognized in the laboratory as the clotting endpoint of the reaction. The fibrinogen value is determined from a standard curve generated by testing of known concentrations of fibrinogen.

Possible Results & Interpretation

Low fibrinogen values may be seen in liver disease, during thrombolytic therapy, in patients with congenital a-, hypo-, or dysfibrinogenemia; in DIC; and in other consumptive states.

In a patient suspected of having a dysfibrinogenemia, an immunologic assay

is essential for documenting a discrepancy between fibrinogen protein levels and actual functionality. Dysfibrinogenemia is characterized by the production of normal or slightly reduced levels of fibrinogen that are functionally abnormal, such that tests of fibrinogen function (e.g., the Clauss fibrinogen assay) are much more severely affected than tests measuring the mere presence of fibrinogen protein (e.g., a fibrinogen antigen assay). An elevated fibrinogen may be seen in acute and chronic stable liver disease. Severe chronic liver disease can be associated with acquired dysfibrinogenemia.

Factors Affecting the Fibrinogen Test

High concentrations of heparin or fibrin degradation products can result in abnormal results. Structurally abnormal fibrinogens can yield falsely low results.

Thrombin Time (TT)

The thrombin time assay evaluates the conversion of fibrinogen to fibrin.

Indications

- Diagnosis of hereditary fibrinogen deficiencies, including a-, hypo-, and dysfibrinogenemia, when used in conjunction with antigen and activity assays
- Diagnosis of acquired thrombin inhibitors
- Detection of the presence of heparin
- Detection of direct thrombin inhibitor effect

Test Principle

A standard concentration of thrombin (10 NIH units/mL) is added to plasma, and the time to clot formation is measured using photo-optical detection. The thrombin clotting time may be used as a qualitative measure of the level of functional fibrinogen.

Possible Results & Interpretation

A prolonged TT generally means there is a reduction in either the amount or the function of fibrinogen. Prolongation can occur because of inherited quantitative defects in production (afibrinogenemia or hypofibrinogenemia) or inherited qualitative defects. Qualitative defects result in dysfibrinogenemia (production of a normal amount of abnormal fibrinogen) or hypodysfibrinogenemia (reduced production of an abnormal fibrinogen).

Acquired quantitative deficiencies of fibrinogen can result from DIC (during which fibrinogen is consumed) or thrombolytic therapy (during which fibrinogen is destroyed). These scenarios lead to high levels of fibrin and fibrinogen degradation products, which can disrupt fibrin polymerization and thus prolong the TT. Acquired dysfibrinogenemia is common in patients with severe chronic liver disease due to the production of abnormally glycosylated forms of fibrinogen. Thrombin inhibitors, such as those seen after exposure to bovine thrombin, and antithrombin-like substances are occasionally identified in association with multiple myeloma.

The thrombin time is also markedly prolonged in the presence of antithrombotic agents, including warfarin, heparin, direct thrombin inhibitors (e.g., dabigatran, bivalirudin), and tissue plasminogen activator (tPA).

A shortened thrombin time is generally of no significance.

Factors Affecting the TT

The standard warnings apply about sample collection, storage, and processing.

Reptilase Time (RT)

This test evaluates the conversion of fibrinogen to fibrin using reptilase, a snake venom.

Indications

- Identify heparin or a heparin-like inhibitor as the cause of a prolonged TT
- Evaluation of patients with quantitative or qualitative fibrinogen disorders.

Test Principle

Plasma is incubated with reptilase, and the time to clot formation is measured through photo-optical detection. Reptilase is a thrombin-like enzyme derived from the venom of the South American pit viper (*Bothrops asper*, aka fer-de-lance) or the common lancehead (*Bothrops atrox*). This enzyme differs from thrombin in its greater specificity and the extent to which it cleaves of the fibrinogen molecule. Reptilase cleaves only fibrinopeptide A, whereas thrombin cleaves fibrinopeptides A and B. Reptilase is inhibited only slightly or not at all by heparin, heparinoids, hirudin, and low levels of fibrin degradation products (FDP), thus making it useful in the differential diagnosis of a prolonged TT.

Possible Results & Interpretation

A prolonged RT may be due to decreased fibrinogen levels, dysfibrinogenemia, fibrin degradation products, soluble fibrin monomer complexes, abnormal antithrombins (e.g., myeloma paraproteins), and uremia.

Factors Affecting the RT

Partially clotted samples (microclots) will cause misleading results.

D-Dimer

This immunoassay provides a rapid quantification of D-dimers in plasma samples.

Indications

- Diagnosis of DIC
- Assist in the diagnosis of venous thromboembolism (VTE)

Test Principle

This is a quantitative immunoassay for factor XIIIa–crosslinked dimers of the D fibrin degradation product. Thus, both clotting and fibrinolysis must be activated. Thrombin is formed, converts fibrinogen to fibrin, and activates XIII to XIIIa, which cross-links fibrin. Under the action of thrombin, fibrinogen is cleaved into fibrin monomers, which tend to self-associate into intermediate polymers. These polymers are subsequently stabilized by thrombin-activated XIIIa through covalent cross-linkages in the region of the D-domain, thus producing the insoluble fibrin clot.

Thrombin also triggers the release of tPA, which activates the fibrinolytic system. Plasmin is formed at the site of the fibrin clot and attacks both the clot and fibrinogen. However, unlike its action on fibrinogen—which leads to fibrinogen degradation products—plasmin acts on the fibrin clot to exclusively generate derivatives of cross-linked fibrin containing D-dimer.

The rapid quantitative D-dimer assay (INNOVANCE® D-Dimer, Siemens, Malvern, PA) uses an enhanced latex turbidimetric method to quantify D-dimer in plasma samples. Polystyrene particles covalently linked to monoclonal antibodies against the D-dimer fragment are added to a specimen. Since the epitope against which the antibodies are directed is present twice in a D-dimer fragment, only one antibody is required to cause the latex particles to agglutinate. Aggregation of the particles increases the turbidity of the sample, allowing quantification of the D-dimer fragment using the Siemens coagulation analyzer.

Possible Results & Interpretation

The D-dimer level is calculated by the analyzer in mg/L using a standard curve. The total measuring range is approximately 0.19 to 4.40 mg/L fibrinogen equivalent units (FEU); however, this range can be extended through automated dilution. The reported range, therefore, is < 0.19 mg/L to > 30.0 mg/L FEU. When used in conjunction with other clinical and radiologic information in the diagnosis of VTE, a cutoff level of 0.5 mg/L FEU should be used. Note that it is possible for patients with a distal deep vein thrombosis to have a normal result. For the diagnosis of DIC, an elevated D-dimer should be interpreted within the clinical context and with the results of additional testing (i.e., PT, PTT, thrombin time, fibrinogen, and platelet count). See sidebar on this page.

Disseminated Intravascular Coagulation (DIC)

DIC is an acquired coagulopathy characterized by excessive activation of the coagulation cascade. This hyperactivity results in thrombocytopenia, coagulation factor deficiencies, and secondary fibrinolysis. DIC is triggered by a wide variety of medical and surgical catastrophes (e.g., sepsis, snake bites, major trauma, amniotic fluid embolism, disseminated cancer, etc.)

In the laboratory, DIC demonstrates prolonged PT, PTT, and thrombin times; decreased fibrinogen levels; and thrombocytopenia. The test of choice for DIC is the D-dimer assay. D-dimers are fragments of cross-linked fibrin that are produced when a fibrin clot is digested by plasmin. It is important to remember that D-dimer levels are a reflection not only of clot dissolution but also of plasma clearance by the liver. Therefore, elevated D-dimer levels can occur in the presence of liver disease as well as DIC. Nevertheless, these competing causes can often be differentiated clinically as liver disease in the absence of accelerated fibrin clot formation generally causes only modest D-dimer elevations, while DIC is associated with moderate to marked elevations. Appendix VI presents the ISTH scoring system for identifying DIC. ♦

Factors Affecting the D-Dimer

In general, monomer D fragments (i.e., non-crosslinked fragments) up to 20 mg/L will not interfere with the assay, and neither will fibrinogen < 1,000 mg/dL. However, high serum levels of certain substances, including hemoglobin, may lead to spurious results (Table 5). This is an immunoassay, so plasma samples containing heterophil antibodies or mouse monoclonal antibodies may lead to inaccurate results. It remains unclear whether human anti-mouse antibodies will interfere.

On the Exclusion of VTE

Numerous studies have demonstrated the utility of the D-dimer in the diagnosis of DVT/PE (collectively, venous thromboembolic disease or VTE), particularly when used with a pretest probability model, such as the Wells criteria or Geneva score (see Appendix VII). In general, the D-dimer helps exclude VTE in patients judged to have a low pretest probability, while radiologic testing is performed in patients with a moderate or high pretest probability.

Most assays have been standardized to use the same cutoff value for exclusion of thrombosis. In our laboratory, this is < 0.5 mg/L FEU. Note that the cutoff value increases with age, starting at age 50. To obtain the correct cutoff, take the patient's age and divide it by 100. For example, a 72-year-old would have a value of 72/100 or 0.72 mg/L.

Finally, be aware that reliable cutoffs have not been established in pregnant patients, whose D-dimer levels naturally increase with each trimester. ♦

Substance	Interference starts at...
monomer D fragments	20 mg/L
fibrinogen	1,000 mg/dL
albumin	6 g/L
bilirubin	60 mg/dL
hemoglobin[1]	0.2 g/dL
heparin	3 IU/mL
rheumatoid factor	1,330 IU/mL
triglycerides	600 mg/dL
urea	500 mg/dL
uric acid	20 mg/dL

Table 5. Substances that may interfere with the D-dimer assay.
[1]e.g., due to hemolysis

Cryofibrinogen

This is a qualitative test that identifies the presence of a cryofibrinogen.

Indications

- Diagnosis of cryofibrinogenemia

Test Principle

Cryofibrinogens are complexes of fibrinogen, fibrin split products, and plasma globulins that precipitate in the cold and can be associated with purpuric thrombosis and hemorrhage. **Citrated blood must be maintained at 37°C (body temperature) and transported immediately to the coagulation laboratory.** The plasma and blood is then separated at 37°C using heated lab equipment and rapid processing techniques.

Once the plasma is separated from the blood, the plasma is divided in half. One half of the plasma is maintained in a 37°C heating block for 24 hours and the other half is placed on ice in the refrigerator for 24 hours. If, at the end of 24 hours, the iced sample has a gel of fibrin-like strands and the 37°C sample does not, the sample is considered positive for cryofibrinogen. The results may be further confirmed by placing the iced sample in a 37°C incubator for 30 minutes; the gel strands should disappear. The absence of cold-precipitable substance in serum provides substantiation that the protein is cryofibrinogen and not cryoglobulin.

Possible Results & Interpretation

Samples are scored as positive or negative. Cryofibrinogenemia has been associated with DIC, myocardial infarction, pregnancy, neoplasm, VTE, connective tissue disease, vasculitis, and infection.

Factors Affecting Cryofibrinogen

Samples must be kept at room temperature until the plasma and red blood cells separate; otherwise, the cryofibrinogen may deposit on the red cells and give a false negative result.

CHAPTER 2

Factor Assays

Factors II, V, VII, and X

These tests are used to determine the functional activity of coagulation factors in the extrinsic and common pathways of coagulation.

Test Principle

Factor assays are performed by determining the extent to which the patient's plasma corrects the clotting time of test plasma that is known to be deficient in the particular factor of interest (e.g., factor VII–deficient plasma is used to measure the VII activity in a patient sample). The PT is used to measure the functional levels of the factors in the extrinsic and common pathways of the coagulation cascade (Figure 1). (The PTT measures the functional levels of the factors in the intrinsic and common pathways.)

Analysis is performed by making serial dilutions of the reference plasma in buffer (with assay values for the factor under analysis). Each dilution is then mixed with an equal volume of "substrate" plasma that is known to contain normal levels of all factors except the factor that is being assayed. A PT is performed on the serial dilutions. The results are plotted on a log-log graph (polynomial curve) with the percent factor on the abscissa and the time in seconds on the ordinate. The results of the above dilutions make a straight line that forms the basis of a standard curve; this curve can then be used to determine the activity of the coagulation factor in patient samples.

To analyze the activity level of a factor on an unknown (i.e., patient) sample, 1:5 (100% activity) and 1:10 (50% activity) dilutions are prepared and mixed with substrate plasma. The percent activity is read from the abscissa of the graph by finding where the clotting time for the unknown sample intercepts the standard curve.

All of this now is in fact programmed into our analyzers, and graph paper can no longer be found in the laboratory.

Indications

- Determination of the functional activity of coagulation factors in the extrinsic and common pathways (factors II, V, VII, and X)

Possible Results & Interpretation

Factor II

Isolated factor II (prothrombin) deficiency may be inherited or acquired. Inherited deficiencies are extremely uncommon, with an incidence of approximately 1 in 2,000,000. The existence of rare inherited dysprothrombinemias suggests that

factor II antigen and activity assays should be used when evaluating patients with prothrombin deficiency.

Acquired factor II deficiency is occasionally seen in patients with antiphospholipid antibody syndrome who develop factor II antibodies. Acquired inhibitors of factor II may be seen in patients exposed to bovine thrombin, which is used to prepare fibrin glue (a local hemostatic agent commonly used in cardiac surgery and neurosurgery).

Factor V

Isolated deficiency of factor V can be inherited (1 in 1,000,000) or, rarely, acquired. Patients with an acquired deficiency generally suffer from amyloidosis. Congenital V deficiency associated with the production of a dysfunctional molecule have been reported. Rare cases of combined factor deficiencies (V and VII, V and VIII) have also been identified. Mutations of ERGIC-53, an endoplasmic reticulum–Golgi intermediate compartment protein, have been noted in some families with combined V and VIII deficiency. Finally, some patients exposed to fibrin glue prepared with bovine thrombin develop inhibitors of factor V.

Note: factor V deficiency is not another name for factor V Leiden! See page 38.

Factor VII

Congenital factor VII deficiency has an incidence of about 1 in 500,000. Both quantitative and qualitative defects have been identified; therefore, activity and antigen assays are useful in determining which type of defect is present. Rare cases of combined factor V and VII deficiency have also been reported.

As with the other vitamin K–dependent coagulation factors (II, VII, IX, X, protein C, protein S), an acquired deficiency of VII can occur if the patient is deficient in vitamin K or is being treated with a vitamin K antagonist. Since all coagulation factors are synthesized in the liver, liver disease can also cause deficiencies. (The exception is VIII, whose synthesis in hepatic sinusoidal endothelial cells and Kupffer cells persists even in the face of advanced liver dysfunction.) Factor VII becomes deficient earlier in liver disease than do other factors due to its short plasma half-life.

Factor X

Factor X deficiency is rare, with an incidence of less than 1 in 500,000. Mutations resulting in qualitative and quantitative defects have been described. Acquired factor X deficiency is most commonly seen in conjunction with vitamin K deficiency or therapy with vitamin K antagonists. Amyloidosis can also cause an acquired deficiency of factor X.

Factors Affecting the Activity Assays for Factors II, V, VII, and X

The presence of an antiphospholipid antibody has been associated with falsely decreased results. This effect can often be identified and overcome by checking test results for a plateau effect during serial dilutions. Factor activity results can also be influenced by the type of coagulometer and the reagents used to perform the test. Dilution errors can adversely affect results as well.

Factors VIII, IX, XI, and XII, prekallikrein, and HMWK

These tests are used to determine the functional activity of coagulation factors in the intrinsic pathway of the coagulation cascade.

Indications

- Diagnosis of deficiency states or inhibitors affecting factors in the intrinsic pathway of the coagulation cascade (VIII, IX, XI, XII, prekallikrein, HMWK)

Test Principle

Plasma samples from patients with a deficiency of any factor in the intrinsic pathway may have prolonged PTT values. These factor assays use the PTT to determine the extent to which a patient's plasma corrects the clotting time of a plasma sample known to be deficient in the particular factor of interest (e.g., factor VIII–deficient plasma is used to perform factor VIII activity assays).

The assay is performed by recalcifying plasma in the presence of a standardized amount of platelet membrane–like phospholipids and an activator of the contact-dependent factors (XII, prekallikrein, HMWK) of the intrinsic pathway. Serial dilutions from 1:5 to 1:320 are made of buffered reference plasma with a known assay value for the factor of interest. Each dilution is mixed with an equal volume of substrate plasma that is known to contain normal levels of all factors except the factor of interest. A PTT is performed on each dilution. The results are plotted on a log-log graph, with the percent factor on the abscissa and the time in seconds on the ordinate. The results from the dilutions form a straight line that is the basis of a standard curve. To analyze activity in an unknown sample, 1:5 (100% activity) and 1:10 (50% activity) dilutions are prepared and mixed with immunoadsorbed, factor-deficient (< 1%) plasma. The percent activity of the unknown is read from the abscissa of the graph by finding where the clotting time intercepts the standard curve.

Factor VIII activity can also be measured using a spectrophotometric chromogenic assay. This assay is based upon the principle that VIII is a cofactor of IX, and the two factors together form a complex called "tenase" (because tenase cleaves and activates factor "ten" in the plasma in vivo). Rather than the clotting endpoint of traditional factor assays, the endpoint of the assay is the amount of colored product formed when tenase cleaves a chromogenic substrate. Since all other components in the chromogenic assay are in excess, the amount of factor VIII provided by the patient sample is rate-limiting. This assay is valuable in patients with antiphospholipid antibodies, which can sometimes interfere with the clotting endpoint of traditional factor assays.

Possible Results & Interpretation

Factor VIII

Low factor VIII activity may be seen in von Willebrand disease, hemophilia A, and hemophilia A carrier states, as well as in the presence of an inhibitor. Heparin therapy and acute DIC may also produce abnormally low VIII activity.

Hemophilia A is a recessive X-linked disease that occurs in approximately 1 in every 5,000 male births. Factor VIII inhibitors develop in 15% to 20% of hemophilia A patients due to exposure to exogenous factor VIII.

The incidence of spontaneously developing an inhibitor of factor VIII is approximately 1 in 1,000,000. These autoantibodies are associated with autoimmune disorders, malignancy, medications, and the peripartum period.

Recall that factor VIII is an acute phase reactant. Therefore, elevated factor VIII activity may be seen secondary to stress, inflammation, exercise, pregnancy, surgery, and estrogen use. Chronically elevated factor VIII levels are associated with a sixfold increase in the risk of venous thrombosis.

Factor IX

Low factor IX activity is seen in hemophilia B, hemophilia B carrier states, vitamin K deficiency, severe liver disease, and oral anticoagulant therapy, and in the presence of circulating inhibitors. Acquired deficiencies have been rarely associated with amyloidosis. Like hemophilia A, hemophilia B is inherited in an X-linked recessive fashion, but it is about fivefold less common, occurring in 1 in 25,000 male births.

Factor IX alloantibodies occur in 2% to 3% of hemophilia B patients and are more common among Scandinavian patients and those with large gene deletions. These antibodies are associated with anaphylactic reactions in hemophilia B patients during factor replacement. Acquired factor IX autoantibodies are extraordinarily rare.

One study suggests that increased factor IX levels are associated with a twofold increase in the risk of venous thrombosis.

Factor XI

Decreased levels of factor XI are seen in hereditary deficiency states and severe liver disease. Congenital factor XI deficiency, previously known as hemophilia C and Rosenthal syndrome, is an autosomal recessive disease. The National Organization for Rare Disorders (NORD) estimates a prevalence of 1 in 1,000,000 in the general population. In Ashkenazi Jewish populations, however, the heterozygote gene frequency can be as high as 6% to 13% and homozygotes can occur in 0.1% to 0.3%. Factor XI deficiency is generally a mild to moderate bleeding disorder, symptomatic only with trauma or surgery, and may therefore be underreported.

Elevated XI has been identified as a risk factor for venous thrombosis.

Factor XII deficiency

Factor XII deficiency is a rare disorder that, despite causing impressive prolongations of the PTT (> 100 seconds), is not associated with clinically important bleeding. Although the index patient succumbed to a pulmonary embolism following trauma, factor XII deficiency probably does not cause significant hypercoagulability.

Prekallikrein and HMWK

Low prekallikrein and HMWK levels are rare congenital deficiency states. As with factor XII, a deficiency in these factors generally has no significant physiologic consequence.

Factors Affecting the Assays for VIII, IX, XI, and XII, prekallikrein, and HMWK

The presence of an antiphospholipid antibody has been associated with falsely decreased results. This effect can often be identified and overcome by checking test results for a plateau effect during serial dilutions. Factor activity results can also be influenced by the type of coagulometer and the reagents used to perform the test. Dilution errors can adversely affect results as well.

Factor XI can be activated by frozen storage, leading to spurious increases in the assayed level.

Factor XIII Activity Assay

Indications

- Diagnosis of factor XIII deficiency or inhibitors directed against factor XIII

Test Principle

Factor XIII, also called fibrin stabilizing factor, is a transglutaminase that helps form covalent bonds between fibrin monomers during clot formation. Routine tests of

hemostasis, including the PT, PTT, and thrombin time, are of course normal. Severe deficiency is associated with spontaneous intracranial bleeding. In cases of suspected factor XIII deficiency, activity levels should be measured.

We no longer perform the factor XIII assay in our laboratory due to low demand. Our reference lab, Quest, uses a chromogenic assay with a normal range of 57% to 192% activity. Of note, there are reports that the thromboelastograph (TEG®) is abnormal in factor XIII deficiency; in these cases, the TEG may show reduced maximum amplitude and strength and increased clot lysis at 30 minutes.

Possible Results & Interpretation

NORD estimates the incidence of congenital factor VIII deficiency at 1 in 2,000,000 to 5,000,000, but it may be more frequent due to under- or misdiagnosis of mild forms. Inhibitors of XIII are rare but have been seen in association with isoniazid. Low factor XIII levels can also be seen in a number of clinical scenarios:

- Henoch-Schönlein purpura (HSP)
- on and following cardiopulmonary bypass
- inflammatory bowel disease
- during pregnancy; severe deficiencies are associated with recurrent miscarriage
- excessive activation, as seen in DIC and on exposure to some snake venoms and caterpillar toxins (e.g., *Lonomia achelous* caterpillar venom)
- in association with sodium valproate; XIII levels returned to normal after the drug was withdrawn

Factors Affecting the Factor XIII Activity Assay

Interpret results from older test methods with caution. The old clot-solubility assay detects only the most severe factor XIII deficiencies. The urea stability test is sensitive to levels of 1% to 5%, while the acetic acid method is sensitive to below 10%.

Factor VIII and IX Inhibitor Assays

The Bethesda assay measures the activity of inhibitors of factors VIII and IX.

Indications

- Diagnosis of factor VIII or IX inhibitors

Test Principle

A factor inhibitor is quantified by mixing dilutions of patient plasma with pooled plasma containing normal levels of the factor under investigation. The activity of the factor is measured immediately after mixing and again after a two-hour incubation at 37°C. By comparing the difference in activity between the patient incubation mixture and a control mixture, the amount of inhibitor present can be calculated; this is expressed in Bethesda units.

During incubation, the pH of the mixture shifts, producing artifactual decreases in factor activity. The Nijmegen modification of the Bethesda assay dilutes the patient sample with buffered plasma, eliminating this decrease. The imprecision due to unbuffered plasma is particularly relevant for lower titer inhibitors.

Possible Results & Interpretation

The result is either "not detected" or "positive." If positive, the titer of the inhibitor is reported in Bethesda units.

Inhibitors of specific coagulation factors can be classified as either autoantibodies or alloantibodies. Autoantibodies arise in patients without a previous history of a coagulation disorder. The most common autoantibody inhibitors are directed against factor VIII and have an annual incidence of 1 in 1,000,000. Factor VIII inhibitors are often associated with certain medications (penicillins, sulfonamide antibiotics) or medical conditions (pregnancy, hematologic malignancies, autoimmune disorders, solid tumors, and many others). Immunosuppressive therapy effectively eradicates these antibodies in up to 60% to 70% of patients. Autoantibodies directed against other coagulation factors are extremely rare.

In contrast to autoantibodies, alloantibodies develop in patients who lack a particular coagulation factor. These patients generate the antibody on exposure to exogenous factor during treatment with coagulation factor concentrates. Factor VIII is the most common target, and approximately 15% to 25% of patients with severe hemophilia A have an alloantibody inhibitor. An inhibitor is usually first suspected when these patients fail to respond to factor VIII concentrate.

The prevalence of an alloantibody inhibitor is much lower among patients with hemophilia B and is estimated to be around 2%. In hemophilia B patients, the development of an inhibitor is often heralded by allergic reactions to factor infusions and by poor hemostatic responses to factor IX concentrates.

Factor VIII and IX alloantibodies are classified by their Bethesda unit titer: a low-titer inhibitor is ≤ 5 Bethesda units and a high-titer inhibitor is > 5. The Bethesda titer influences the selection of treatments during bleeding episodes. Patients with low-titer inhibitors can be effectively treated with factor concentrates by doubling or tripling the dose. The advantage of increasing the dose is that factor activity levels can be used as an objective assessment of the adequacy of therapy. This is very useful in situations where such information is critical, such as during major surgical procedures or life-threatening bleeds. The disadvantage is that further exposure to factor VIII or IX concentrates may lead to a subsequent increase in the titer, complicating future therapy.

Factors Affecting the Factor VIII and IX Inhibitor Assays

The inhibitor assay should not be performed on patients with normal factor activity levels (i.e., you should not look for a factor VIII inhibitor if VIII activity is normal).

If a patient has had prolonged treatment with factor VIII or IX concentrate and has a sample drawn post-infusion, transient saturation of the inhibitor by the infused factor may result in artifactually reduced levels.

This assay cannot distinguish between specific factor inhibitors and other causes of a positive mixing test result, such as fibrin degradation products, heparin, and heparin-like inhibitors.

CHAPTER 3

von Willebrand Disease

Ristocetin cofactor activity
von Willebrand factor antigen
von Willebrand factor multimers
Collagen-binding assay

About von Willebrand Disease

von Willebrand factor (vWF), a plasma glycoprotein produced by endothelial cells and megakaryocytes, plays two key roles in hemostasis: It participates in platelet adhesion and aggregation, and it serves as a carrier protein for factor VIII, protecting VIII from inactivation by activated protein C. von Willebrand disease (vWD) is a bleeding disorder caused by inherited defects in vWF. The non-inherited form is called acquired von Willebrand syndrome (AvWS).

The classification of vWD was updated in 2006 by the ISTH. The new criteria relaxed the previous requirement that "vWD" be reserved for patients with a mutation in the *VWF* gene on chromosome 12. This was done not only because demonstrating such a mutation is impractical in many cases but also because a wide variety of defects, including protein processing errors, can lead to disease. The three major categories of vWD are partial quantitative deficiency (type 1), qualitative deficiency (type 2), and total deficiency (type 3) (Table 6).

vWD Type 1

The most common form of vWD is type 1, a partial quantitative deficiency resulting in mild to moderate bleeding. Approximately 75% of patients with vWD have this form. There is a modest and equivalent reduction of both the vWF activity and protein levels, which is reflected in the ristocetin cofactor (vWF:RCo) and vWF antigen (vWF:Ag) assays. Factor VIII activity may be reduced because of its shortened half-

Type	Description	Inheritance	Bleeding propensity
1	partial quantitative deficiency	AD	mild to moderate
2	qualitative vWF defect		usually moderate
2A	decreased vWF-dependent platelet adhesion; selective HMWM deficiency	AD and AR	
2B	increased affinity for platelet GPIb	AD	
2M	decreased vWF-dependent platelet adhesion; no HMWM deficiency	AD and AR	
2N	markedly decreased factor VIII affinity	AR	
3	near-complete vWF deficiency	AR	severe

Table 6. Classification of von Willebrand disease. Adapted from Nichols WL, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines. Haemophilia. 2008;14(2):171-232. HMWM, high-molecular-weight multimers; AD, autosomal dominant; AR, autosomal recessive.

life in the absence of sufficient vWF. Analysis of vWF multimers via SDS gel electrophoresis demonstrates a normal pattern, with all sizes of vWF multimers present.

vWD Type 2

In type 2 vWD, patients produce qualitatively defective vWF molecules. Four major variants are recognized: 2A, 2B, 2M, and 2N. In type 2A, mutations in the vWF molecule either prevent the normal assembly of vWF multimers or produce multimers that are unusually susceptible to in vivo proteolysis. The result is a loss of large and intermediate multimers, the fractions that are the most active in platelet adhesion.

In type 2B vWD, mutations result in an increased affinity of vWF for platelets. This leads to spontaneous platelet aggregation, the selective loss of high-molecular-weight vWF multimers, and, often, mild to moderate thrombocytopenia. Recent studies indicate the thrombocytopenia associated with type 2B vWD is also due to reduced platelet production secondary to abnormal megakaryocyte function.

Type 2M vWD is characterized by mutations reducing the interaction between vWF and platelets and endothelium. It differs from type 2A in that type 2M does not demonstrate a loss of high-molecular-weight vWF multimers.

Each of these type 2 disorders is distinguished from type 1 vWD by much larger reductions in the activity of vWF (as measured by the vWF:RCo activity assay) than in the levels of vWF protein (vWF:Ag). In addition, type 2A and 2B are characterized by the loss of large and intermediate and large vWF multimer fractions, respectively, in multimer analysis. Platelet aggregation studies using low doses of ristocetin are useful in demonstrating the hyper-responsive phenotype of type 2B vWD.

Type 2N vWD results from mutations in the factor VIII binding site on the vWF protein. As a result, type 2N behaves much like a mild autosomal form of hemophilia A, with factor VIII activity levels in the 5% to 15% range. vWF antigen and activity levels and multimer patterns are normal.

vWD Type 3

The most severe form of vWD is type 3. It is an autosomal recessive disease in which individuals have undetectable levels of vWF protein and function along with markedly reduced factor VIII levels. A small proportion of patients (2.6% to 9.5%) have been reported to develop alloantibodies to vWF after exposure to plasma products.

Acquired von Willebrand Syndrome

Three primary mechanisms for AvWS have been proposed: autoimmunity against vWF; shear-induced proteolysis of vWF; and increased binding of vWF to platelets and cell surfaces. Some reports have also implicated non-immune-related hypothyroidism and certain medications.

The laboratory findings in AvWS are similar to those in vWD. Although multimer analysis may be normal, it often shows a decrease in large multimers, similar to that seen in type 2A vWD.

Tests for von Willebrand Disease

When any bleeding disorder is suspected, a thorough clinical and family history should be taken. The clinical history is generally the most important part of pursuing a diagnosis if vWD due to the wide range of defects that may be present. For example, the PTT is typically normal in mild type 1 and type 2 vWD.

The initial tests for the workup of suspected vWD are the vWF antigen (vWF:Ag) test, the ristocetin cofactor (vWF:RCo) activity assay, and the factor VIII activity assay (Chapter 2). A platelet function test (PFA-100®; p. 55), the collagen-binding

assay (p. 30), and multimer analysis are helpful in diagnosis as well as in differentiating between types. Figure 3 shows a suggested laboratory evaluation algorithm from the National Heart, Lung, and Blood Institute (NHLBI), and Table 8 shows the expected results of various laboratory tests for each of the types of vWD. See Appendix VIII for notes on the use of desmopressin to diagnose and manage vWD.

Ristocetin Cofactor Activity Assay

This assay measures the functional level of von Willebrand factor in plasma.

Indications

- Diagnosis of von Willebrand disease

Test Principle

This assay is based on the observation that the antibiotic ristocetin can induce platelet agglutination in a vWF-dependent fashion (through binding of the platelet GPIb receptor by vWF). Following reconstitution, lyophilized platelets are treated with ristocetin in the presence of dilutions of normal standardized human plasma with a known level of vWF:RCo activity. The ensuing agglutination decreases the turbidity of the suspension, and the vWF:RCo activity is determined from the change in absorbance. A standard curve is prepared, after which patient plasma is then used as the source of vWF:RCo activity. The vWF:RCo activity of the patient sample is inter-

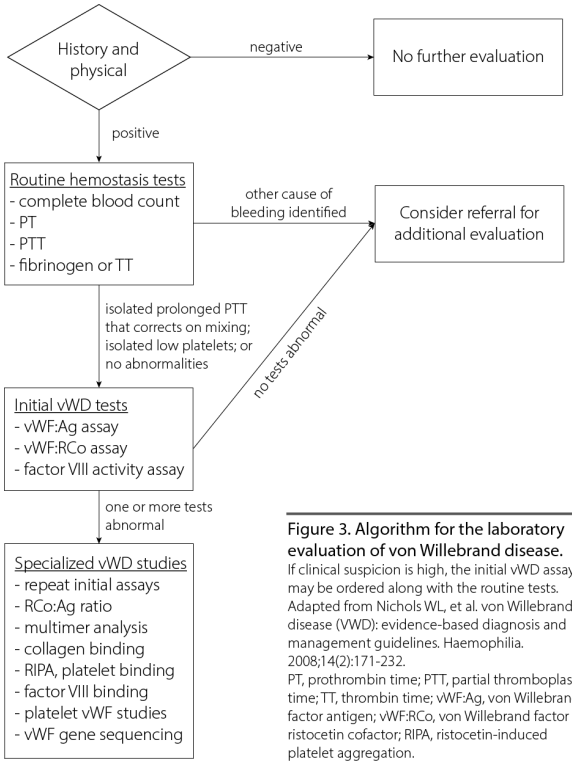

Figure 3. Algorithm for the laboratory evaluation of von Willebrand disease.
If clinical suspicion is high, the initial vWD assays may be ordered along with the routine tests. Adapted from Nichols WL, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines. Haemophilia. 2008;14(2):171-232.
PT, prothrombin time; PTT, partial thromboplastin time; TT, thrombin time; vWF:Ag, von Willebrand factor antigen; vWF:RCo, von Willebrand factor ristocetin cofactor; RIPA, ristocetin-induced platelet aggregation.

polated from the standard curve. Note that while platelets play a passive role in such agglutination, there is an absolute requirement that the GPIb receptor be intact.

Although the vWF:RCo activity assay is considered by many investigators to be the single most important assay for diagnosing vWD, a complete evaluation also requires the determination of the vWF:Ag level, factor VIII activity, vWF multimer analysis, and clinical and family history. Recently, the platelet function analyzer (PFA-100®) assay has been demonstrated to provide useful diagnostic information in the workup for vWD. The bleeding time is ordered less frequently nowadays.

Possible Results & Interpretation

vWF activity is reduced in type 1 and 2 vWD and absent in type 3. In type 1 vWD, vWF:RCo activity and vWF antigen levels are typically similarly decreased, reflecting the fact that type 1 is due to a mutation that reduces the production of an otherwise structurally normal vWF molecule. By contrast, type 2 vWD is caused by a mutation in the coding region of the *VWF* gene, leading to a dysfunctional vWF molecule (see p. 26 for details). As a consequence, type 2 vWD is usually associated with significantly greater reductions in vWF:RCo activity than in vWF:Ag levels.

These differences between type 1 and type 2 vWD are captured in the vWF:RCo to vWF:Ag ratio (RCo:Ag), which is reported with the results of the ristocetin cofactor and vWF antigen assays. The RCo:Ag ratio is generally > 0.6 in type 1 and < 0.5 in type 2. One exception is type 2N vWD, in which ristocetin cofactor activity and vWF antigen levels are both normal. The mutation in the vWF molecule is isolated to the factor VIII binding pocket, so the only abnormalities seen are in the patient's factor VIII activity and vWF factor VIII binding activity (see p. 26). It is therefore prudent to follow up RCo:Ag ratios of 0.5 to 0.7 with additional tests.

In type 3 vWD, wherein homozygous mutations in the *VWF* gene lead to severely decreased or absent vWF, both vWF:RCo and vWF:Ag levels are extremely low.

Factors Affecting the Ristocetin Cofactor Activity Assay

Larger vWF multimers are able to mediate platelet adhesion more effectively than smaller multimers. Consequently, patients with selective loss of the larger, more active vWF multimers (e.g., type 2 vWD) are likely to have greater reductions in vWF function (and thus ristocetin cofactor activity) than would otherwise be predicted by their vWF:Ag levels. This loss also manifests as an abnormal distribution of multimers (a predominance of low-molecular-weight multimers, with high-molecular-weight multimers decreased or absent) rather than an absolute deficiency of vWF.

Delayed delivery of samples to the laboratory or inadequate sample preparation (e.g., failure to remove platelets from the patient sample) can cause false-positive results due to platelet binding of vWF. Alternative functional assays of vWF function, such as the collagen-binding assay should be considered in the event of borderline vWF:RCo activity results.

von Willebrand Factor Antigen (vWF:Ag)

This test measures the quantity of von Willebrand factor protein in patient plasma.

Indications

- Diagnosis of von Willebrand disease

Test Principle

This is a turbidimetric assy measured by photometry. A suspension of latex microparticles covalently bound to antibodies specific for vWF:Ag is mixed with patient

plasma. An antigen-antibody reaction leads to agglutination of the microparticles, increasing the turbidity of the solution, which is reflected by an increase in the absorption of a beam of monochromatic light. The resulting increase in absorbance is proportional to the amount of antigen present in the specimen.

Possible Results & Interpretation

In type 1 vWD, there is a mild to moderate reduction in the amount of vWF:Ag. The amount of vWF:Ag may be normal or low in type 2 variants of vWD. This quantity can be used in conjunction with the functional level of vWF (measured using the ristocetin cofactor assay) to differentiate between the types of vWD (Table 8). vWF:Ag is absent or greatly diminished in patients with severe (type 3) vWD.

Factors Affecting the vWF Antigen Assay

vWF:Ag levels are influenced by the patient's ABO blood group (Table 7). Therefore, physicians should take into account the patient's ABO group as well as the clinical symptoms and laboratory test results when making a diagnosis of vWD. In general, patients should have both clinical symptoms of bleeding as well as abnormal laboratory results, particularly if they are of blood type O: Type O patients tend to have lower vWF:Ag levels.

ABO Type (n)	mean[1] vWF:Ag, U/dL	mean ± 2 SD
O (456)	75	36 - 157
A (340)	106	48 - 234
B (196)	117	57 - 241
AB (109)	123	64 - 238

Table 7. The influence of ABO group on vWF:Ag levels. Group O differed from A, B, and AB ($p < 0.01$). Group A differed from B ($p < 0.05$) and AB ($p < 0.01$). Adapted from Gill JC, et al. The effect of ABO blood group on the diagnosis of von Willebrand disease. Blood. 1987;69(6):1691-5.
vWF:Ag, von Willebrand factor antigen; U, units (as reported by Gill, et al); sd, standard deviation. [1]geometric mean

Recall that vWF is an acute-phase reactant. The following conditions may elevate vWF:Ag and thus confound efforts to diagnose vWD: pregnancy, oral contraceptives, liver disease, inflammation, exercise, stress, traumatic venipuncture, the post-operative state, and fainting. In extremely rare instances, anti-rabbit antibodies can lead to aberrant test results in affected individuals.

von Willebrand Factor Multimer Analysis

This test demonstrates the concentrations of the different vWF multimer fractions.

Indications

- Diagnosis of von Willebrand disease

Test Principle

The subclassification of von Willebrand disease is based on the distribution of vWF multimers in patient plasma. vWF multimers are detected using SDS-agarose gel electrophoresis combined with radiolabeled antibody detection or transblotting and enzyme-linked antibody detection. Our laboratory sends out plasma to the Blood Center of Wisconsin for multimer analysis.

Possible Results & Interpretation

All normal multimer sizes are present in type 1 vWD. Type 2A is associated with the selective loss of large and intermediate multimers. Type 2B is characterized by the loss of high-molecular-weight multimers. Both a normal multimer pattern and

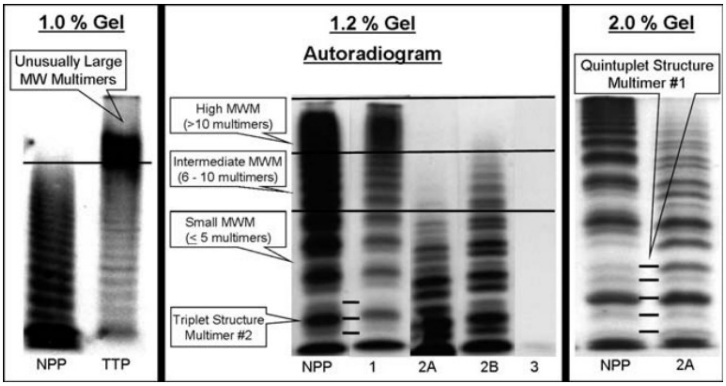

Figure 4. An image of multimer gels found online. These von Willebrand gels are good for teaching purposes. However, rarely do they look as pristine as this picture from the Internet. Ledford-Kraemer MR. Analysis of von Willebrand factor structure by multimer analysis. Am J Hematol. 2010;85(7):510-4.

an accumulation of ultra-large vWF multimers (Vicenza subtype) can be seen in type 2M. Type 2N demonstrates a normal vWF multimer pattern. Type 3 vWD is associated with the complete absence of multimers, congruent with the severity of this vWF deficiency.

Factors Affecting von Willebrand Factor Multimer Analysis

Standard gels resolve the lower molecular-weight multimer bands into three to five satellite bands but are less sensitive to changes in high-molecular-weight multimers. Low-resolution gels, by contrast, detect

- loss of high-molecular-weight multimers as seen in some variants of vWD (i.e., type 2B);
- acquired losses secondary to DIC, hemolytic uremic syndrome (HUS), acute thrombotic thrombocytopenic purpura (TTP), and cardiac lesions, such as atrial and ventricular septal defects; and
- larger than normal multimers as seen in newborns, after desmopressin (DDAVP) infusion, recurrent TTP during remission, and in patients with the type 2M Vicenza variant

Platelet contamination of plasma will show an artificial loss of high-molecular-weight multimers.

Collagen-Binding Assay

This test quantifies the collagen-binding capacity of von Willebrand factor.

Indications

- Diagnosis of von Willebrand disease
- Distinguishing subtypes of von Willebrand disease

Test Principle

The collagen-binding test is an enzyme immunoassay. The patient sample is incubated in microwells in which collagen has been bound. Any von Willebrand factor

			Type 2				
Assay	Control	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	Type 3
Screening tests							
vWF activity (vWF:RCo)	N	B or ↓ or ↓↓	↓ or ↓↓	↓↓	↓ or ↓↓	N or B	↓↓↓
vWF antigen	N	B or ↓ or ↓↓	B or ↓	B or ↓	B or ↓	N or B	↓↓↓
Factor VIII activity	N	N or ↓	N or ↓	N or ↓	N	↓↓	↓↓↓
PFA-100 COL/EPI[1]	N	N or ↑	↑	↑	↑	N	↑↑↑
Structure analysis							
vWF multimer analysis	N	N	lack of HMWM ↓ IMWM	lack of HMWM	N	N	absent
Supplemental tests							
RIPA, high-dose	N	N or ↓	↓	N	↓	N	nonreactive
RIPA, low-dose	nonreactive	nonreactive	nonreactive	↑	nonreactive	nonreactive	nonreactive
Factor VIII binding (vWF:FVIIIB)	N	N	N	N	N	↓↓↓	absent

Table 8. How to use laboratory tests for the diagnosis of von Willebrand disease.

Adapted from Nichols WL, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines. Haemophilia. 2008;14(2);171-232.

vWF, von Willebrand factor; RCo, ristocetin cofactor; N, normal; B, borderline; PFA, platelet factor analyzer; HMWM, high-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers; RIPA, ristocetin-induced platelet aggregation.

[1]collagen/epinephrine cartridge

(vWF) multimers present in the sample bind to the collagen. After washing away unbound plasma proteins, anti-vWF antibodies conjugated to horseradish peroxidase bind the captured vWF multimers. Excess antibody is washed off and a chromogenic substrate is added. The resulting color intensity is proportional to the amount of high-molecular-weight vWF multimers present in the sample; this amount can then be quantified using the test kit's calibrated standards.

Possible Results & Interpretation

The reference range is 50% to 110%. In patients with qualitative deficiencies of vWF molecules—that is, type 2 von Willebrand disease—those with type 2A or 2B will demonstrate loss of high-molecular-weight vWF multimers. Since these are generally the multimers that bind collagen, these patients will show a decrease in collagen-binding percentage.

Factors Affecting the Collagen-Binding Assay

Lipemic, hemolyzed, and icteric samples should not be used. Samples should not undergo more than one freeze-thaw cycle.

CHAPTER 4

Hypercoagulability

Antiphospholipid Antibodies

Antiphospholipid antibodies (APLAs) are autoantibodies directed against negatively charged phospholipids (cardiolipin, phosphatidylserine, etc.), phospholipid-binding proteins (β2-glycoprotein I, annexin V, prothrombin, etc.), or a combination of the two. Clinical conditions associated with APLAs include infection (syphilis, HIV), exposure to certain drugs (phenothiazines, hydralazine, interferon, etc.), neoplasms, and autoimmune diseases (systemic lupus erythematosus). Patients demonstrating persistent APLAs and hypercoagulability may be diagnosed with antiphospholipid syndrome (APS), as defined by specific clinical and laboratory criteria.

Current classification criteria recognize three major classes of APLAs: lupus anticoagulants (LAs), anticardiolipin IgG/IgM, and anti-β2-glycoprotein I IgG/IgM. LAs are a class of APLAs that prolong the clotting time in phospholipid-dependent in vitro tests, such as the PTT. (Like the other APLAs, LAs exert a primarily prothrombotic effect in vivo.) LA antibodies may target cardiolipin or β2-glycoprotein I (β2GPI) but may also target other epitopes; it is the functional effect in the laboratory that defines an antibody as a lupus anticoagulant. Enzyme-linked immunosorbent assay (ELISA) is frequently used to identify anticardiolipin and anti-β2GPI.

The diagnostic strategy for evaluating a patient should be based on a consensus between the laboratory and the clinicians treating these disorders. We rely on the dilute Russell viper venom time for initial evaluation of a suspected APLA because of its strong association with thrombosis. Since APLAs may vary over time and the degree of and relationship to pathogenicity is dependent on their persistence beyond eight to twelve weeks, we recommend repeating tests at that time.

Partial Thromboplastin Time (PTT)

The PTT is one of the two clot-based assays—the other is the dilute Russell viper venom test, below—used to screen for lupus anticoagulants. Only a PTT test that

uses a low phospholipid reagent is sensitive to the presence of LAs. For more information about this assay, see Chapter 1, p. 11.

Dilute Russell Viper Venom Time (dRVVT)

This test measures the direct activation of factor X and is useful in the diagnosis of a lupus anticoagulant.

Indications

- Diagnosis of an antiphospholipid antibody

Test Principle

The Russell viper (*Vipera russelii*) is a common Asian pit viper whose venom can act as a partial thromboplastin that directly activates factor X to Xa. In the Russell viper venom test (RVVT), an extract of this venom is added to the patient's plasma along with calcium and a platelet substitute (i.e., a source of phospholipid), and the time to clotting is measured. This direct activation of factor X bypasses both the intrinsic and extrinsic pathways in the coagulation cascade, thereby excluding interferences from deficiencies of factors VII, VIII, IX, XI, and XII and their respective inhibitors.

A modified test, the dilute RVVT (dRVVT), has been developed as a sensitive screening test for the presence of LA. By using more-dilute phospholipid than the RVVT, the dRVVT significantly increases the sensitivity of the test for the presence of LA. The dRVVT is also useful for the diagnosis of LA in patient samples with a normal PTT, as the dilution and type of phospholipid contained in the dRVVT reagent makes it more sensitive for LA than the PTT is.

Possible Results & Interpretation

If the test plasma gives a dRVVT that is within the established normal reference range, the test is negative for LA. An abnormally long dRVVT may be caused by LA, a deficiency of II, V, X, or fibrinogen; liver disease; or vitamin K deficiency. Warfarin may also cause an abnormally long result and is in fact the most common reason for an abnormal dRVVT in our hospital. If the dRVVT is prolonged, a dRVVT confirmatory test (below) and mixing studies with normal plasma are performed to determine if the prolongation of dRVVT is due to LA or another cause.

Factors Affecting the dRVVT

The dRVVT test incorporates an agent that neutralizes heparin ≤ 1.0 IU/mL. Plasma with a heparin level > 1.0 IU/mL may give elevated results and should not be evaluated with this test. If the dRVVT is performed on plasma that contains inhibitor(s) to factor II, V, or X, the reagent may not identify the presence of LA due to the prolongation of the dRVVT by the factor inhibitors.

dRVVT Confirmation Test

This companion test to the dRVVT is used to confirm the presence of LA.

Indications

- Evaluation of an abnormal dRVVT

Test Principle

The dRVVT confirmation test is specifically designed for use in conjunction with the dRVVT. It compares the results of a dRVVT performed with and without

concentrated phospholipids. Plasma with a prolonged dRVVT (> 2 SD from the established normal reference mean) is retested using a second reagent. This reagent is supplemented with excess phospholipids, which reverse (correct) the inhibition of anticoagulation caused by antibodies with LA properties.

Possible Results & Interpretation

The result of this test is a ratio: the clotting time of the dRVVT test divided by the clotting time of the dRVVT confirmation test.

$$\frac{\text{dRVVT test (seconds)}}{\text{dRVVT confirmation test (seconds)}}$$

This ratio corresponds to the magnitude to which the addition of concentrated phospholipids corrects the dRVVT. If the ratio is greater than 1.4, the test is positive for LA. If the ratio results are within the established laboratory normal reference ratio range (≤ 1.4), the test is negative.

The dRVVT and the dRVVT confirmation assays may be prolonged in patients with deficiencies of factors II, V, or X or fibrinogen. However, the final ratio result will be normal unless LA is present. If LA is present, the dRVVT confirmation assay will shorten significantly with the addition of excess phospholipids, producing an abnormal dRVVT-test:dRVVT-confirmation ratio. Warfarin and other vitamin K antagonists may prolong both the dRVVT and the dRVVT confirmation time; again, however, the ratio result will be normal if no LA is present.

The presence of a factor deficiency or warfarin can also be identified by reviewing the results of mixing studies of the dRVVT performed with normal plasma. In patients with a factor deficiency or on warfarin therapy, the dRVVT will correct with 1:1 and 4:1 mixes with normal plasma (see "Mixing Studies," p. 13). By contrast, if a lupus anticoagulant is present, the 1:1 and 4:1 mixes will not correct (Table 9).

dRVVT		dRVVT Confirmation		Interpretation
Patient	1:1 mix of patient:normal	Patient	1:1 mix of patient:normal	
N	N	N	N	LA not detected
A	A	N	N	LA probably present
A	N	A	N	Possible factor deficiency or anticoagulant therapy
A	A	A	N	Possible factor deficiency
A	A	A	A	Other inhibitors may be present

Table 9. Interpreting the dRVVT confirmation assay. A, abnormal; N, normal.

Factors Affecting the dRVVT Confirmation Assay

The dRVVT confirmation reagent contains agents that neutralize ≤ 1.0 IU/mL of heparin. Plasma containing heparin levels > 1.0 IU/mL may give elevated results for the dRVVT, and this confirmation test should not be performed. If the dRVVT confirmation test is performed on plasma with a suspected LA that may also contain

inhibitors of factors II, V, or X, the reagent may not identify the presence of LA, due to the prolongation of the dRVVT confirmation assay by these inhibitors.

Direct thrombin inhibitors and anti-Xa anticoagulants will prolong the dRVVT, and adding plasma will not correct it. The result of the confirmation phase (with excess phospholipid) is variable. We therefore recommend that the interfering drug be removed with addition of charcoal. In our lab, this reagent is called DOAC-Stop. To ensure that we have absorbed all the drug, we measure a chromogenic factor X. This value should be normal after absorption.

IgG, IgA, and IgM Anticardiolipin Antibody ELISA

This semiquantitative assay measures the amount of IgG, IgA, and IgM antibodies in a sample. Most reports of anticardiolipin antibodies (ACAs) focus on the IgG and IgM subtypes, which are included in the criteria of APS. Several recent studies, however, indicate that elevated levels of IgA ACA are frequently found in patients with systemic lupus erythematosus (SLE) and related disorders. In these studies, IgA levels were higher in patients with vascular complications and thrombocytopenia.

Indications

- Diagnosis of antiphospholipid antibody syndrome (APS)
- Assess risk of thrombosis in patients with SLE and lupus-like disorders

Test Principle

We use the QUANTA Lite® IgG, IgA, and IgM ACA ELISA kits (Inova Diagnostics, San Diego, CA). Serum samples are incubated in microplate wells coated with anticardiolipin. After washing away unbound protein, an anti-human IgG, IgA, or IgM secondary antibody labeled with alkaline phosphatase (AP) is added. After a second washing step to remove excess antibody, an AP substrate is added, which generates a colored product in the presence of AP. The amount of colored reaction product formed is measured photometrically at 405 nM and directly corresponds to the amount of antibody present in the patient serum sample.

Possible Results & Interpretation

Antibody levels for this assay are reported in "phospholipid units" (PL), which represent the binding activity of the antibodies in a sample. For example, 20 μg/mL of IgG anticardiolipin is reported as 20 GPL. Samples with antibody levels less than 20 GPL, MPL, or APL are considered negative.

Although the correlation between ACA results and clinical events has varied from study to study, low positive results have generally not been associated with an increased risk of clinical events. Moderately increased and high antibody levels have been associated with clinical symptoms. Clinical studies indicate that elevated IgG ACA levels are most consistently associated with an increased risk of thrombosis, while elevated IgM levels have been less consistently associated with clinical sequelae. Several studies suggest that moderate to high levels of IgA ACA may confer an increased risk of venous thrombosis, thrombocytopenia, and fetal loss.

Since ACAs have been associated with a wide variety of illnesses, their presence must be integrated with clinical information for optimal clinical decision-making. A complete evaluation for APS usually includes a PTT, mixing studies (if the PTT is prolonged), a dRVVT performed with and without purified phospholipids, and an anti-β2GPI antibody ELISA. The diagnosis of APS requires clinical and laboratory

evidence of the disease. Patients with positive test results who are asymptomatic do not fulfill criteria for APS and thus should not be treated for this disease. Since transient anticardiolipin antibodies can be seen in conjunction with many viral infections, patients should have positive test results confirmed by repeat testing. Syphilis and HIV infection are associated with antiphospholipid antibodies; therefore, patients with positive tests should also be evaluated for these infections.

Factors Affecting the Anticardiolipin Antibody ELISA

Rheumatoid factor may interfere with the measurement of IgM ACAs. Specifically, when IgG ACAs are present, high levels of rheumatoid factor IgM can cause a positive bias in the measurement of IgM anticardiolipin.

IgG, IgA, and IgM Anti-β2-Glycoprotein I Antibody ELISA

This semi-quantitative test measures the amount of anti-β2-glycoprotein I (anti-β2GPI) antibody in serum. In the early 1990s, several investigators demonstrated that β2GPI, a phospholipid-binding protein also known as apolipoprotein H, is the target of many of the anticardiolipin antibodies in patients with autoimmune disorders. β2GPI may function physiologically as an antithrombotic agent, providing one potential reason why patients with these antibodies are at increased risk of developing clots.

Indications

- Diagnosis of antiphospholipid antibody syndrome (APS)
- Assess risk of thrombosis in patients with SLE and lupus-like disorders

Test Principle

We use the QUANTA Lite® β_2 GPI ELISA kits (Inova Diagnostics, San Diego, CA). Patient serum is added to microplate wells that have been precoated with β2GPI. A secondary antibody against human immunoglobulin labeled with horseradish peroxidase is added next. Washes before and after the addition of the secondary antibody eliminate excess unbound protein and antibody. The amount of β2GPI antibody in the patient sample is determined by the amount of colored product generated after the addition of the peroxidase substrate.

Possible Results & Interpretation

Results are reported in standard Ig units (e.g., standard IgG units, standard IgM units). Samples with values greater than 20 standard units are considered positive. The clinical significance of positive test results must be interpreted in conjunction with the patient's clinical findings, and treatment should not be based upon test results alone. In particular, syphilis and other infections should be ruled out, and testing should be repeated after an appropriate interval.

Patients with a positive β2GPI ELISA may not always have a positive anticardiolipin ELISA result and vice versa. A complete evaluation for the antiphospholipid antibody syndrome usually includes a PTT, mixing studies (if the PTT is prolonged), a dRVVT performed with and without purified phospholipids, and mixing studies and anticardiolipin antibody testing.

Factors Affecting the Anti-β2-Glycoprotein I ELISA

Rheumatoid factor may interfere with the measurement of IgM anti-β2GPI (see "Factors Affecting the Anticardiolipin Antibody ELISA," this page).

Factor V Leiden

Factor V Leiden (FVL) is an inherited thrombophilia that may be associated with as much as 40% of unexplained venous thromboses in selected patient populations from thrombosis clinics. By way of comparison, deficiencies of antithrombin, protein S, and protein C each account for no more than 5% of patients with a hypercoagulable state.

The *F5* point mutation (R506Q) responsible for the abnormal FVL protein alters one of the sites where factor V is cleaved and inactivated by activated protein C. The presence of this mutation is responsible for the vast majority of cases of activated protein C resistance. See Figure 5 for a review of the role of protein C.

Activated Protein C Resistance Assay

This clot-based assay is used to identify resistance to activated protein C. Since FVL is the cause of resistance in more than 90% of cases, it becomes an effective screening test for that mutation. This test is performed prior to genetic testing for FVL as it is highly sensitive and specific for the mutant protein and is much less expensive.

Indications

- Diagnosis of factor V Leiden

Test Principle

Resistance to activated protein C (APC) is the most common inherited risk factor for thrombosis, occurring in 5% of Caucasian European Americans, 20% of unselected patients with venous thromboembolism (VTE), and 40% of selected patients with early onset VTE, a positive family history of VTE, recurrent VTE, or idiopathic VTE. Resistance is often associated with other inherited risk factors for thrombosis.

In normal plasma, activated protein C prolongs the PTT time because it inactivates factors Va and VIIIa. This assay increases the selectivity for FVL (and other

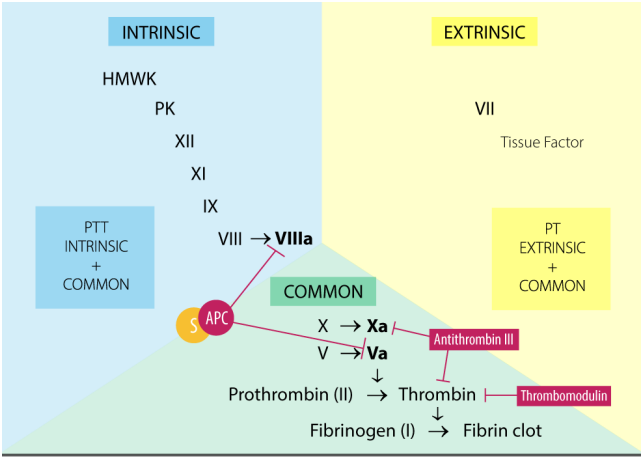

Figure 5. The "anti-coagulation" cascade. This simplified cartoon shows the major components of the anticoagulation system. Thrombomodulin binds thrombin, inhibiting its activity. The thrombin-thrombomodulin complex binds protein C and activates it. With its cofactor protein S, activated protein C inactivates factors Va and VIIIa. APC, activated protein C.

F5 mutations that produce proteins resistant to APC) by normalizing the concentrations of the other plasma proteins involved in the formation and regulation of thrombin. The assay is thus performed in the presence of an excess of factor V–deficient plasma, and the sensitivity and specificity for FVL is significantly increased. An additional benefit of this modification is that the plasma of patients on oral anticoagulant therapy can also be accurately tested.

Possible Results & Interpretation

The result is a ratio of the patient sample clotting time to the control clotting time. A value less than 2.2 indicates a high likelihood of true APC resistance, and DNA-based testing for FVL should be performed.

Factors Affecting the Activated Protein C Resistance Assay

Although the standard dilution used by the test (1:4) strongly decreases interference, the assay may give misleading results in patients with high-titer inhibitors (e.g., lupus anticoagulants). In such cases, increasing the dilution to 1:8 may correct the result. As with any PTT-based assay, contact activation may activate factors V and VIII. Elevated factor VIII levels alone, however, do not interfere with the assay.

As noted above, the sensitivity and specificity of the assay is unaffected by plasma samples obtained from patients on warfarin. The prescribed assay procedure also allows for the accurate analysis of plasma with heparin levels (unfractionated or low molecular weight) < 1.0 IU/mL.

Factor V Leiden Mutation Analysis

This is a polymerase chain reaction (PCR) assay for the *F5* R506Q mutation that causes factor V Leiden.

Indications

- Confirmation of factor V Leiden after an abnormal APC resistance assay

Test Principle

This test is sent out to a reference lab. Generally speaking, genomic DNA is amplified using PCR, and the sequence of interest is identified and compared with a wild-type sequence.

Possible Results & Interpretation

Results are reported as negative, heterozygous, or homozygous for the mutation. Studies have found that heterozygous patients have a three- to sevenfold higher risk of venous thrombosis compared with those negative for the mutation. In homozygotes, this risk increases to fifty- to eightyfold. The frequency of the factor V Leiden mutation varies substantially by the ethnic background of the individual.

Factors Affecting Factor V Leiden Mutation Analysis

The assay detects only the presence of the mutation in the DNA; it says nothing about whether or not the mutant gene is expressed.

Elevated Factor VIII, IX, or XI

Case-control studies, such as the Leiden Evaluation of Thrombophilia Study (LETS), have identified elevated levels of factors VIII, IX, and XI as risk factors for the development and recurrence of venous thromboembolism (VTE).

Increased factor VIII activity has traditionally been of the greatest clinical concern. Several studies have found that VIII activity that is greater than two standard deviations above the population mean is associated with an increased risk of venous thromboembolism. One large single-center cohort study estimated these patients to have a sixfold increase in their risk of recurrent VTE. Although family studies have demonstrated that elevated VIII is genetically determined, the molecular mechanisms responsible remain to be completely clarified.

Since factor VIII is an acute-phase reactant, clinical studies have generally avoided measuring these levels during infectious or inflammatory events; they also wait at least six months after an episode of VTE. These strict requirements and the high prevalence of elevated VIII in thrombosis clinic populations (about 20% to 25%) have limited the usefulness of factor VIII activity tests in decision-making about the duration of anticoagulation.

LETS also identified elevated factor IX and XI levels as being risk factors for VTE, with each being associated with a twofold increase in risk. Confirmation of these findings and integration of this information into clinical decision algorithms for anticoagulation has yet to be performed.

Indications

- Diagnosis of thrombophilia

Test Principle

See p. 20, "Factors VIII, IX, XI, and XII, prekallikrein, and HMWK."

Possible Results & Interpretation

Factor VIII, IX, and XI activity levels above the 95th percentile are considered elevated and represent an independent risk factor for VTE. Since these levels can be influenced by heparin, direct thrombin inhibitors, and thrombolytic therapy, samples should not be drawn in patients receiving these medications. Factor VIII is an acute-phase reactant, so VIII activity should not be measured in the setting of acute thrombosis, inflammation, or infection. Vitamin K antagonists, such as warfarin, reduce factor IX levels, so IX levels cannot be measured accurately in patients receiving such medications.

Factors Affecting the Activity Assays for Factors VIII, IX, and XI

Instrument and reagent differences as well as dilution errors can cause aberrant results. The presence of lupus anticoagulants has been associated with falsely decreased results; this effect can often be identified and overcome by performing serial dilutions of the sample and examining test results for a plateau effect. Finally, factor XI can be activated by freezing, causing a falsely increased activity level.

Protein C Deficiency

Protein C is a vitamin K–dependent serine protease that regulates hemostasis through both anticoagulant and profibrinolytic effects. Protein C circulates in plasma in an inactive form until it is activated by the endothelium-bound thrombin-thrombomodulin complex. Activated protein C (APC) complexes with protein S to proteolytically inactivate factors Va and VIIIa, thus downregulating the coagulation cascade (Fig. 5). APC plays a minor role in the regulation of the fibrinolytic cascade by inactivating plasminogen activator inhibitor-1.

Protein C deficiency can be inherited or acquired. Congenital heterozygous

protein C deficiency leads to a tenfold increased risk of venous thrombosis. The prevalence of heterozygous protein C deficiency is 0.2% to 0.4%. Its prevalence is also low in thrombosis clinics, where it accounts for only 4% of patients. Congenital homozygous deficiency is extremely rare; it is associated with a severe thrombotic disorder in neonates known as purpura fulminans.

Acquired deficiency may be due to liver failure, prematurity, or vitamin K deficiency (malabsorption, oral anticoagulant therapy). In vitamin K deficiency, since the activity of other vitamin K–dependent coagulation factors is also diminished, the risk of thrombosis under these conditions is small.

Protein C has a short half-life. If warfarin is initiated (particularly with a large loading dose) in a patient with protein C deficiency without concomitant heparin therapy, very low levels of protein C activity may precipitate coumarin skin necrosis, which is caused by thrombosis of dermal blood vessels.

Protein C Activity Assay

This test is used to measure the activity of protein C in a patient sample.

Indications

- Diagnosis of congenital or acquired protein C deficiency
- Differentiation between protein C deficiency states, when used in conjunction with other methods (e.g., antigenic determination, protein C coagulometry)
- Monitor replacement therapy with protein C concentrates in patients with congenital deficiency

Test Principle

We use the Berichrom® Protein C kit (Siemens, Malvern, PA) for detection of protein C activity. This method detects the amidolytically active portion of activated protein C (APC), including the non-carboxylated version synthesized during vitamin K deficiency. This means that when vitamin K deficiency is present, this test will identify a higher level of protein C activity than a coagulometric test will. Therefore, to obtain a complete picture of the cause of protein C deficiency, it is advisable to complement this test with coagulometry and the antigenic determination method.

The Berichrom method uses an enzyme isolated from the venom of the Southern copperhead (*Agkistrodon contortrix*) to activate the protein C in a patient sample. The enzymatic activity of APC is measured through spectrophotometric evaluation of its effect on a chromogenic substrate, p-Glu-Pro-Arg-monoacetate. The assay is based on the following reactions:

$$\text{protein C}_{\text{sample}} \xrightarrow[\text{VENOM}]{\text{SNAKE}} \text{APC}$$

$$\text{p-Glu-Pro-Arg-monoacetate} \xrightarrow{\text{APC}} \text{p-Glu-Pro-Arg-OH + monoacetate}$$

Possible Results & Interpretation

Hereditary protein C deficiency is a heterozygous disorder that results in half-normal plasma levels of protein C. Protein C levels of 55% to 70% may be due to a true deficiency but could also represent the lower end of the normal distribution. Causes of protein C deficiency include hereditary deficiency, liver disease, DIC,

acute thrombosis, and the post-operative state. Although vitamin K deficiency and warfarin therapy can cause reductions in protein C activity when measured using coagulometric assays, our current assay is not affected by these conditions.

Factors Affecting the Protein C Activity Assay

The assay is not affected by heparin at concentrations of less than 1.0 IU/mL of plasma; higher levels may result in an overestimation of protein C activity. Warfarin therapy can cause decreased protein C antigen levels; therefore, if possible, discontinue warfarin at least two weeks before testing. Abnormal proteins produced during therapy with vitamin K antagonists (including warfarin) result in protein C activity levels of < 5% to 30% in normal individuals. Falsely low levels may also been in patients being treated with aprotinin, an antifibrinolytic.

Protein C Antigen Assay

This test measures the concentration of protein C.

Indications

- Confirmation of low protein C activity
- Identification of a dysfunctional protein C molecule when used in conjunction with the protein C activity assay

Test Principle

The protein C antigen test, which is sent to our reference lab, is an immunoassay.

Possible Results & Interpretation

Decreased protein C antigen may occur in hereditary protein C deficiency, liver disease, DIC, acute thrombosis, vitamin K deficiency, therapy with warfarin, and in the post-operative state. Hereditary protein C deficiency has been described in some patients with recurrent venous thrombosis; family studies may be diagnostically useful. Patients with heterozygous deficiencies of protein C here approximately half of normal levels.

Factors Affecting the Protein C Antigen Assay

In extremely rare instances, patients may have anti-rabbit antibodies that lead to aberrant results.

Protein S Deficiency

Protein S, a vitamin K–dependent plasma protein, is the cofactor of activated protein C (APC). Under normal conditions, approximately 40% of protein S circulates as free antigen and 60% is bound to C4b binding protein (C4bBP).

Protein S stimulates the proteolytic inactivation of factors Va and VIIIa by APC, thereby stimulating APC's coagulation-inhibiting effects. Decreased protein S activity increases thromboembolic risk. As with homozygous protein C deficiency, homozygous protein S deficiency leads to purpura fulminans in neonates.

In addition to hereditary protein S deficiency, a wide range of clinical situations can lead to diminished protein S, including hepatic disorders, acute and/or chronic inflammation, acute thrombotic events, oral anticoagulation, treatment with L-asparaginase, pregnancy, oral contraceptives, estrogen therapy, and elevated

Type	PS Activity	Free PS antigen	Total PS antigen	Estimated incidence
I	↓	↓	↓	up to 80%
II (IIb)	↓	↔	↔	0.1% to 5%
III (IIa)	↓	↓	↔	up to 20%

Table 10. Results of laboratory tests, by protein S deficiency type.
Adapted from Marlar RA, Gausman JN. Protein S abnormalities: a diagnostic nightmare. Am J Hematol. 2011;86:418-21. PS, protein S.

plasma levels of C4bBP. Recall that C4bBP is an acute-phase protein that normally functions as a regulator of the complement pathway. When C4bBP is bound to protein S, protein S cannot complex with protein C and thus cannot function as a cofactor in anticoagulation. These reductions of free functional protein S contribute to the thrombophilic state that has been associated with pregnancy, estrogen therapy, and inflammation. Newborns have low levels of C4bBP; thus, it is possible for tests to show normal levels of protein S activity despite reductions in total S in this population.

Hereditary protein S deficiency is associated with mutations in the *PROS1* gene on chromosome 3. This is a heterozygous disorder that results in half-normal plasma levels of protein S. It is associated with a tenfold increased risk of venous (and perhaps arterial) thrombotic events and may occur in as many as 0.1% of the general population. Family studies may be diagnostically useful.

Patients may be diagnosed with a specific type of protein S deficiency based on their laboratory testing phenotype: Types I and III (formerly IIa) present as quantitative defects and Type II (formerly IIb) presents as a qualitative defect. See Table 10 for the results of laboratory tests for the different types. At present, studies have found that these types confer no differences in thromboembolic risk.

Since the results of protein S activity assays can be influenced by multiple preanalytical variables, some laboratories have begun to use total and free protein S antigen testing preferentially in the diagnosis of protein S deficiency.

Protein S Activity Assay

This test is used to measure the activity of protein S in a patient sample.

Indications

- Diagnosis of protein S deficiency
- Subtyping protein S deficiency states, when used in conjunction with protein S antigen tests

Test Principle

Activated protein C proteolytically cleaves factor Va, which is generated during the activation of the coagulation cascade by Russell viper venom (RVV). In this reaction, protein S acts as a cofactor that accelerates the reaction. As a result, the coagulation time increases proportionally to the activity of protein S in the sample. The addition of deficient plasma ensures that the test mixture has a sufficient supply of fibrinogen, factor V, and the other necessary coagulation factors. Coagulation is triggered at the level of factor X by RVV. Activated X forms thrombin from prothrombin under the action of the remaining factor Va.

The protein S activity assay measures the ability of protein S to function as a co-factor for protein C. Dilutions of normal plasma are mixed with protein S–depleted plasma. A reagent containing factor Xa, activated protein C, and phospholipid is then added to activate the mixed plasma. After a five-minute activation time, clot formation is initiated by the addition of calcium chloride. Under these conditions, the prolongation of the clotting time is directly proportional to the concentration of protein S in the patient plasma. The use of factor Xa as the activator minimizes the potential interference by high levels of factor VIII that can be found in some patients. The value for protein S of a patient sample is determined by comparing the clotting time of the patient sample with the time obtained for dilutions of pooled plasma used to construct a standard curve.

Possible Results & Interpretation

Protein S activity levels of 55% to 65% are consistent with either a deficiency state or the lower end of the normal distribution. See "Protein S Deficiency," p. 42, for the causes of deficiency.

Factors Affecting the Protein S Activity Assay

The presence of heparin greater than 3.0 IU/mL may interfere with assay results by prolonging the clotting time and giving an artificially high protein S value. Lupus-type anticoagulants may lead to increased or decreased protein S results. The presence of factor V with a mutation at its protein Ca cleavage site may lead to diminished recovery of protein S.

A decrease in protein S activity does not necessarily indicate a decrease in plasma concentration, since protein S is nonfunctional when bound to C4bBP. Decreased levels of protein S activity should generally be evaluated further with a protein S antigen study.

Protein S Antigen, Total and Free

These tests measure the concentration of protein S in a patient sample.

Indications

- Diagnosis of protein S deficiency
- Subtyping protein S deficiency states, when used in conjunction with protein S activity tests

Test Principle

This test, which is sent to our reference lab, uses immunoturbidimetry to measure the formation of antigen-antibody complexes in solution. In general, this method measures the absorbance of light passing through a sample containing microlatex particles bound to specific antibodies. When the antigen in question is present, antigen-antibody complexes form; these complexes have diameters greater than the wavelength of the light passing through and therefore absorbance is reduced. There is a direct relationship between the observed absorbance value and the concentration of the antigen being measured.

Possible Results & Interpretation

A low level of protein S antigen indicates a reduction in the total protein S in the plasma sample. Reductions in free protein S, which can result in reduced protein

S activity in the presence of normal levels of total protein S antigen, can occur in conditions of inflammation, pregnancy, and estrogen use. See "Protein S Deficiency," p. 50, for additional information.

Factors Affecting the Protein S Antigen Assay

High levels of heparin, hemoglobin, bilirubin, rheumatoid factor, triglycerides, and platelets may interfere with this test. Elevated levels of C4bBP will reduce free protein S levels.

Homocysteine Concentration Assay

This test measures the concentration of homocysteine in a patient sample.

Indications

- Diagnosis of homocystinuria
- Diagnosis of hyperhomocysteinemia
- Evaluation of suspected deficiency of vitamin B6 or B12 or folate

Test Principle

Homocysteine is a thiol-containing intermediary amino acid formed by the conversion of methionine to cysteine. It can be remethylated back to methionine by methionine synthase (cobalamin and folate are cofactors) or converted to cysteine by cystathionine-β-synthase (pyridoxine is a cofactor) in a transsulfuration step. Homocysteine exported into plasma circulates mostly in its oxidized form, bound to plasma proteins. Smaller amounts of reduced and disulfide forms are also present. Total homocysteine is the sum of all homocysteine forms present in blood.

This test, which is performed in the Hopkins Core Lab, uses the ST AIA-PACK Homocysteine test kit (Tosoh Bioscience, South San Francisco, CA). Oxidized homocysteine is reduced by to the free form and converted to S-adenosyl-L-homocysteine (SAH) by SAH hydrolase and excess adenosine prior to the immunoassay. SAH present in the pretreated sample competes with immobilized SAH on magnetic beads for binding sites on the enzyme-labeled anti-SAH mouse monoclonal antibody. The magnetic beads are washed to remove unbound anti-SAH mouse monoclonal antibody and are then incubated with a fluorogenic substrate.

The rate of fluorescence produced by the enzyme reaction indicates the amount of enzyme-labeled anti-SAH mouse monoclonal antibody. The amount of antibody that binds to the beads is inversely proportional to the homocysteine concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve.

Possible Results & Interpretation

Homocystinuria is a rare autosomal recessive disorder characterized by severe elevations in plasma and urine homocysteine concentrations. Clinical manifestations of homocystinuria include developmental delay, Marfanoid appearance, osteoporosis, ocular abnormalities, thromboembolic disease, and severe premature atherosclerosis. Less marked elevations in plasma homocysteine are much more common, occurring in 5% to 7% of the population. Deficiency of vitamins can cause elevated homocysteine levels in plasma or serum.

Although unassociated with the clinical stigmata of homocystinuria, moderate hyperhomocysteinemia has been considered an independent risk marker for

atherosclerotic vascular disease and venous thromboembolism (VTE). However, the relationship between plasma homocysteine levels and vascular complications, particularly VTE, is unclear, and associations may have been due to failure to take into account confounding risk factors. Hyperhomocysteinemia may occur as a result of inherited disorders that alter the enzyme activity in the remethylation and transsulfuration pathways or by nutritional deficiencies of cobalamin (vitamin B12), folate, or pyridoxine (vitamin B6). Plasma homocysteine is increased in patients with renal failure.

Factors Affecting the Homocysteine Concentration Assay

The assay is sensitive and specific with no interfering reagent peaks. However, differences in sample handling can cause significantly different results in homocysteine measurement. Samples must be taken in gold-top or red-tops (no EDTA or heparin), and *the sample must be placed and kept on ice immediately after collection***.** If not, red blood cells will continue to synthesize homocysteine, leading to 10% to 50% increases in homocysteine concentration.

This test is not affected by anticoagulant therapy. Samples from patients receiving alkaline phosphatase inhibitors will show erroneous results. Samples from patients receiving S-adenosyl-methionine, intravenous fluorescein, methotrexate, carbamazepine, phenytoin, nitrous oxide, or 6-azauridine triacetate may have elevated levels of homocysteine.

Factor II (Prothrombin) G20210A Mutation Analysis

This is a DNA-based assay for the factor II G20210A mutation, a mutation in the 3'-untranslated region of the prothrombin gene. Factor II G20210A is found in 18% of patients with a family history of thrombosis. Recent evidence suggests that this point mutation improves the efficiency of RNA processing, resulting in elevated prothrombin levels, the presumed cause of the hypercoagulable state.

Indications

- To test for the relatively common hypercoagulable state due to the prothrombin mutation, factor II G20210A.

Test Principle

This test, which is sent to a reference lab, is performed by polymerase chain reaction.

Possible Results & Interpretation

Calculation of a wild-type:mutant ratio allows the identification of patients who are heterozygous or homozygous for the G20210A mutation. Heterozygotes have a three- to fourfold increased risk of thrombosis, regardless of sex or age. Factor II G20210A frequently occurs in patients with factor V Leiden (FVL); the risk of thrombosis increases to 20 to 25 times normal when FVL is present. As with FVL, the frequency of the factor II G20210A mutation is likely to vary substantially according to the ethnic background of the individual. Due to the genetic nature of the disorder, family counseling may be appropriate.

Factors Affecting the Factor II G20210A Mutation Analysis

The assay detects only the presence of the mutation in the DNA; it says nothing about whether or not the mutant gene is expressed.

Antithrombin Deficiency

Antithrombin (AT; previously called antithrombin III) is synthesized in the liver and functions as an inhibitor of thrombin. It also inhibits factor Xa and, to a lesser extent, IXa, XIa, XIIa, plasmin, urokinase, kallikrein, and trypsin. Heparin dramatically enhances the activity of AT.

Congenital deficiency of AT is rare (0.02% to 0.2% of the population) but is associated with a twentyfold increase in the risk of venous thromboembolism (VTE). Up to 7% of people with VTE have an inherited deficiency of AT. It is inherited in an autosomal dominant fashion and affected patients typically have half-normal levels. Causes of acquired deficiency include liver disease, protein-wasting states, DIC, acute thrombosis, and heparin or estrogen therapy.

Testing for suspected deficiency should include the antigen test, which measures the concentration of AT, and the activity assay, which determines the functionality of the AT present.

Antithrombin Activity Assay

This test measures the activity of antithrombin (AT; previously "antithrombin III").

Indications

- Diagnosis of AT deficiency; follow with antigen assay if result is abnormal

Test Principle

We use the INNOVANCE® Antithrombin assay (Siemens, Malvern, PA). An excess of factor Xa is added to citrated plasma. In the presence of heparin, a portion of the enzyme is complexed and inactivated by the antithrombin present in the patient sample. Excess (i.e., uninhibited) Xa then cleaves a chromogenic substrate, releasing a dye. The rate of the release of dye is inversely proportional to the inhibiting activity of antithrombin the patient sample—that is, the lower the concentration of functionally active antithrombin, the higher the absorbance signal per time unit.

Possible Results & Interpretation

Levels of 60% to 80% are consistent with either a deficiency state or the lower end of the normal distribution. These and lower levels should be investigated with an antigen assay. Patients with hereditary heterozygous AT deficiency typically have half-normal levels. Some patients have mutations that result in a quantitative defect that is characterized by equivalently decreased functional and antigenic levels. Others produce a defective molecule with reduced function but normal antigen levels. Acquired AT deficiency can be seen in liver disease, the post-operative state, nephrotic syndrome (usually with loss of > 4 grams of protein per day), DIC, extensive thrombosis, women using oral contraceptives, and during heparin or L-asparaginase therapy.

Studies have not demonstrated pathologic effects from increased levels of AT.

Factors Affecting the Antithrombin Activity Assay

Therapeutic doses of direct oral factor Xa inhibitors may cause erroneously increased antithrombin activity values. Very rare antithrombin gene variants with reduced functional activity may yield results within the reference range.
The test is not affected by hemoglobin up to 1,000 mg/dL, conjugated bilirubin up to 40 mg/dL, unconjugated bilirubin up to 60 mg/dL, and triglycerides up to 425

mg/dL (normal AT activity levels) and 338 mg/dL (high AT activity levels). The test procedure described here is not affected by therapeutic doses of heparin.

Antithrombin Antigen Assay

This test measures the concentration of antithrombin (AT).

Indications

- Diagnosis of AT deficiency; use with the activity assay

Test Principle

This test, which is sent to a reference lab, uses an immunoturbidimetric method. In general, this method measures the absorbance of light passing through a sample containing microlatex particles bound to specific antibodies. When the antigen in question is present, antigen-antibody complexes form; these complexes have diameters greater than the wavelength of the light passing through and therefore absorbance is reduced. There is a direct relationship between the observed absorbance value and the concentration of the antigen being measured.

Possible Results & Interpretation

Since AT deficiency can be due to quantitative (decreased production of a normally functioning protein) or qualitative defects (production of a dysfunctional protein), antigen determination can in conjunction with the activity assay help determine if the deficiency is due to decreased production or a dysfunctional protein.

Factors Affecting the Antithrombin Antigen Assay

Fasting samples should be used for this assay. In addition, patient should abstain from anabolic steroids, gemfibrozil, warfarin, heparin, asparaginase, estrogens, gestodene, and oral contraceptives for three days prior to specimen collection. Cloudy or lipemic samples may lead to an overestimation of AT levels. The presence of rheumatoid factor may cause erroneous results.

Dysfibrinogenemia

Dysfibrinogenemia is characterized by the synthesis of an abnormal fibrinogen molecule that can predispose to abnormal bleeding, thrombosis, or both. Congenital dysfibrinogenemia is a rare cause of thrombophilia that accounts for fewer than 1% of patients seen in thrombosis clinics. Dysfibrinogenemia should always be considered in any patient who appears to have a predisposition to form venous or arterial thromboses.

Laboratory testing for dysfibrinogenemia should include a functional fibrinogen test (p. 14), thrombin time (p. 15), fibrinogen antigen level, and a reptilase time (p. 16). Samples should not be collected during therapy with heparin, direct thrombin inhibitors, or thrombolytic agents. The fibrinogen antigen test is available from our reference laboratory. This test is performed by nephelometry and overnight fasting is required. Lipemia and hemolysis can cause erroneous results.

CHAPTER 5

The Fibrinolytic System

Plasminogen activity and antigen
Tissue plasminogen activator antigen
Plasminogen activator inhibitor-1
α2-antiplasmin (α2-plasmin inhibitor)

Fibrinolysis

In addition to the anticoagulant elements described in Chapter 4, several enzymes contribute to hemostasis by breaking down the end product of coagulation, the fibrin clot (see Fig. 6). Plasmin is the major protease responsible for fibrinolysis. It is formed from the liver proenzyme plasminogen through the action of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The presence of fibrin enhances this activity by positioning and stabilizing plasminogen and tPA. Plasmin then cleaves fibrin into smaller polymers, known as fibrin degradation products (FDP) or fibrin split products (FSP). D-dimers are a type of FDP (see p. 22). FDP are then cleared by phagocytes and hepatic metabolism.

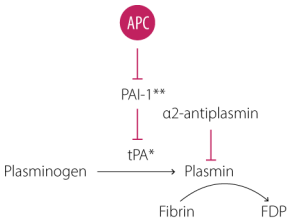

Figure 6. Fibrinolysis. Plasmin breaks down fibrin into fibrin degradation products (FDP). It is activated from its zymogen form by tissue plasminogen activator (tPA) and *urokinase plasminogen activator (uPA; not shown) and is inhibited by α2-antiplasmin. Plasminogen activator inhibitor-1 (PAI-1) blocks tPA and uPA. **PAI-2 (not shown) is active during preganancy and mainly targets uPA. Activated protein C (APC) inhibits PAI-1 and PAI-2.

Thrombin-activatable fibrinolysis inhibitor (TAFI) modifies fibrin to reduce its ability to recruit plasminogen and tPA. Fibrinolysis is also regulated by plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2), which inhibit tPA and uPA. PAI-1 is normally of greater significance, as PAI-2 is thought to be active primarily during pregnancy. The PAIs are inhibited by activated protein C. Plasmin itself is primarily inhibited by α2-antiplasmin (also called α2-plasmin inhibitor).

Deficiencies of any of these components may result in bleeding or thrombosis. However, disorders of fibrinolysis are rare, and hereditary disorders exceedingly so.

Plasminogen Activity and Antigen Assays

These tests measure the activity and quantity of plasminogen in plasma.

Indications

- Evaluation of unusual hemorrhagic or thrombotic disorders
- Monitoring hepatic regeneration during thrombolytic therapy

Test Principle

These tests are sent to a reference lab. The activity test is performed using spectrophotometry and the antigen test is performed using nephelometry.

Possible Results & Interpretation

Low plasminogen activity may be seen in liver disease, DIC, and primary fibrinolysis; after cardiopulmonary bypass; and during therapy with streptokinase, urokinase, or tPA.

Hereditary plasminogen deficiency is an autosomal recessive disorder with a prevalence of 1.6 per 1,000,000. It is categorized as quantitative (Type I) or qualitative (Type II). Type I deficiencies demonstrate a simultaneous decrease of both functional and antigenic levels of plasminogen. The rarer Type II deficiency is characterized by a functional level that is significantly lower than the antigenic level. Whether a deficiency of plasminogen is associated with an increased risk for venous thromboembolic disease is controversial. Some feel that plasminogen deficiency in isolation is a risk factor for thromboses. Conversely, others suggest that this deficiency is not in itself thrombogenic but rather constitutes a factor that enhances the clinical risk of other hemostatic disorders. A rare ocular disorder, ligneous conjunctivitis, is associated with severe plasminogen deficiency.

Factors Affecting the Plasminogen Activity and Antigen Assays

Overnight fasting is required for the antigen assay. These assays are insensitive to plasma inhibitors. Both assays may be affected by heparin, cryoprecipitate products, and elevated levels of hemoglobin, bilirubin, or triglycerides.

Tissue Plasminogen Activator (tPA) Antigen Assay

This test measures the concentration of tissue plasminogen activator (tPA).

Indications

- Evaluation of unusual hemorrhagic or thrombotic disorders

Test Principle

This send-out test uses the enzyme-linked immunosorbent assay method (ELISA).

Possible Results & Interpretation

Endothelial cells release tPA in response to numerous physiologic stimuli, including thrombin and epinephrine. Abnormally elevated tPA activity may be seen following venous occlusion, exercise, or desmopressin infusion. Levels may increase in the setting of hepatic dysfunction, since the liver eliminates tPA.

High plasma levels have been found in some patients with bleeding tendencies, and decreased levels may be associated with a predisposition toward venous thrombosis. These associations are tenuous due to confounding. There have also been reports of patients with antiphospholipid syndrome developing antibodies to tPA.

Factors Affecting the Tissue Plasminogen Activator Antigen Assay

Traumatic venipuncture or prolonged stasis may invalidate results.

Plasminogen Activator Inhibitor-1 Activity Assay

Plasminogen activator inhibitor-1 (PAI-1) is a fast-acting inhibitor of tPA that is released into circulation by endothelial cells and other cell types. The concentration

of PAI-1 is normally much higher than tPA activity levels. An increased plasma level of PAI-1 can lead to impaired fibrinolytic function.

Indications

- Evaluation of unusual hemorrhagic or thrombotic disorders

Test Principle

This send-out test is performed as an enzyme immunoassay.

Possible Results & Interpretation

Elevated PAI-1 levels have been associated with thrombosis and septic shock in a number of studies, although an actual pathogenic mechanism is unclear. Elevated levels can persist for months after a thrombotic event.

PAI-1 behaves as an acute-phase reactant, and levels rapidly increase in the setting of major surgery, myocardial infarction, severe trauma, or sepsis. PAI-1 also gradually increases with age and during pregnancy. Postpartum PAI-1 rapidly returns to pre-pregnancy levels. Diurnal variation has been reported.

Low PAI-1 activity may be seen in liver disease, amyloidosis, and, rarely, as a congenital deficiency, and may be associated with clinically apparent bleeding.

Factors Affecting the Plasminogen Activator Inhibitor-1 Activity Assay

PAI-1 levels will be undetectable and inaccurate in patients receiving therapy with tPA, streptokinase, or urokinase. Extremely hemolyzed or icteric samples may yield unreliable results.

α2-Antiplasmin Activity Assay

This test measures the activity of α2-antiplasmin (α2-AP; also called α2-plasmin inhibitor), a fast-acting inhibitor of plasmin (see Fig. 6).

Indications

- Evaluation of unusual hemorrhagic or thrombotic disorders
- Monitoring fibrinolytic treatment

Test Principle

This chromogenic assay is sent to a referral lab.

Possible Results & Interpretation

Congenital deficiency of α2-AP is a rare, autosomal codominant disorder. It typically manifests as delayed bleeding after trauma, although prolonged and unusual sites of bleeding have also been reported. Severe bleeding is primarily associated with homozygous deficiency. Heterozygotes will have half-normal levels. Acquired α2-AP deficiency has been reported in conjunction with many conditions, including DIC, liver disease, amyloidosis, nephrotic syndrome, malignancy, and injury.

Elevated α2-AP levels have been observed during the post-operative period. Elevations may be associated with reduced efficacy of thrombolytic agents.

All results must be interpreted in the context of anticoagulant therapy.

Factors Affecting the α2-Antiplasmin Activity Assay

When α2-AP activity is tested by an amidolytic method, slight interference by α2-macroglobulin (a slower-acting plasmin inhibitor) cannot be precluded. In

particular, this may occur when the α2-AP level is greatly decreased or when the α2-macroglobulin level is very high.

Plasma collected in samples containing aminocaproic acid, aprotinin, or heparin should not be used; therapeutic doses of these substances, however, have little or no effect. Lipemic plasma and plasma with increased bilirubin or hemoglobin may interfere with the assay.

CHAPTER 6

Qualitative Platelet Dysfunction Testing

Platelet count
Bleeding time
Platelet aggregation panel
PFA-100®
Thrombotic thrombocytopenic purpura
  ADAMTS13 activity
  ADAMTS13 inhibitor
  ADAMTS13 antibody
Heparin-induced thrombocytopenia
  Platelet factor 4 antibody
  ^{14}C-serotonin release

Platelet Count

This test measures the number of platelets in a sample.

Indications

- Evaluation of hemostatic disorders
- Evaluation of other disorders that may affect platelet quantity or quality
- Monitoring response to blood transfusion therapy

Test Principle

This measurement is usually performed as part of a complete blood count using flow cytometry. This method uses scattered light and fluorescence from a semiconductor laser to determine differences in cell size, complexity, and RNA and DNA content.

Possible Results & Interpretation

This test is very accurate but may require further investigation, such as a peripheral blood smear, to interpret high and low counts. A peripheral smear not only allows a manual estimate of the count, it can also be used to evaluate platelet morphology.

Factors Affecting the Platelet Count

Giant platelets and clumped platelets may falsely decrease the platelet count. Clumping may occur in samples collected in EDTA. Sample clotting will yield inaccurate results.

Bleeding Time

The bleeding time, which measures the time it takes to stop bleeding after a standard cut is made in the skin, was thought to reflect the integrity of the platelet and vascular components of hemostasis. This test is no longer performed at most institutions—including ours—due to a lack of correlation between bleeding time

and surgical bleeding, the availability of functional platelet tests that do not involve cutting the patient, and inconsistency in test administration.

Platelet Aggregation Panel

This test evaluates the response of platelets in platelet-rich plasma or whole blood to various aggregating reagents.

Indications

- Diagnosis of a qualitative platelet defect
- Differentiation of the cause of a platelet dysfunction
- Monitoring therapy with antiplatelet agents

Test Principle

This in vitro test of platelet function can detect and diagnose acquired or congenital qualitative platelet defects. The basis of the test is the ability or inability of platelets to respond to a particular aggregating reagent. The reagents used to initiate aggregation in this panel include thrombin, adenosine diphosphate, collagen, ristocetin, arachidonic acid, and epinephrine.

We now use chemi-luminescence and light transmission aggregation methods for this test, whereas for several years we did impedance aggregation. The change was based upon our frequent need to do aggregation studies on patients with low platelet counts, standardization of normal range, and technologist preference. In general, we follow the CLSI guidelines, which Dr. Kickler co-authored.

The basic principle of the chemi-luminescence method is to initiate the aggregation of platelets, then measure the amount of ATP released by the platelets and the amount of aggregation that takes place. The ATP is measured through the luminescence produced when it reacts with a firefly luciferin-luciferase reagent. Aggregation is measured using light transmission.

Keep in mind that you must coordinate with the Special Coagulation Lab to order this test. It requires sixs of blood and must be hand-carried to the lab. The test also carries specific requirements for patient activity and medications (see "Factors Affecting the Platelet Aggregation Panel," next page).

Possible Results & Interpretation

The interpretation of this test is based on the results of normal controls and the changes in luminescence and light transmission over time. These results should be assessed in conjunction with the patient history and other laboratory findings.

Although an interpretation is provided with this test, here are some rules of thumb to keep in mind:

- Absent or decreased secretion of ATP in response to thrombin may indicate either a storage pool deficiency or a secretion defect. Absence of a secondary wave in collagen aggregation and a failure to respond to ADP will also be present.
- No aggregation and no secretion in response to arachidonic acid may confirm impaired thromboxane synthesis (for example, from aspirin administration) Aggregation but no secretion may indicate either a storage pool deficiency or a secretion defect.
- von Willebrand disease (vWD) is characterized by a deficiency of von Willebrand factor (vWF), a plasma cofactor required for platelet adhesion. The

antibiotic ristocetin induces platelet adhesion in the presence of vWF and the GPIb platelet receptor. Thus, failure to aggregate in response to ristocetin suggests either von Willebrand disease or absence of the GPIb receptor (Bernard-Soulier syndrome). Repeating ristocetin aggregation with the addition of normal plasma or concentrated vWF will correct the aggregation abnormality in vWD but not in Bernard-Soulier syndrome. In some cases, a prolonged lag time may be the only indication of abnormality. Hyper-responsiveness to ristocetin may be seen in type 2B and platelet vWD and can be confirmed by testing at a final concentration of 0.3 mg/mL ristocetin.

- Glanzmann's thrombasthenia, a rare abnormality of platelet GPIIb/IIIa (the fibrinogen receptor), is characterized by abnormal aggregation and secretion in response to all agonists except ristocetin.

Factors Affecting the Platelet Aggregation Panel

Patients for whole blood aggregation tests should be resting but need not be fasting. They should be nonsmoking. They should avoid taking any prescription or over-the-counter medications known to affect platelet function for ten days to two weeks prior to the test; if you are unsure about any medication (as the list extends beyond targeted antiplatelet therapies), contact the Hemostasis laboratory.

Platelet Function Analyzer 100 (PFA-100®)

This test is an in vitro assay of platelet function that uses citrated whole blood.

Indications

- Screening for disorders of platelet function

Test Principle

The PFA-100® (Siemens, Malvern, PA) is a an instrument that simulates and measures in vitro the process of platelet adhesion and aggregation following a vascular injury.

The system uses a test cartridge with a reservoir for the sample, a capillary channel, and a membrane into which a small aperture has been created. The membrane is coated with collagen and ADP or epinephrine. Citrated whole blood is aspirated through the capilllary and membrane under high shear flow conditions. The collagen coating simulates in vivo subendothelium and the ADP/epinephrine acts as an additional physiologic platelet agonist. Platelets adhere to the membrane, become activated, and secrete their granule constituents. Platelet aggregates form that occlude the membrane aperture and arrest the flow of blood

The analyzer into which the cartridge is placed measures the time required for the membrane aperture to become occluded with a platelet plug. This event is sensed by the analyzer and recorded as the closure time (CT); that is, the time elapsed from the start of the test until aperture occlusion occurs.

Possible Results & Interpretation

The CT is an indicator of platelet function in the analyzed whole blood sample. Consequently, the CT is affected by thrombocytopenia as well as by congenital and acquired disorders of platelet function, including Bernard-Soulier syndrome, Glanzmann's thrombasthenia, storage pool disorders, von Willebrand disease,

cartridge	mean CT (s)	reference (s)
COL/ADP	92	71 - 118
COL/EPI	132	94 - 193

Table 11. Closure times, by PFA-100® cartridge type.
COL/ADP, collagen/ADP; COL/EPI, collagen/epinephrine; CT, closure time.

uremia, and exposure to antiplatelet drugs, such as aspirin, nonsteroidal anti-inflammatory agents, clopidogrel, ticlopidine, etc. Deviations of the hematocrit from the normal range can also influence the assay.

As mentioned, different test cartridges are available. The collagen/ADP (COL/ADP) cartridge is primarily used to detect intrinsic platelet defects, von Willebrand disease, and exposure to platelet inhibitors. If an abnormal result is obtained using the COL/ADP cartridge, the collagen/epinephrine (COL/EPI) cartridge is then used to determine if aspirin or aspirin-containing medications may be present. See Table 11 for a comparison of CTs by cartridge type.

Factors Affecting the PFA-100® Assay

Hemolysis, by reducing the hematocrit and increasing the ADP content of the patient sample, may interfere with this test. Microthrombi and particulate matter in a sample can cause a flow obstruction, leading to automatic test cancellation.

Specimens with high sedimentation properties may give misleading results: Since samples are tested sequentially in the COL/ADP and COL/EPI cartridges, it is conceivable that some settling could occur by the second cartridge and affect the closure time. The manufacturer recommends that such samples be tested in each cartridge separately.

As with any test of platelet function, a thorough medication history is essential to avoid misleading results due to medications that influence platelet function. Likewise, platelet counts less than 150,000/µL and hematocrit values less than 35% may influence closure times. The effect on closure times of hematocrit values above 50% or platelet counts greater than 500,000/µL is unknown. Certain fatty acids and lipids found in the diet are known to inhibit platelet function, and physicians may wish to advise patients to refrain from fatty foods prior to testing.

Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disorder characterized by microangiopathic hemolytic anemia, thrombocytopenia, neurologic symptoms, and ischemic organ failure. TTP can be congenital or acquired. In congenital TTP, a mutation of the *ADAMTS13* gene results in the accumulation of large von Willebrand factor (vWF) multimers. These endothelium-bound multimers promote microthromboses by binding circulating platelets. Acquisition of autoantibodies against the ADAMTS13 protein, whose function is to cleave the vWF multimers, can also lead to TTP.

Other disorders, such as hemolytic-uremic syndrome and HELLP (hemolysis, elevated liver enzymes, and low platelets), demonstrate similar symptoms. Laboratory testing can be useful in distinguishing among these conditions. Our tests for ADAMTS13 activity and inhibitors use a method of enzyme-linked immunosorbent assay (ELISA) that employs fluorescence resonance energy transfer (FRET) technology. ADAMTS13 antibody testing is performed at the Blood Center of Wisconsin.

It is important to keep in mind that the activity and inhibitor assays are specific but not sensitive. Any negative result must be considered within the patient's unique clinical context.

ADAMTS13 Activity Assay

This test measures the functional activity of the ADAMTS13 cleaving protease.

Indications

- Diagnosis of thrombotic thrombocytopenic purpura

Test Principle

We measure the functional activity of the cleaving protease using a recombinant von Willebrand protein joined to a fluorescent tag. This fluorescence is quenched as long as the protein remains intact. With ADAMTS13-mediated proteolysis, the fluorescent tag is released, allowing precise measurement of enzyme activity.

Possible Results & Interpretation

The result of the test is reported as a percent of normal expected activity; therefore, a value of "> 100%" is possible. Several different calibration controls are used, so the upper limit may appear as any number, for example "> 98%." The normal reference range is currently 70% to 150%. For all patient samples falling below the reference range, we will order and perform the ADAMTS13 inhibitor assay (below).

Factors Affecting the ADAMTS13 Activity Assay

Only samples collected in 3.2% sodium citrate are accepted for this assay. Severe hemolysis or bilirubinemia may affect the assay result. Treatment with blood products or factor concentrates could mask a deficiency.

ADAMTS13 Inhibitor Assay

This test measures the functional activity of an inhibitor of the ADAMTS13 cleaving protease.

Indications

- Diagnosis of thrombotic thrombocytopenic purpura due to an antibody

Test Principle

This test is performed if the ADAMTS13 functional activity assay (above) is abnormal. The patient sample is heated to inactivate any existing ADAMTS13 protein, then incubated with normal pooled plasma whose percent of ADAMTS13 activity is known. The functional ADAMTS13 in the normal plasma forms complexes with any anti-ADAMTS13 antibodies present in the patient sample. The resulting mixture and a control containing the same amount of normal pooled plasma as was added to the patient sample are run through the functional activity assay. The results of each sample are compared in order to establish the percent inhibitor activity.

Possible Results & Interpretation

The result of the test is reported as the percent inhibition. A result of < 45% inhibition is within the reference range.

Factors Affecting the ADAMTS13 Inhibitor Assay

Only samples collected in 3.2% sodium citrate are accepted for this assay. Severe hemolysis or bilirubinemia may affect the assay result. Treatment with blood products, such as plasma exchange or transfusion, could cause falsely normal results.

ADAMTS13 Antibody Assay

This test detects the presence of an IgG antibody against ADAMTS13.

Indications

- Diagnosis of thrombotic thrombocytopenic purpura due to an antibody

Test Principle

This send-out test uses the enzyme-linked immunosorbent assay (ELISA) method.

Possible Results & Interpretation

The result of the test is reported in arbitrary units; the reference range will be included in the reference laboratory's report. This assay is less specific than the inhibitor and activity assays.

Factors Affecting the ADAMTS13 Antibody Assay

False positive results have been described in healthy patients and in patients with immunologic disorders. Recent plasma exchange or blood transfusion could cause inaccurate results.

Heparin-Induced Thrombocytopenia

Patients uncommonly develop thrombocytopenia during the administration of heparin (unfractionated or low molecular weight). Two different forms of heparin-induced thrombocytopenia (HIT) have been described. Type I HIT is characterized by the development of minimal thrombocytopenia (platelet nadir generally no less than 100,000/μL) during the first few days of intravenous heparin administration. It is non-immunologic and is due to the platelet-aggregating properties of heparin when administered in high intravenous doses. Type I HIT is of little clinical significance and resolves spontaneously with continued therapy.

Type II HIT—nowadays simply referred to as HIT—typically develops four to fourteen days after starting therapy in heparin-naïve patients and can occur regardless of the size of the dose or the route of administration. Platelet counts typically fall below 100,000/μL or by 50% from the baseline (median count, 60,000/μL). In the majority of cases, this drop is due to antibodies to a complex of heparin and platelet factor 4 (PF4), a protein stored in platelet α-granules. These patients are at extremely high risk for venous and/or arterial thrombosis; heparin must be promptly discontinued and alternative anticoagulation agents employed. The tests most frequently used to investigate cases of suspected HIT are functional assays (serotonin release, platelet aggregation) and immunoassays for anti-PF4 antibodies.

Platelet Factor 4 Assay

This enzyme-linked immunosorbent assay is used to detect anti-platelet factor 4 antibodies.

Indications

- Diagnosis of heparin-induced thrombocytopenia

Test Principle

The antibodies in HIT recognize sites on platelet factor 4 (PF4) that are created when PF4 is complexed with heparin or other linear polyanionic compounds, such as polyvinyl sulfonate (PVS). PF4 is a constituent of platelet α-granules and is

released upon platelet activation and secretion.

In this assay, PF4:PVS complexes are immobilized in microwells. Patient serum is added, and if antibodies to PF4 are present, they will bind the complexes. After washing away unbound immunoglobulin, the serum is incubated with an alkaline phosphatase–linked anti-human IgG reagent. This reagent is washed away, and the substrate p-nitrophenyl phosphate is added. After 30 minutes of incubation, the reaction is stopped by the addition of sodium hydroxide. The amount of color generated is proportional to the amount of PF4 antibody present in the sample.

This assay detects only IgG, decreasing the number of false positives.

Possible Results & Interpretations

A mean optical density (OD) ≥ 0.400 is regarded as positive. Positive samples are confirmed through a heparin neutralization test. Any positive test result is a critical action value at our hospital.

This assay, which is intended for use as a screening test, has about 85% sensitivity for immune-mediated HIT. If clinical suspicion is high, the patient should be treated, regardless of a negative result. It is possible that the antibody titer may have been too low for detection initially; repeating the assay after several days can be useful in some cases. In addition, some patients with HIT have antibodies directed at other (non-PF4) antigens, such as IL-8 or neutrophil activating peptide 2 (NAP-2).

False positives are also possible. Some antibodies that recognize sites on the PF4:PVS complex may not bind the PF4:heparin complex (the reverse is of course also true). Therefore, a positive PF4 ELISA is not in itself sufficient for a diagnosis of HIT. Surveillance studies of have demonstrated that as many as 61% of cardiac surgery patients receiving heparin develop PF4:heparin antibodies without any clinical evidence of HIT. This test should only be performed when clinical suspicion is present, and positive or negative results must be interpreted in conjunction with clinical findings and other laboratory data.

Factors Affecting the Platelet Factor 4 Assay

Samples anticoagulated with heparin should not be used in this assay. Microbially contaminated, hemolyzed, lipemic, icteric, or heat-inactivated serum samples may give inconsistent test results and should be avoided. The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause increased nonspecific binding and produce false positives.

^{14}C-Serotonin Release Assay

This functional assay measures heparin-dependent platelet activation.

Indications

- Diagnosis of heparin-induced thrombocytopenia

Test Principle

The serotonin release assay (SRA), which is sent to a referral laboratory, has poor sensitivity but is more specific than the PF4 ELISA.

In this assay, donor platelets are labeled with ^{14}C-serotonin and allowed to incubate with patient serum at low and high concentrations of heparin. The amount of ^{14}C released into the supernatant is measured. If > 20% is released at 0.1 IU/mL of heparin and < 20% at 100 IU/mL heparin, the sample is reported as positive for

heparin antibodies. The inhibition of serotonin release at a high concentration of heparin is consistent with an immune complex-mediated disorder. A second sample may be required to confirm the absence of heparin-dependent antibodies if the clinical findings indicate heparin-induced thrombocytopenia or if the initial test result is a borderline reaction.

Appendix I: Hemostasis factors and molecules

	SYNONYM(S)
procoagulant factors	
fibrinogen	factor I
prothrombin	factor II
tissue factor	factor III; tissue thromboplastin
Ca^{2+}	factor IV
factor V	proaccelerin; labile factor; accelerator globulin
factor VI (term no longer used)	activated factor V
factor VII	stable factor; proconvertin
factor VIII	antihemophilic factor
factor IX	Christmas factor; plasma thromboplastin component
factor X	Stuart-Prower factor
factor XI	plasma thromboplastin antecedent
factor XII	Hageman factor
factor XIII	fibrin stabilizing factor
high molecular weight kininogen	HMWK; Fitzgerald factor
plasma prekallikrein	Fletcher factor
von Willebrand factor	vWF
anticoagulant factors	
tissue factor pathway inhibitor	TFPI
antithrombin	AT; antithrombin III; ATIII
thrombomodulin	
protein C	
protein S	
fibrinolytic factors	
tissue plasminogen activator	tPA; tissue-type plasminogen activator
urokinase plasminogen activator	uPA; urokinase-type plasminogen activator
urokinase plasminogen activator receptor	uPAR; urokinase receptor
plasminogen	
plasmin	fibrinolysin
plasminogen activator inhibitor 1	PAI-1
plasminogen activator inhibitor 2	PAI-2
α2-antiplasmin	α2-AP; α2-plasmin inhibitor

Table 12. Factors and molecules involved in hemostasis. Adapted from Boulpaep E. Blood. In: Boron WF, Boulpaep EL, eds. *Medical Physiology*. 3rd ed. Philadelphia, PA: Elsevier; 2017: 429-466.

Appendix II: Patients with bleeding problems

There is no substitute for a history to assess a bleeding diathesis and in determining what tests should be ordered.

Suggested topics to cover

- ☐ What was the age at initial presentation?
  early: inherited; older: acquired
- ☐ How did the bleeding present?
  petechiae: vascular or platelet
  ecchymoses/purpura: defect in primary hemostasis
  spontaneous; deep tissue hemorrhage; delayed onset; bleeding into tissues, joints, or body cavities: defect in coagulation factor
- ☐ Does the patient have a bleeding history?
- ☐ Is there a family history of bleeding?
  in addition to patterns of inheritance (e.g., sex-linked, factors VIII and IX; autosomal dominant, von Willebrand disease), ask about consanguinity
- ☐ What is the patient's ethnic origin?
- ☐ Has the patient undergone surgery without excessive bleeding?
- ☐ Has the patient had dental procedures without bleeding?
- ☐ Is the bleeding typically from one site or multiple sites?
- ☐ Is the bleeding prolonged, delayed, or recurrent?
- ☐ Could any medications have made the bleeding worse?
  aspirin, aspirin-containing medications, nonsteroidal anti-inflammatory drugs, antibiotics, anticoagulants
- ☐ Does the patient follow any unusual dietary practices?
  unusual or excessive intake; alcohol, vitamins, herbs, supplements
- ☐ Has the patient required transfusions?
- ☐ Does the patient have any systemic illnesses that could cause hemorrhage?
  renal disease, liver disease, myeloproliferative disorders, plasma cell dyscrasias, heart murmurs

General considerations

- Certain clinical scenarios provide insight into the type of disorder (if any). Intervention with surgery or transfusion indicates a more severe presentation. See Table 1 for an assessment tool.
- Epistaxis is common in von Willebrand disease and qualitative platelet disorders. Determine whether these have been spontaneous or post-traumatic, bilateral or unilateral.
- Menorrhagia can also be a clue to a platelet disorder or von Willebrand disease.
- A lack of significant bleeding after dental extraction supports normal hemostasis.
- Don't forget to ask about smaller surgical procedures, such as circumcision, tonsillectomy, and skin biopsies.
- Some types of bleeding have limited usefulness when documenting bleeding disorders: gastrointestinal and genitourinary bleeding, hemoptysis, and childbirth.

Appendix III: Patients with thrombosis problems

Suggested questions

- ☐ What was the age at initial presentation?
  infants to young adults, most likely inherited
  adults, inherited or acquired
  elderly, most likely acquired
- ☐ Has the patient had a venous thrombosis or clot in the past?
- ☐ Was the thrombosis provoked or unprovoked?
- ☐ Is there a family history of venous thrombosis?
  a negative history does not rule out the presence of an inherited disorder of thrombosis; sometimes, one or more environmental factors is needed
- ☐ What is the patient's ethnic origin?
- ☐ Does the patient have any risk factors for thrombosis?
  - *trauma*
  - *malignancy*
  - *immobilization*
  - *inflammatory conditions*
  - *autoimmune disorders*
  - *nephrotic syndrome*
  - *surgery*
  - *obesity*
  - *pregnancy*
  - *hormone use (e.g., estrogens)*
  - *chemotherapy, including tamoxifen*
  - *antithrombotic agents*

Appendix IV: Effects of anticoagulants on testing

Assay	heparin	LMWH	warfarin	DTIs	comments
Clot-based tests					
dRVVT	—	—	↑	↑	warfarin will correct on mixing; DTIs will not
reptilase time	—	—	—	—	
protein C or S activity	—*	—*	↓	↓ (spurious)	DTIs impair thrombin formation; generally, no clot forms
activated protein C	—*	—*	—	may ↑	lupus anticoagulants may cause low ratio
thrombin time (including dilute)	↑	— or slight ↑	—	↑	
Chromogenic tests					
antithrombin activity	↓	may ↓	—	—	
protein C or S activity	—	—	↓	—	
factor VIII activity	—	—	—	—	
Immunologic assays					
antithrombin antigen	↓	may ↓	—	—	↓
protein C or S antigen	—	—	—	—	protein S decreases with pregnancy and estrogen use
antiphospholipid antibodies[1]	—	—	—	—	titers may vary over time
D-dimer	may ↓	may ↓	—	—	

Table 13. Expected change in selected hemostasis tests due to anticoagulant therapy.

— no effect

—* no effect due to neutralizers in the assay.

[1] anticardiolipin antibodies, anti-β2-glycoprotein I antibodies

LMWH, low molecular weight heparin; DTI, direct thrombin inhibitor; dRVVT, dilute Russell viper venom time.

Appendix V: List of coagulation tests

NAME	EPIC ID	SPECIMEN	LAB
Activated Partial Thromboplastin Time	LAB325	blue top	JHH
Activated Protein C-Resistance	LAB20176	blue top	QUEST
ADAMTS13 Activity	LAB20199	blue top	JHH
ADAMTS13 Inhibitor	LAB20174	blue top	JHH
ADAMTS13 Antibody	LAB20616	call lab	BCW
Alpha -2- Antiplasmin Assay	LAB1123	blue top	QUEST
Anticoagulants, Nonspecific	LAB20216	blue top	JHH
Antiphospholipid Antibody Panel	LAB20388	blue top	QUEST
Antithrombin Antigen	LAB759	blue top	JHH
Antithrombin III, Functional Activity	LAB311	blue top	JHH
Anti-Xa (LMWH)	LAB20226	blue top	JHH
Anti-Xa (Unfractioned Heparin)	LAB20196	blue top	JHH
APTT- Heparin	LAB20208	blue top	JHH
Beta Thromboglobulin	LAB20187	call lab	ESOTERIX
Beta-2 Glycoprotein Antibodies	LAB1179	red top	JHH
Cardiolipin Antibody Panel (IgG, IgM, IgA)	LAB464	red top	JHH
CBC Without Differential	LAB294	lavender top	JHH
Chromogenic Factor X Activity	LAB24725	blue top	JHH
Cryofibrinogen	LAB569	blue top	JHH
Dabigatran (Reflex to Thrombin Time)	LAB25938	blue top	QUEST
D-Dimer, Quantitative	LAB313	blue top	JHH
Factor II (Prothrombin) 20210 Mutation	LAB834	lavender top	QUEST
Factor II Activity	LAB303	blue top	JHH
Factor V Activity	LAB304	blue top	JHH
Factor V Inhibitor Screen	LAB20178	blue top	QUEST
Factor VII Activity	LAB305	blue top	JHH
Factor VII Inhibitor Screen	LAB20179	blue top	JHH
Factor VIII Activity	LAB306	blue top	JHH
Factor VIII Antigen	LAB26198	blue top	QUEST
Factor VIII Chromogenic	LAB46291	blue top	JHH
Factor VIII Gene Inversion Analysis	LAB21499	lavender top	BCW
Factor VIII Inhibitor Screen	LAB335	blue top	JHH
Factor IX Activity	LAB308	blue top	JHH
Factor IX Antigen	LAB20191	blue top	QUEST
Factor IX Inhibitor Screen	LAB20222	blue top	JHH
Factor X Activity	LAB758	blue top	JHH
Factor X Inhibitor Screen	LAB20182	blue top	JHH

NAME	EPIC ID	SPECIMEN	LAB
Factor XI Activity	LAB309	blue top	JHH
Factor XII Activity	LAB310	blue top	JHH
Fibrinogen Antigen	LAB1121	blue top	QUEST
Fibrinogen, Quantitative	LAB314	blue top	JHH
Fondaparinux (Xa inhibition)	LAB45904	blue top	QUEST
Lupus Anticoagulant Panel	LAB42620	call lab	JHH
P2Y12 Assay	LAB20209	call lab	JHH
Patelet Factor 4- IgG ELISA Antibody	LAB20293	red top	JHH
Plasminogen Activator Inhibitor Antigen	LAB20565	blue top	QUEST
Plasminogen Activity	LAB847	blue top	JHH
Platelet Aggregation Panel	LAB20406	call lab	JHH
Platelet Antibody, Heparin-Dependent	LAB20612	call lab	BCW
Platelet Count, Citrated	LAB20156	blue top	JHH
Platelet Function Test (PFA-100®)	LAB318	blue top	JHH
Platelet Glycoprotein Expression	LAB20579	call lab	BCW
Prekallikrein Factor Activity	LAB23509	blue top	ARUP
Protein C Activity	LAB489	blue top	JHH
Protein S Activity	LAB491	blue top	JHH
Protein S Antigen, Total	LAB493	blue top	JHH
Protein S, Antigen and Free	LAB760	blue top	JHH
Prothrombin Fragment 1.2	LAB20312	blue top	JHH
Prothrombin Time + INR	LAB320	blue top	JHH
Reptilase Time	LAB1136	blue top	JHH
Ristocetin Cofactor	LAB331	blue top	JHH
Russell's Viper Venom, Diluted	LAB319	blue top	JHH
Serotonin Release Assay, UFH	LAB20294	red top	QUEST
Thrombin Time	LAB324	blue top	JHH
Thromboelastograph	LAB1122	blue top	JHH
Thromboxane B2 Urine	LAB20198	Urine	ESOTERIX
Tissue Plasminogen Activator	LAB20310	blue top	JHH
TTP Panel (ADAMTS13)	LAB23558	blue top	JHH
Viscosity, Plasma	LAB23009	lavender top	JHH
Von Willebrand Antigen	LAB757	call lab	JHH
Von Willebrand Collagen Binding	LAB25249	blue top	JHH
Von Willebrand Factor Multimers	LAB1111	blue top	QUEST
VWD Type 2N Binding (VWF-FVIII)	LAB45480	call lab	QUEST
VWF Propeptide Antigen	LAB20581	call lab	BCW

Table 14. List of coagulation tests at the Johns Hopkins Hospital.

Appendix VI: Evaluation of DIC

In 2001, an ISTH subcommittee presented this framework to help identify overt disseminated intravascular coagulation (DIC) as well as possible early DIC. This framework has been validated in multiple analyses. It should be used with caution in patients with underlying hematologic malignancies.

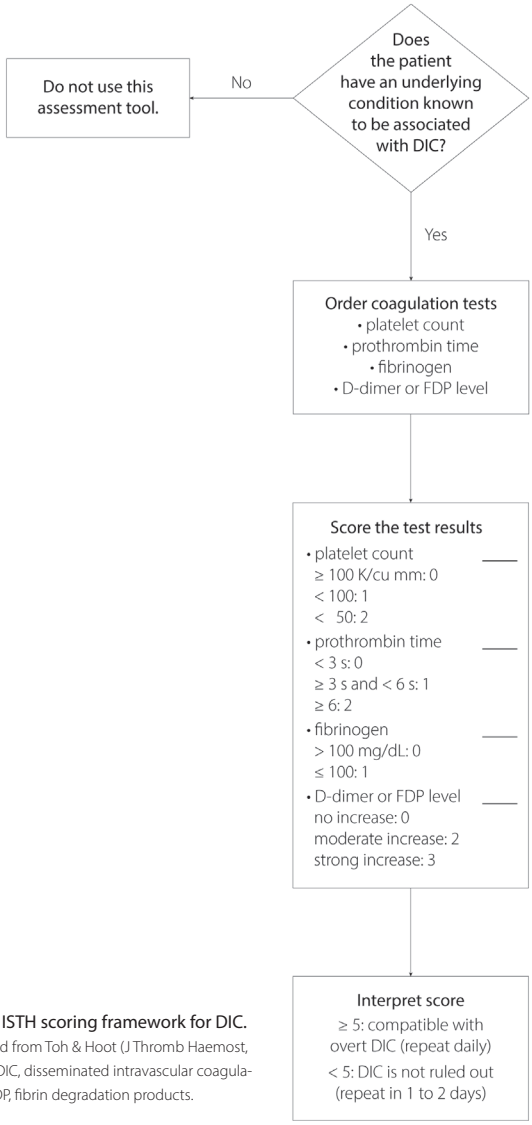

Fig. 7. ISTH scoring framework for DIC.
Adapted from Toh & Hoot (J Thromb Haemost, 2007). DIC, disseminated intravascular coagulation; FDP, fibrin degradation products.

Appendix VII: Wells criteria for VTE

The Wells criteria are probably the most widely used tools for determining the pre-test probability of venous thromboembolism (VTE).

Clinical characteristic	Score
Active cancer (treatment within the previous six months or currently receiving palliative treatment)	1
Paralysis, paresis, or recent plaster immobilization of the lower extremities	1
Recently bedridden for ≥ 3 days or major surgery (requiring general or regional anesthesia) within the previous twelve weeks	1
Localized tenderness along the distribution of the deep venous system	1
Entire leg swollen	1
Calf swelling ≥ 3 cm as compared with the asymptomatic side (measured 10 cm below the tibial tuberosity)	1
Pitting edema, confined to the symptomatic leg	1
Collateral superficial veins (nonvaricose)	1
Previously documented deep-vein thrombosis	1
Alternative diagnosis at least as likely as deep-vein thrombosis	-2

Table 15. Wells criteria for determining the pre-test probability of a deep vein thrombosis. If the sum of the scores is ≥ 2, a deep-vein thrombosis is likely; < 2, it is unlikely. If both legs are affected, use the more symptomatic leg. Adapted from Wells PS, et al. Evaluation of D-dimer in the diagnosis of suspected deep-vein thrombosis. N Engl J Med. 2003.

Clinical characteristic	Score
Clinical signs and symptoms of DVT (must have at least leg swelling and pain on palpation of deep veins)	3
An alternative diagnosis is less likely than pulmonary embolism	3
Heart rate > 100 beats per minute	1.5
Immobilization or surgery within the previous four weeks	1.5
Previous DVT or pulmonary embolism	1.5
Hemoptysis	1
Malignancy (current treatment or treatment, including palliative, within the previous six months)	1

Table 16. Wells criteria for determining the pre-test probability of a pulmonary embolism. If the sum of the scores is > 6, the probability of pulmonary embolism is high. If the sum is between 2 and 6 inclusive, the probability is moderate. Scores < 2 are low probability. Adapted from Wells PS, et al. Derivation of a simple clinical model to categorize patients probability of pulmonary embolism. Thromb Haemost. 2000. DVT, deep-vein thrombosis.

Appendix VIII: Notes on the DDAVP challenge

Desmopressin (trade name DDAVP), a synthetic analogue of the natural pituitary hormone 8-arginine vasopressin, is a common therapy for von Willebrand disease (vWD), as it causes the release of endogenous stores of von Willebrand factor (vWF) and factor VIII.

To perform a desmopressin trial, take baseline measurements of vWF antigen and factor VIII. Repeat these measurements one and four to six hours following the administration of DDAVP.

The activity levels of vWF and factor VIII generally rise by three to five times the baseline levels, peaking at about one hour post-infusion. A clinical response to desmopressin can be defined as maximum levels of vWF:RCo and factor VIII of more than 50 IU/dL.

Once administered, desmopressin's effects should last eight to ten hours. Evaluation of vWF and factor VIII activity at four to six hours post-administration, as well as the use of different assays, can help identify patterns of release and clearance peculiar to certain vWD variants. This information may suggest alternate forms of therapy.

For example, the pattern in mild type 1 vWD is fairly characteristic: vWF increases by two to four times the baseline, then begins to decrease, returning to baseline within 24 hours. The vWF initially detected by an optimized vWF collagen-binding (vWF:CB) assay tends to increase more so than that detected by vWF:Ag and vWF:RCo, since vWF:CB best reflects the ultra-high-molecular-weight vWF antigen initially released by desmopressin. In other words, in type 1 vWD, the vWF:CB/vWF:Ag ratio tends to rise to a higher level (generally > 1.0) than that of the vWF:RCo/vWF:Ag ratio. The patterns for type 2A and 2B are different, with desmopressin tending to increase vWF:Ag to much higher levels than either vWF:RCo or vWF:CB. Thus, neither the vWF:CB/vWF:Ag nor the vWF:RCo/vWF:Ag ratio tends to rise, remaining < 0.7 for most cases. The pattern for type 2M vWD (GPIb binding defect) is different again, with vWF:CB/vWF:Ag ratios remaining normal post-desmopressin and vWF:RCo/vWF:Ag ratios remaining low (generally < 0.7).

Pre- and post-desmopressin evaluation of vWF test parameters can also uncover vWD-related defects in vWF clearance. For example, in vWD Vicenza, tests for vWF:Ag versus the vWF propeptide shows accelerated clearance in two to four hours.

Appendix IX: Normal ranges (adult)

TEST	Reference range
activated protein C resistance	≥ 2.1
ADAMTS13 activity	70% to 150%
α2-antiplasmin	85% to 156%
anti-β2-glycoprotein I	≤ 20
antithrombin III activity	80% to 120% (adult)
antithrombin III antigen	80% to 120%
cardiolipin antibodies	≤ 11 (IgA); ≤ 14 (IgG); ≤ 12 (IgM)
cryofibrinogen	negative
D-dimer, quantitative	0.00 to 0.49 mg/L FEU
dilute Russell viper venom time	27.0 to 45.0 sec
factor inhibitors	< 0.5 Bethesda Units
factor V Leiden mutation analysis	negative
factors II, V, VII, VIII, IX, X, XI, XII	50% to 200%
factor X activity, chromogenic	72% to 138%
factor XIII, activity	57% to 192% activity
fibrinogen activity	170 to 422 mg/dL
fibrinogen antigen	130 to 350 mg/dL
heparin anti-Xa (for LMWH)	0.6 to 1.0 IU/mL
heparin anti-XAa (for UFH)	0.3 to 0.7 IU/mL
heparinase (Hepzyme)	negative
homocysteine	female: 4.6 to 12.1 µmol/L; male: 5.0 to 15.6 µmol/L
INR	0.9 to 1.1 sec
plasminogen activator inhibitor	4 to 43 ng/mL
plasminogen activity	65% to 176%
plasminogen antigen	8 to 14 mg/dL
platelet aggregation	Interpretation provided
platelet count	150 to 350 K/cu mm
Platelet factor 4 antibody	negative
prekallikrein activity	55% to 207%
protein C activity	42% to 142%
protein C antigen	70% to 140%
protein S activity	65% to 140% (adult)
protein S antigen (total)	70% to 140%
prothrombin fragment 1.2	41 to 372 pmol/L
PT	9.3 to 11.7 sec
PTT	23.1 to 30.9 sec
reptilase time	14.2 to 20.6 sec
ristocetin cofactor assay	50% to 150%
serotonin release assay	negative
thrombin time	17.5 to 20.6 sec
tissue plasminogen activator	≤ 12.8 ng/mL
von Willebrand collagen binding	50% to 110%
von Willebrand disease Type 2N binding	0.73 to 1.42
von Willebrand factor antigen	50% to 150%
von Willebrand factor multimers	normal pattern and distribution

Table 17. Adult reference ranges for selected tests. Check Epic for the most up-to-date values.

Appendix X: Normal ranges (pediatric)[1,2]

Test	1 to 7 days	1 to 4 weeks	1 to 3 months	3 to 6 months	> 6 months
antithrombin	39 to 93%	39 to 93%	41 to 108%	50 to 120%	73 to 120%
PT	10.0 to 15.9 sec	10.0 to 15.9 sec	10.0 to 14.9 sec	10.0 to 14.9 sec	9.3 to 11.7 sec
PTT	28.0 to 50.0 sec	22.4 to 56.8 sec	29.0 to 52.2 sec	25.1 to 47.1 sec	23.1 to 30.9 sec
factor II	26% to 127%	33% to 140%	34% to 144%	45% to 159%	50% to 200%
factor V	34% to 193%	50% to 187%	48% to 179%	48% to 176%	50% to 200%
factor VII	28% to 191%	35% to 191%	39% to 191%	39% to 191%	50% to 200%
factor VIII	50% to 239%	50% to 211%	50% to 210%	50% to 168%	50% to 200%
factor IX	15% to 111%	15% to 108%	21% to 139%	25% to 169%	50% to 200%
factor X	12% to 104%	20% to 114%	31% to 141%	35% to 155%	50% to 200%
factor XI	10% to 137%	23% to 137%	27% to 153%	41% to 211%	50% to 200%
factor XII	11% to 113%	11% to 101%	17% to 133%	25% to 140%	50% to 200%
protein C	17% to 90%	20% to 90%	21% to 112%	28% to 112%	80% to 120%
protein S	12% to 88%	22% to 105%	33% to 133%	54% to 134%	65% to 140%
vWF antigen	50% to 287%	50% to 254%	50% to 240%	50% to 200%	50% to 150%

Table 18. Pediatric reference ranges for selected tests. Normal ranges for studies may vary slightly over time. For the most up-to-date values, see the ranges listed in the electronic medical record. For Special Coagulation studies not listed here, normal values have not been established in this laboratory for pediatric patients. PT, prothrombin time; PTT, partial thromboplastin time; vWF, von Willebrand factor.

[1] Normal values have not been established in this laborator for premature infants.

[2] These ranges were established using this article as a guide: Andrew M, Paes B, Johnston M. Development of the hemostatic system in the neonate and young infant. Am J Pediatr Hematol Oncol. 1990;12(1):95-104.

Appendix XI: Thromboelastography

The thromboelastograph (TEG®) is an in vitro test that measures the speed of clot formation, clot strength, and clot lysis. This test is used to optimize the use of blood products in the operating room, in patients using extracorporeal membrane oxygenation or a ventricular assist device, and in monitoring certain blood dyscrasias.

A patient sample of citrated whole blood is added to a heated cuvette into which a pin is suspended via a wire (Fig. 8). The cup is rotated back and forth through an arc (measuring 4° 45'). As the clot develops, it impedes the progress of the rotation,

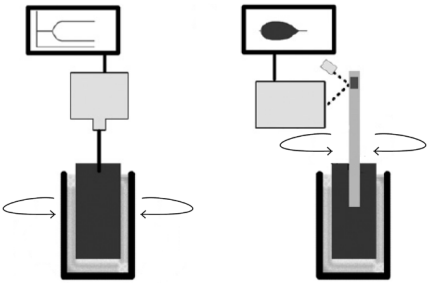

Fig. 8. TEG and ROTEM. This cartoon shows the designs of the TEG and ROTEM systems. The two mechanisms measure similar parameters but use different terms and different reference ranges. Note that reference ranges also change based on the reagents added. Adapted from Bolliger D, et al. Principles and practice of thromboelastography. Transfus Med Rev. 2012;26(1):1-13.

and the resistance is translated through the wire into a visual tracing that reflects the strength of the clot being formed (Fig. 9).

The components of this tracing represent different stages of clot formation, allowing the clinician to identify coagulation abnormalities. Reagents—usually kaolin and calcium—are typically added to speed the process of coagulation. Rotational thromboelastometry (ROTEM) is a variation of TEG that uses optical instead of mechanical signals to produce a tracing.

In addition to the graphical tracing, TEG results are given numerically (Table 19).

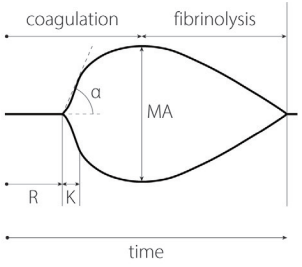

Fig. 9. Normal TEG tracing. The x-axis represents time and the y-axis (not shown) clot firmness, in millimeters (mm). R is the time to initial clot (fibrin) formation. K is the speed with which the clot strength reaches 20 mm. The α angle represents the kinetics of clot formation. The coagulation phase stops at the maximum amplitude (MA), and fibrinolysis begins.

This quantification is useful, in particular for monitoring hemostatic changes over time. If you order TEG frequently, however, and have access to the visual tracings, it is worthwhile to become familiar with the distinct graphical patterns associated with a range of coagulation deficits (Fig. 10).

Notes on the Interpretation of the Thromboelastograph

R, the rate or reaction time, is the time from the addition of blood to the analyzer cuvette to the start of fibrin formation. R is prolonged by anticoagulants and is shortened by hypercoagulable states.

Parameter	Description	Reference range
R	Rate: time to initial fibrin formation (CF = 2 mm)	3.7 to 8.6 min
K	Clot kinetics: time to reach CF 20 mm from 2 mm	0.9 to 2.6 min
α angle	Clot strengthening: rate of clot strengthening	57.4 to 75.6°
MA	Maximum amplitude (strength) of clot	52.2 to 74.9 mm
LY30	Lysis: rate of amplitude change at 30 minutes after MA	0 to 10.2%

Table 19. TEG parameters provided in the test report. CF, clot firmness. Keep in mind that rapid TEG uses different reference ranges.

K measures the speed to reach a clot strength of 20 mm, while the α angle represents the rapidity of fibrin buildup and cross-linking. K and the α angle measure similar information, although α is more comprehensive. Each is affected by the availability of fibrinogen, which determines the rate of clot buildup; factor XIII, which enables the cross-linking of fibrin to form a stable clot; and, to a lesser extent, platelets. Since factor XIII is rarely deficient, a prolonged K with reduced α almost always represents low fibrinogen and can be corrected by administering cryoglobulin or fresh frozen plasma. K is prolonged by anticoagulants that affect fibrinogen and platelet function, while α is decreased by such anticoagulants.

The maximum amplitude (MA), which represents the ultimate strength of the clot, is a function of the dynamic properties of fibrin and platelet bonding. In contrast to K/α, the MA is affected more by the platelet count than the fibrinogen level. However, the MA and K/α are correlated through the fibrin-platelet bonding needed to produce the final clot. Because platelets are affected by most—if not all—cardiac surgeries, in these patients, a coagulopathy with small MA could be corrected by transfusion of platelets alone.

The LY30 is the rate of reduction of the amplitude as measured at 30 minutes after the MA. This variable represents the ultimate stability of the clot. An LY30 greater than 7.5% suggests hyperfibrinolysis.

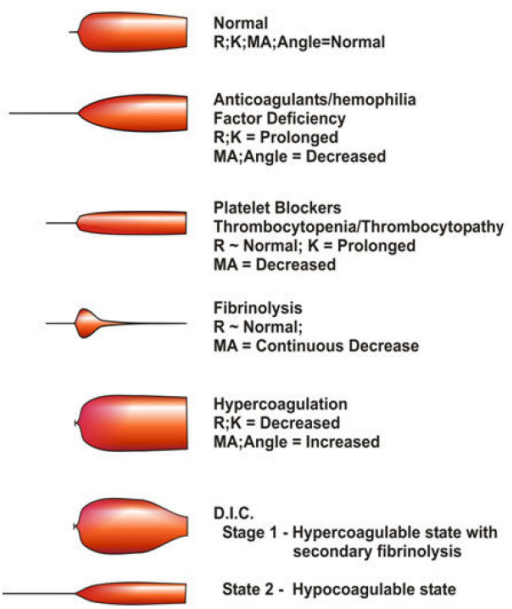

Fig. 10. Common TEG patterns. Many articles have been published about thromboelastography and the meaning of certain patterns. This image is an adaptation from the original package insert.

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Index

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