# Laboratory Analysis of Coagulation

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## List of Abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ACA | Anticardiolipin antibody |
| ADP | Adenosine diphosphate |
| APA | Antiphospholipid antibody |
| APC | Activated protein C |
| APC-R | APC resistance |
| APS | Antiphospholipid syndrome |
| aPTT | Activated partial thromboplastin time |
| AR | Autosomal recessive |
| AS | Allele-specific |
| ASA | Aspirin (acetyl salicylic acid) |
| AT | Antithrombin |
| ATP | Adenosine triphosphate |
| B2GPI | Beta2 glycoprotein 1 |
| BT | Bleeding time |
| BU | Bethesda unit |
| C4bBP | C4b-binding protein |
| CAP | College of American Pathologists |
| CLIA | Clinical Laboratory Improvement Amendments |
| COX1 | Cyclooxygenase 1 |
| CT | Closure time |
| DIC | Disseminated intravascular coagulation |
| DRVVT | Dilute Russell's viper venom test |
| DTI | Direct thrombin inhibitor |
| DVT | Deep vein thrombosis |
| ELISA | Enzyme-linked immunosorbent assay |
| ELT | Euglobulin lysis time |
| EM | Electron microscopy |
| ET | Essential thrombocythemia |
| FDP | Fibrin degradation product |
| FII | Prothrombin |
| FIIa | Thrombin |
| FVIIa | Activated factor VII |
| FVIII | Factor VIII |
| FVL | Factor V Leiden |
| FRET | Fluorescence resonance energy transfer |
| GP | Glycoprotein |
| HMWK | High-molecular-weight kininogen |
| INR | International normalized ratio |
| ISI | International sensitivity index |
| ISTH | International Society for Thrombosis and Haemostasis |
| LA | Lupus anticoagulant |
| LMW | Low molecular weight |
| MPN | Myeloproliferative neoplasms |
| MPV | Mean platelet volume |

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|---|---|
| MTHFR | Methylenetetrahydrofolate reductase |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| PAI | Plasminogen activator inhibitor |
| PCR | Polymerase chain reaction |
| PDW | Platelet distribution width |
| PE | Pulmonary embolism |
| PFA | Platelet function analyzer |
| PK | Prekallikrein |
| PRP | Platelet rich plasma |
| PT | Prothrombin time |
| RFLP | Restriction fragment length polymorphism |
| RIPA | Ristocetin-induced platelet aggregation |
| RT | Reptilase time |
| SLE | Systemic lupus erythematosus |
| SNP | Single nucleotide polymorphism |
| TAFI | Thrombin-activatable fibrinolysis inhibitor |
| TAR | Thrombocytopenia with absent radii |
| TF | Tissue factor |
| TFPI | Tissue factor pathway inhibitor |
| TM | Thrombomodulin |
| tPA | Tissue plasminogen activator |
| TT | Thrombin time |
| $TxA_2$ | Thromboxane $A_2$ |
| uPA | Urokinase plasminogen activators |
| VTE | Venous thromboembolism |
| VWD | von Willebrand disease |
| VWF | von Willebrand factor |
| XR | X-linked recessive |

## Introduction of Hemostasis and Thrombosis

The goal of physiologic hemostasis is to stop any bleeding that occurs and, ultimately, to return the vessel wall back to its original state. This is achieved through a dynamic interaction of pro- and anticoagulant elements. Early studies of hemostasis focused primarily on the process of clot formation. Originally described as a coagulation "cascade," the model for in vivo hemostasis subsequently evolved to incorporate the more complex contributions of elements beyond the traditional coagulation factors (Roberts et al. 1998; Hoffman and Monroe 2001; Schmaier and Miller 2011). Although it is now well established that the classic coagulation cascade does not accurately depict in vivo events, it remains particularly relevant with regard to understanding the in vitro process of hemostasis reflected by widely used coagulation screening tests such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT).

## Physiology of Hemostasis

Following an insult to the vascular wall, hemostasis is initiated by platelet adhesion at the site of injury. This is followed by platelet aggregation and degranulation, with release of multiple mediators and procoagulant factors by the activated platelets. At the same time, tissue factor expressed at the site of injury initiates serial activation of coagulation factors. These events culminate in the formation of a fibrin thrombus which incorporates the activated platelets into its structure. In order to prevent the clot from growing uncontrollably, antithrombotic mechanisms are activated to maintain the balance of pro- and anticoagulant processes. Clot remodeling by fibrinolysis occurs over time, while cellular elements move in to repair the underlying tissue damage. The remainder of the clot is eventually eliminated and vascular patency and integrity restored. Thrombin plays a key role in virtually every step of the hemostatic process. Derangements of one or more pro- or anticoagulant elements of hemostasis may result in an increased risk of bleeding, an increased risk of clotting, or, rarely, both.

### Initiation of Hemostasis by Platelet Plug Formation

The role of platelets in hemostasis and laboratory evaluation of platelet function are discussed in section of this chapter.

### Initiation and Propagation of Clotting Through Activation of Coagulation Factors

Clotting factors are proenzymes or inactive precursor proteins (zymogens), enzyme cofactors, and substrates that are sequentially activated to form a fibrin clot. All of these factors are made

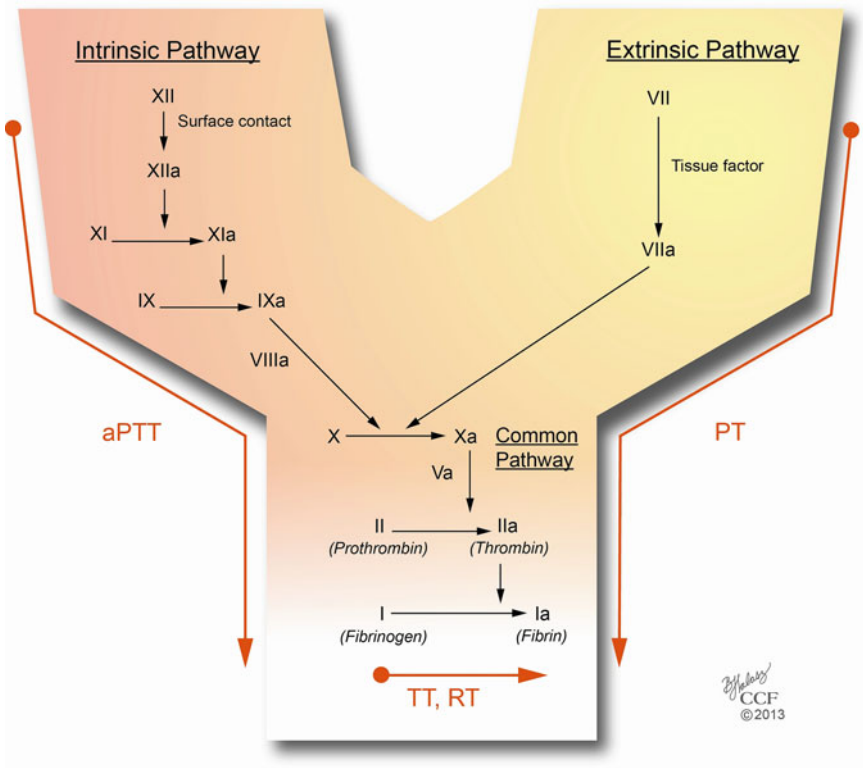


**Fig. 1.1** Classic coagulation cascade. This model of coagulation depicts extrinsic, intrinsic, and common pathways of coagulation. Calcium ions and phospholipids, which are not depicted for simplicity, are necessary cofactors in several steps of this process (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013. All Rights Reserved). *aPTT* activated partial thromboplastin time, *PT* prothrombin time, *TT* thrombin time, *RT* reptilase time

in the liver by hepatocytes, except for factor VIII (FVIII) which may be made by the reticuloendothelial system (Shovlin et al. 2010; Schmaier and Miller 2011). Some of the procoagulant factors (II, VII, IX, and X) undergo vitamin K-dependent gamma-carboxylation, which allows them to bind to the phospholipid surfaces where they are activated. Nutritional vitamin K deficiency and oral anticoagulation with warfarin, a vitamin K antagonist, anticoagulate by disrupting this process (Ageno et al. 2012; Schmaier and Miller 2011).

## Classic Coagulation Cascade

The classic coagulation cascade (Fig. 1.1) illustrates intrinsic and extrinsic pathways of clotting which converge in a common pathway ending in clot formation. The extrinsic pathway, which is assessed by the PT, starts with activation of FVII by tissue factor (TF), followed by direct activation of the common pathway by activated factor VII (FVIIa). The intrinsic pathway, which is assessed by the aPTT, starts with activation of the contact factor XII, followed by a cascading activation of factors XI then IX. Activated factor VIII serves as a cofactor for activation of the common pathway by FIXa. Once the common pathway is initiated by activation of FX by either FVIIa or FIXa, activated factor V serves as a cofactor for FXa to activate prothrombin (FII) to thrombin (FIIa), which in turn cleaves fibrinogen (factor I) to fibrin. Calcium is a necessary cofactor for nearly all of the above steps, while phospholipid is required for activation events in the intrinsic pathway and for activation of FII (Mann 2003; Hoffman and Monroe 2007; Schmaier and Miller 2011; Leung 2013).

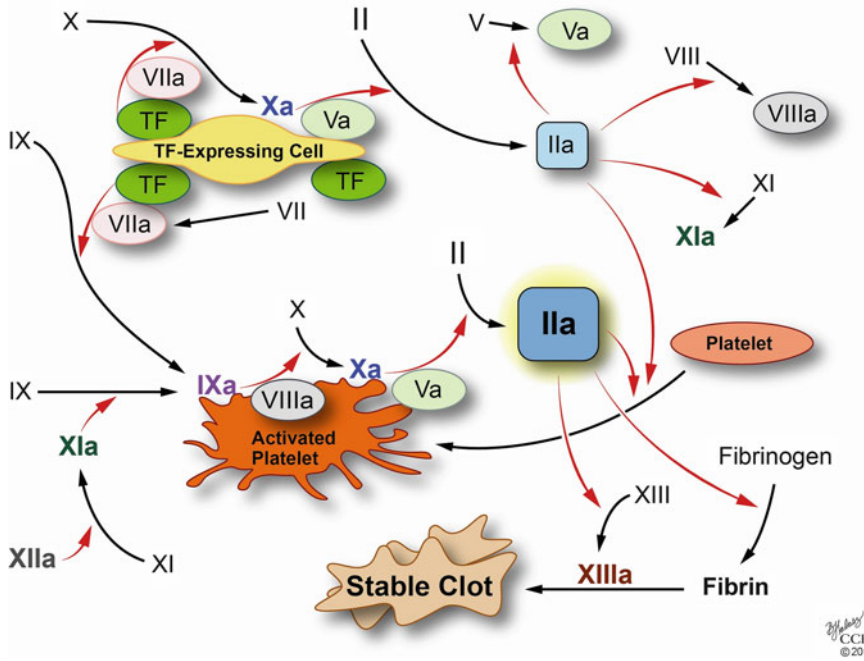


**Fig. 1.2** Cell-based model of coagulation. This model of coagulation incorporates some of the cellular elements involved in coagulation and better reflects the complexity and interdependence of the elements of in vivo coagulation. Calcium ions, which are not depicted for simplicity, are necessary cofactors in several steps of this process. In the cell-based model of coagulation, FVII is activated to FVIIa by tissue factor (TF). The TF–FVIIa complex activates FX to FXa, which together with its cofactor FVa activates prothrombin (FII) to thrombin (FIIa). In addition to activating factors V, VIII, and XI, the FIIa generated by this mechanism also activates platelets. Factor IX is activated by both the TF–FVIIa complex and FXIa. Together with its cofactor FVIIIa, FIXa activates FX on the surface of activated platelets. FXa, together with its cofactor FVa, activates prothrombin (FII) to thrombin (FIIa). The thrombin (FIIa) generated at this point converts fibrinogen (FI) to fibrin (FIa) and factor XIII to the clot-stabilizing FXIIIa. The end result of this process is a stable, cross-linked polymerized fibrin clot. *Black arrows* indicate transition of inactivated factors to their activated forms. *Red arrows* indicate an activating effect, with cofactor contribution by the factors tangential to the *red arrows* (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013. All Rights Reserved)

## Cell-Based Model of Coagulation

Activation of circulating FVII to FVIIa by tissue factor is the primary initiator of the coagulation cascade in both the classic and cell-based models of coagulation. However, the cell-based model of coagulation better reflects the complexity and interdependence of the in vivo events resulting in clot formation (Fig. 1.2).

Tissue factor (TF) is a transmembrane glycoprotein expressed in a variety of cells which are not typically in direct contact with the blood flow, including vascular smooth muscle cells, adventitial fibroblasts, and pericytes. Endothelial cells do not normally express TF. In the event of an injury to a vascular wall resulting in endothelial damage or disruption, expression of TF is increased and TF-bearing cells are exposed to circulating blood. TF activates FVII to FVIIa (Rapaport and Rao 1995; Hoffman and Monroe 2007; Breitenstein et al. 2009; Leung 2013).

The TF–FVIIa complex activates FX to FXa. Activated factor V acts as a cofactor for FXa to activate prothrombin (FII) to thrombin (FIIa). The FVa needed for this process may be released directly from activated platelets or activated by FXa or non-coagulation proteases. The small amount of thrombin generated by TF–FVIIa activation stimulates upregulation of TF expression and activates platelets, resulting in exposure of the platelet phospholipid surfaces needed

for assembly of intrinsic factor activating complexes. Thrombin also directly activates factors V, VIII, and XI, which, together with activation of factor IX to FIXa by the TF–FVIIa complex, facilitates clotting through the intrinsic pathway. The hemostatic response is markedly amplified at this point given the ability of FIXa to diffuse to adjacent platelet surfaces, as opposed to the FXa generated by the TF–FVIIa complex which is localized to TF-expressing cell due to inhibition of FXa by antithrombin (AT) and tissue factor pathway inhibitor (TFPI) (Mann 2003; Hoffman and Monroe 2007; Schmaier and Miller 2011; Leung 2013).

Once activated, FVIIIa and FIXa quickly become localized to the surface of activated platelets and activate FX. The prothrombinase complex, consisting of FXa and its cofactor FVa, is a very potent thrombin generator in the common pathway. The enhanced thrombin generation by this mechanism results in conversion of fibrinogen to fibrin. The fibrin monomers undergo polymerization and the clot is then cross-linked and stabilized by FXIII, which is also activated by thrombin (Mann 2003; Hoffman and Monroe 2007; Schmaier and Miller 2011; Leung 2013).

FXII (Hageman factor) autoactivates in association with negatively charged surfaces, such as exposed collagen at the site of a vascular injury and polysomes released by activated platelets. FXII, prekallikrein (PK or Fletcher factor), and high-molecular-weight kininogen (HMWK, Fitzgerald, or Williams factor) comprise the contact system which can also activate FIX. The contact system factors are redundant in vivo; deficiencies in these factors are not associated with bleeding but may instead be associated with an increased risk of thrombosis (Gallimore et al. 2004; Girolami et al. 2011; Schmaier and Miller 2011).

Binding of thrombin to thrombomodulin (TM) induces a conformational change in thrombin whereby it ceases to activate platelets and cleave fibrinogen. The thrombin–TM complex activates protein C to decelerate the clotting process. In addition to its role in slowing down the clotting process, the thrombin–TM complex also activates thrombin-activatable fibrinolysis inhibitor (TAFI) to further stabilize the clot and protect it from rapid lysis by plasmin (Hoffman and Monroe 2007; Schmaier and Miller 2011; Leung 2013).

In summary, although the classic coagulation cascade might imply that the extrinsic and intrinsic pathways are redundant, the cell-based coagulation model makes it clear that they are not. Extrinsic pathway activities are limited to TF-expressing surfaces and result in initiation of clotting and activation of the platelets and coagulation factors needed for amplification of the hemostatic response. On the other hand, intrinsic pathway activities take place on the phospholipid surface of the activated platelets and generate the thrombin burst which facilitates formation and stabilization of the fibrin clot. Thus, the intrinsic and extrinsic coagulation pathways each play a unique and vital role in achieving hemostasis (Hoffman and Monroe 2007).

## Termination of Clotting by Antithrombotic Mechanisms

The three main antithrombotic mechanisms involved in terminating clotting are tissue factor pathway inhibitor (TFPI), antithrombin (AT), and activated protein C (APC). Deficiencies of these natural anticoagulants, or their cofactors, can result in an increased risk of thrombosis. The function of natural anticoagulants is not captured by coagulation screening tests such as the PT and aPTT.

TFPI is the most potent inhibitor of TF–FVIIa complex and inhibits TF–FVIIa by forming a quaternary complex with FVIIa, TF, and FXa (Breitenstein et al. 2009; Leung 2013). Although AT inhibits most of the activated coagulation factors, including thrombin (FIIa) and factors IXa, Xa, XIa, and XIIa, it exerts its primary effect through inhibition of factors IIa and Xa. Endogenous and exogenous heparin and heparin-like substances can significantly potentiate the anticoagulant effect of AT, in some cases by 1,000-fold or greater (Schmaier and Miller 2011; Leung 2013). The structure of the different types of heparin plays a role in determining their effect through interaction with AT; for example, unfractionated heparin exerts its primary anticoagulant effect through AT-mediated inactivation of FIIa, whereas low molecular weight (LMW) heparins exert their primary anticoagulant effect through

AT-mediated inactivation of FXa (Garcia et al. 2012). The thrombin–TM complex activates protein C, which, in association with its cofactor protein S, inactivates factors Va and VIIIa. Activated protein C (APC) has also been found to play a role in other associated processes including inflammation and stimulating fibrinolysis (Schmaier and Miller 2011; Griffin et al. 2012; Leung 2013).

### Clot Lysis

After hemostasis is achieved, it is important to remove the clot and restore the patency of the blood vessel as part of the wound healing process. Tissue plasminogen activator (tPA) converts fibrin-bound plasminogen to plasmin which cleaves the fibrin strands releasing fibrin degradation products (FDPs). D-dimer is a major FDP consisting of two D domains from adjacent fibrin monomers that had been cross-linked by FXIIIa. tPA is primarily responsible for initiating intravascular fibrinolysis, while urokinase plasminogen activators (uPA) perform this function in the extravascular compartment. Plasminogen activator inhibitors (PAI-1 and PAI-2) inhibit tPA and uPA, while alpha-2-antiplasmin inhibits plasmin (Hoffman and Monroe 2007; Schmaier and Miller 2011; Leung 2013). To further maintain the balance of pro- and antifibrinolytic processes, thrombin, plasmin, and the thrombin–TM complex may all activate thrombin-activatable fibrinolysis inhibitor (TAFI) to TAFIa, which inhibits fibrinolysis and protects the clot from premature degradation by plasmin (Hoffman and Monroe 2007; Schmaier and Miller 2011; Colucci and Semeraro 2012; Leung 2013).

The activity of pro- and antifibrinolytic factors is not captured by coagulation screening tests such as the PT and aPTT, or even by the thrombin time. The euglobulin lysis time (ELT) can be used as a screening test to assess global fibrinolytic function; assays for individual factors may also be performed. The ELT is expected to be shortened in hyperfibrinolysis and may be prolonged with hypofibrinolysis. However, the usefulness of ELT is limited by its relative insensitivity and a broad variation in results among normal individuals (Glassman et al. 1993).

## Laboratory Assays for Evaluation of Coagulation Disorders

### Commonly Used Laboratory Assays Related to Hemostasis

The most widely used screening tests of coagulation function are the prothrombin time (PT), the international normalized ratio (INR), and the activated partial thromboplastin time (aPTT). Unless otherwise specified, samples used for coagulation testing are collected in 3.2 % sodium citrate (light blue top) test tubes. The anticoagulant effect of citrate is exerted by chelating calcium, which is a required cofactor for most steps in the hemostatic process (Adcock et al. 1998).

### Prothrombin Time (PT) and International Normalized Ratio (INR)

The PT assesses the extrinsic and common pathways (factors VII, X, V, II, and I) (Fig. 1.1). Patient plasma is incubated for a short time with thromboplastin, a source of phospholipid and tissue factor, and then recalcified. The PT is the time (in seconds) that it takes to form a fibrin clot after adding the calcium.

Variable prolongation of the PT was noted in patients on warfarin therapy depending on the thromboplastin reagent and test system used (Bailey et al. 1971). The INR was conceived to provide a standardized approach to therapeutic monitoring of warfarin, whereby an international sensitivity index (ISI) is determined by the manufacturer for each reagent/test system combination relative to the international standard for thromboplastin. The INR is then calculated as follows:

$$\text{INR} = (\text{Patient PT}/\text{Mean normal PT for the laboratory})^{\text{ISI}}$$

(Ageno et al. 2012). The INR has only been validated for patients on oral anticoagulant therapy with a vitamin K antagonist, in other words, those in whom only the vitamin K-dependent factors are expected to be decreased (Loeliger et al. 1985). The widespread adoption of the INR as a general indicator of coagulation function, and its incorporation into the model for end-stage liver disease (MELD) scoring system for prioritization

of patients for liver transplant, should be viewed with caution as the INR is not reliably reproducible across reagents, tests systems, and laboratories in settings other than vitamin K deficiency or antagonism. For example, patients with liver disease tested by different reagents or laboratories may have enough variation in their INR results to impact their MELD score without having had any significant change in their underlying condition (Sermon et al. 2010). In addition, utilizing the INR as a presumptive marker of coagulation hypofunction in patients with liver disease can be particularly misleading given the complexity of their underlying hemostatic derangements (Tripodi et al. 2011).

Also of note, as a PT-derived parameter, the INR may not be reliable for monitoring warfarin therapy in patients with lupus anticoagulants that are associated with a baseline prolongation of the PT. Although PT reagents are typically selected to be insensitive to the effect of lupus anticoagulants, some lupus anticoagulants and antiphospholipid antibodies with antiprothrombin activity may prolong the PT. Retesting the sample using a different PT reagent and performing factor II assays can be diagnostically helpful (Tripodi et al. 2001; Mazodier et al. 2012). Alternative tests that are insensitive to the effect of lupus anticoagulants, such as a chromogenic factor X assay, may be used to monitor the warfarin anticoagulant effect in such cases (Moll and Ortel 1997; Kasthuri and Roubey 2007).

### Activated Partial Thromboplastin Time (aPTT)

The aPTT assesses the intrinsic and common pathways (factors XII, XI, IX, VIII, X, V, II, and I) (Fig. 1.1). Patient plasma is incubated for a short time with a partial thromboplastin, which is a tissue factor-free source of phospholipid, and a negatively charged surface, such as kaolin or silica, which can activate the contact factors. The sample is then recalcified. The aPTT is the time (in seconds) that it takes to form a fibrin clot after adding the calcium.

aPTT reagents differ in their sensitivity to heparin, coagulation factor deficiencies, and lupus anticoagulants (Bowyer et al. 2011; Fritsma et al. 2012; Gouin-Thibaut et al. 2012). Two main methods of clot detection, mechanical and optical, are used in coagulation testing with no clear advantage to one method over the other (McCraw et al. 2010). In some cases it may be necessary to recheck questionable aPTT (or other coagulation test) results by retesting the sample by both methods. For example, occasionally, an otherwise unexplained result of an aPTT that appears to be prolonged beyond the limit of detection actually reflects a markedly shortened aPTT. This occurs when the initial testing is performed using an optical detection method. Because the sample had already clotted before the optical method, which relies on a change in light transmittance, set its baseline, no change in light transmittance occurred over time and the result was erroneously interpreted as an aPTT prolonged beyond the limit of detection. In this case, the true shortened aPTT can be revealed by using a mechanical clot detection method (Milos et al. 2010).

### Thrombin Time (TT) and Reptilase Time (RT)

The TT measures the time (in seconds) that it takes to convert fibrinogen to fibrin after adding exogenous thrombin to the patient sample (Fig. 1.1). The RT also measures the time it takes to convert fibrinogen to fibrin but differs from the TT in that the activation is thrombin independent and relies instead on an enzyme derived from a snake's venom (Zehnder 2013). Both the TT and RT are prolonged by fibrinogen abnormalities such as hypofibrinogenemia and dysfibrinogenemia, whereas the TT is prolonged and the RT is normal in the presence of unfractionated heparin or a direct thrombin inhibitor (DTI). LMW heparin does not typically prolong the TT or the RT. Additionally, the TT may be prolonged by high concentrations of FDPs and shortened in the presence of plasma volume expanders such as dextran and hydroxyethyl starch (Cunningham 2008).

### Mixing Studies

aPTT mixing studies are utilized more frequently than PT mixing studies but all mixing studies share a common principle: the patient sample is mixed with normal plasma at a ratio of 1:1 and

the test of interest is performed. If the prolonged test "corrects" into the normal reference range, the result suggests a deficiency of one or more coagulation factors in the patient plasma. If the prolonged test does not correct, the result suggests the presence of an inhibitor. Weak inhibitors, however, can be diluted by the mixing study resulting in an apparent "correction" of the test (Moore and Savidge 2006). Additionally, significant deficiencies of multiple coagulation factors may not be completely corrected by the addition of normal plasma in a 1:1 ratio.

Mixing studies may be assessed at immediate and delayed phases. The delayed or time- and temperature-dependent phase reflects a period of incubation at 37° Celsius for 1–2 h prior to test performance. An immediate acting inhibitor typically suggests the presence of heparin, a DTI, or a nonspecific inhibitor such as a lupus anticoagulant. Specific factor inhibitors (such as a factor VIII inhibitor) may be associated with complete or partial correction at the immediate phase and a marked inhibitor effect at the delayed or time-dependent phase (Verbruggen et al. 2009).

### Coagulation Factor Assays

Coagulation factors can be assessed by antigenic/immunologic or functional tests. It is usually most appropriate to test a factor's functional activity first and perform subsequent antigenic/immunologic testing only if there is clinical suspicion of qualitative factor abnormalities, which are far less common than quantitative factor deficiencies. The functional activity of a factor is typically determined by its ability to correct the clotting time of a standard plasma deficient in only the factor of interest (Mackie et al. 2013).

Similar to the aPTT, clot-based factor assays are subject to interference by inhibitors such as heparin, DTIs, and lupus anticoagulants. Normally, serial dilution of a patient sample is expected to result in decreased levels of factor activity with each dilution; however, in the presence of an inhibitor, a dilutional effect is noted whereby the factor activity level increases, rather than decreases, with dilution because of dilution of the inhibitor effect (Mackie et al. 2013).

### Bethesda Assay

Although most commonly used to evaluate the potency of factor VIII inhibitors, the Bethesda assay can be used to assess the magnitude of the effect of any specific factor inhibitor by measuring the ability of the patient's plasma to neutralize the factor of interest in normal plasma. By definition, one Bethesda unit (1 BU) is the quantity of inhibitor that neutralizes 50 % of the factor of interest in normal plasma. Since the Bethesda assay is clot based, it is subject to interference by lupus anticoagulants, heparin, and DTIs, the presence of which may overestimate the effect of the specific factor inhibitor under study (Verbruggen 2010; Kershaw and Favaloro 2012).

## Preanalytic Variables and Other Test Considerations

Coagulation tests are particularly sensitive to the effects of preanalytic variables. Extra care should be taken when collecting samples through indwelling lines due to the risk of contamination with heparin or other medications (Preston et al. 2010; Mackie et al. 2013). Sample collection tubes should be completely filled to ensure an appropriate ratio of plasma to anticoagulant (Adcock et al. 1998; McCraw et al. 2010; Preston et al. 2010; Mackie et al. 2013). Polycythemic patients with a hematocrit >55 % require an adjustment of the amount of anticoagulant in the test tube to avoid obtaining spuriously prolonged clotting times given the relatively low plasma to anticoagulant ratio in this case (Marlar et al. 2006; Preston et al. 2010; Mackie et al. 2013). Other considerations include handling, transport and storage conditions, and temperatures. Thawing and mixing of frozen samples prior to testing should also be performed with caution (McCraw et al. 2010; Preston et al. 2010; Mackie et al. 2013). Severely hemolyzed, turbid, or lipemic samples may impact coagulation test results, particularly when optical clot detection methods are used (Laga et al. 2006; Preston et al. 2010; Mackie et al. 2013).

## Effect of Anticoagulants on Coagulation Assays

Anticoagulants can significantly affect the results of coagulation assays. Some of these effects are consistent and may aid in therapeutic drug monitoring; others merely complicate the laboratory evaluation of the patient's underlying hemostatic function. The effect of warfarin, heparin, and DTI on coagulation assays is relatively well established, whereas the effect of several new anticoagulants is still being evaluated (Favaloro et al. 2011).

The PT and INR are the first to prolong with oral vitamin K antagonist therapy (Ageno et al. 2012); prolongation of the aPTT is also typically observed in established warfarin therapy, particularly in case of INR greater than 1.5. In this setting, prolonged PTs and aPTTs are expected to correct fully on mixing studies. Warfarin therapy does not affect the thrombin time (TT) and is not expected to affect coagulation factor assays for procoagulant factors other than those which are vitamin K dependent (factors II, VII, IX, and X).

Unfractionated heparin exerts its primary anticoagulant effect by potentiating antithrombin (AT) (Garcia et al. 2012). The presence of unfractionated heparin would therefore be expected to prolong all clot-based assays due to its impact on the common pathway. The aPTT and TT are indeed prolonged in the presence of unfractionated heparin; however, in clinical laboratory practice, the PT appears to be unaffected by unfractionated heparin. This is because PT reagents contain a heparin-neutralizing agent that eliminates the heparin effect in nearly all cases. Valid results by other clot-based assays may also be obtained after treatment of the sample with a heparin-neutralizing agent, such as an enzyme or absorbing resin, as long as the heparin concentration in the sample does not exceed the neutralizing capability of these agents.

LMW heparins typically exert their primary anticoagulant effect through inhibition of FXa. The aPTT is variably prolonged in the presence of LMW heparins, whereas the PT and TT are typically unaffected by its presence. Heparin-neutralizing agents tend to be more effective at neutralizing unfractionated heparin than they are at neutralizing LMWH, both in vivo and in vitro (Garcia et al. 2012).

Oral and parenteral DTIs are currently available (Ageno et al. 2012; Garcia et al. 2012). DTIs act on thrombin and are associated with marked prolongation of the TT and variable prolongation of the PT and aPTT. DTIs have also been reported to interfere with several other coagulation assays, including mixing studies, factor assays, Bethesda assays, and tests for lupus anticoagulant. At the present time, no in vivo or in vitro DTI-neutralizing agents have been identified (Dager et al. 2012; Halbmayer et al. 2012; Adcock et al. 2013).

## General Approach for Evaluation of Prolonged PT and/or aPTT Results

Prolongations of the PT and aPTT tend to garner the most amount of attention in clinical practice. Table 1.1 illustrates a broad approach to the differential diagnosis of various combinations of normal and prolonged PT and aPTT test results.

Shortened PT and aPTTs are evaluated less frequently than prolonged ones. In most cases, shortened PTs and aPTTs are noted in clinical settings physiologically associated with increased coagulation factor levels, such as pregnancy and acute phase reactions. Shortened PTs and/or aPTTs may also be seen in some cases of dysfibrinogenemia. A shortened aPTT is not currently considered to be a definitive indicator of hypercoagulability, but both shortened aPTTs and increased levels of some coagulation factors, including FVIII and FIX, which can cause a shortened aPTT, have been associated with an increased risk of thrombosis (Tripodi et al. 2004; Jenkins et al. 2012).

## Laboratory Assays for Evaluation of Hypercoagulability

Hypercoagulable status, also called *thrombophilia*, is described as a group of hereditary or acquired conditions with the propensity to

**Table 1.1** General approach to the differential diagnosis of combinations of normal and prolonged PT and aPTT results

| PT | aPTT | aPTT mixing study | Diagnostic possibilities |
|---|---|---|---|
| Normal | Normal | Not indicated | Normal |
| | | | Coagulation abnormalities not assessed by the PT and aPTT (e.g., factor XIII deficiency) |
| Prolonged | Normal | Not indicated | Factor VII deficiency |
| | | | Mild deficiencies of common pathway factors (X, V, II, fibrinogen) |
| | | | Early warfarin/vitamin K deficiency effect, early liver disease, early disseminated intravascular coagulation (DIC) |
| Prolonged | Prolonged | Corrects | Congenital deficiency of a single coagulation factor in the common pathway, or combined deficiencies of factors in the common and/or intrinsic and extrinsic pathways |
| | | | Acquired deficiency of a single factor in the common pathway (e.g., factor X deficiency in amyloidosis) |
| | | | Acquired deficiencies of multiple coagulation factors (e.g., established warfarin/vitamin K deficiency effect, established liver disease, consumptive coagulopathy such as DIC, dilutional coagulopathy) |
| | | | Dysfibrinogenemia |
| | | | Weak inhibitor effect that is diluted out |
| | | Does not correct | Inhibitors: inhibitor of a specific coagulation factor in the common pathway (e.g., factor V inhibitor); direct thrombin inhibitor (DTI); lupus anticoagulant (rarely associated with prolonged PT); high heparin (very rarely associated with prolonged PT) or combined heparin and warfarin therapy |
| | | | Profound deficiency of multiple coagulation factors that is not completely corrected by mixing with normal plasma |
| Normal | Prolonged | Corrects | Deficiencies of one or more of the intrinsic pathway factors (XII, XI, IX, and VIII) or the contact factors (prekallikrein and high-molecular-weight kininogen) |
| | | | Weak inhibitor effect that is diluted out |
| | | Does not correct | Non-specific inhibitors: lupus anticoagulants; heparin; DTI |
| | | | An inhibitor of a specific factor in the intrinsic pathway (e.g., factor VIII inhibitor) |

**Table 1.2** Summary of risk factors for acquired and hereditary thrombophilia

| Acquired thrombotic risk factors | Hereditary thrombotic risk factors |
|---|---|
| Major surgery/trauma | Activated protein C resistance/factor V Leiden |
| Immobilization | Prothrombin gene G20210A mutation |
| Solid or hematologic malignancies (e.g., myeloproliferative neoplasm) | Protein C deficiency[a] |
| Pregnancy | Protein S deficiency[a] |
| Oral contraceptives | Antithrombin deficiency[a] |
| Estrogen replacement therapy | Hyperhomocysteinemia[a] |
| Limb immobilization (e.g., hip/knee replacement, prolonged cast, stroke) | Elevated factor VIII activity[a] |
| Bedridden due to illness | Dysfibrinogenemia[a] |
| Antiphospholipid antibody syndrome | |
| Heparin-induced thrombocytopenia | |
| Paroxysmal nocturnal hemoglobinuria | |
| Obesity | |
| Nephrotic syndrome | |
| Smoking | |

[a]Can be hereditary or acquired

develop venous thrombosis, arterial thrombosis, or both. Although acquired or hereditary thrombotic risk factors are not completely understood, the prevalence of factor V Leiden mutation, prothrombin gene G20210A mutation, elevated factor VIII, and hyperhomocysteinemia is higher than antithrombin, protein C, or protein S deficiencies in general population. The prevalence of thrombosis in individuals with a personal and/or family history of thrombosis is higher than in the general population. Acquired and hereditary risk factors for thrombophilia are summarized in Table 1.2 (Eby and Olson 2008a; Margetic 2010). Although patients with hypercoagulable risk factors are at a great risk for developing a thrombotic event, not all patients with hypercoagulable risk factors will develop an overt thrombosis and not all patients with thrombosis will have an identifiable hypercoagulable state (Kottke-Marchant 1994; Khor and Van Cott 2009; Margetic 2010).

Diagnostic thrombophilia testing is indicated in patients with idiopathic or recurrent venous thromboembolism (VTE), a first episode of VTE at a young age (<40 years), VTE in the setting of a strong family history, VTE in an unusual vascular site (cerebral, hepatic, mesenteric, or renal veins), neonatal purpura fulminans, warfarin-induced skin necrosis, and recurrent pregnancy loss (Heit 2007; Baglin et al. 2010). No single laboratory test is available to identify all hypercoagulable defects. Selection for thrombophilia testing differs depending on location and type of thrombosis. Many tests currently used to detect thrombophilia can be often affected by concurrent clinical conditions. In selected patients with thrombophilia, it is best to test for all recognized hereditary risk factors, both common and uncommon (Heit 2007; Eby and Olson 2008a; Middeldorp 2011). Figure 1.3 outlines a testing algorithm to maximize diagnostic potential in patients with thrombophilia. Testing should be performed in a stepwise manner beginning with high-yield screening tests followed by appropriate specific confirmatory tests. These comprehensive panels generate multiple test results which can each be affected by a variety of clinical conditions and drugs. Comprehensive narrative interpretation by coagulation specialists is necessary to synthesize the test results and correctly interpret them in the clinical context (Eby 2008b; Margetic 2010).

Appropriate timing for diagnostic thrombophilia testing is of critical importance. Tests should be performed at least 4–6 weeks after an acute thrombotic event or discontinuation of anticoagulant therapies including warfarin, heparin, DTIs, and fibrinolytic agents (Eby and Olson

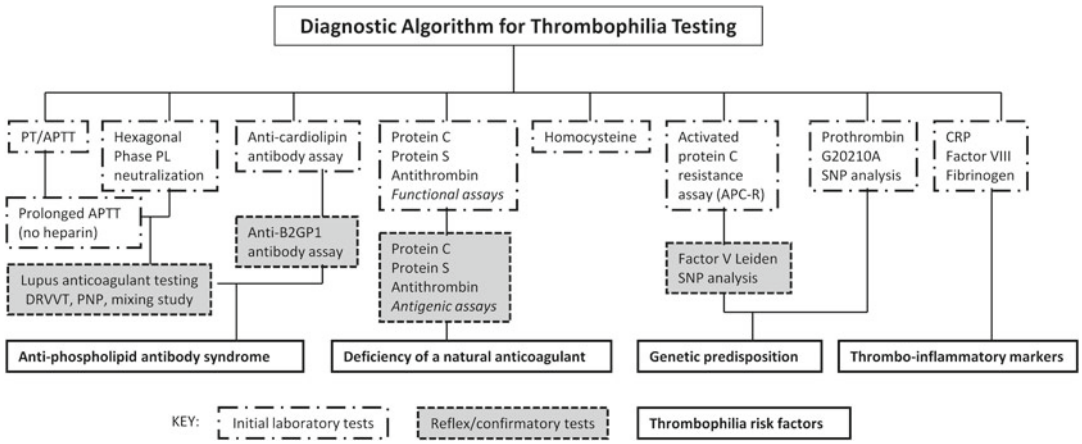


**Fig. 1.3** Comprehensive diagnostic interpretive panel of laboratory tests for thrombophilia (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013. All Rights Reserved). *aPTT* activated partial thromboplastin time, *B2GP1* β2 glycoprotein 1, *CRP* C-reactive protein, *DRVVT* dilute Russell's viper venom test, *PL* phospholipid, *PNP* platelet neutralization procedure, *PT* prothrombin time, *SNP* single nucleotide polymorphism

2008a; Heit 2007; Khor and Van Cott 2009). Acute thrombosis can cause elevation of acute phase reactants including factor VIII, C-reactive protein, beta chain of C4b-binding protein (C4bBP), fibrinogen, and IgM anticardiolipin antibodies. Warfarin therapy can lower protein C and protein S activity levels. Unfractionated or LMW heparins can affect antithrombin and lupus anticoagulant assays in addition to aPTT- and clot-based assays. Protein C, protein S, and antithrombin levels can be affected by liver dysfunction. Combined deficiency of protein C, protein S, and antithrombin can be observed in a consumptive coagulopathy, disseminated intravascular coagulation (DIC), liver disease, after recent thrombotic event, during the postoperative state, or with implantation of cardiovascular devices such as ventricular assist devices. Information about clinical conditions such as liver disease, pregnancy, or systemic inflammation should be provided to the laboratory to assure accurate test interpretation. Abnormal results should be repeated in a new specimen when the patient is in stable health and after anticoagulant therapy is discontinued. Alternatively, thrombophilia testing may be delayed until those clinical conditions have subsided. The one exception is DNA analysis for genetic mutations, which is not affected by anticoagulant therapy.

## Activated Protein C Resistance and the Factor V Leiden Mutation

Activated protein C (APC) degrades activated coagulation factors Va and VIIIa in the presence of its cofactor protein S. APC resistance (APC-R) is observed in approximately 20 % of patients with first episode of deep vein thrombosis (DVT) and 50 % of familial thrombosis. Greater than 90 % of APC-R patients have a point mutation in the factor V gene, known as factor V Leiden (FVL) mutation (Rosendaal et al. 1995; Zivelin et al. 1997; Margetic 2010). FVL mutation causes a DNA polymorphism (G1691A) substituting amino acid arginine to glutamine at position 506 (R506Q), one of the three arginine sites (R306, R506, and R679) cleaved by APC (Eby and Olson 2008a). FVL is present in heterozygous form in approximately 3–5 % of the general Caucasian population and is rare in African, Australian, or South Asian populations (Ricker et al. 1997; Margetic 2010). The FVL mutation is the most common known hereditary risk factor for venous thrombosis. However, its risk in arterial thrombosis is not yet clear. The risk of venous thrombosis is increased four- to eightfold in individuals heterozygous for FVL and 80-fold in homozygotes (Greengard et al. 1994; Baglin et al. 2010). This thrombotic risk is further

increased in the presence of a second risk factor. Women with FVL mutation (heterozygous) using oral contraceptives appear to have a 30- to 60-fold increased risk of thrombosis. Some studies show that the risk of pulmonary embolism (PE) is not as great as the risk of DVT in FVL mutation patients (Rosendaal et al. 1995). In addition to FVL mutation, various less common FV mutations including FVR2 haplotype (H1299R), FV Liverpool (I359T), FV Cambridge (R306T), and FV Hong Kong (R306G) also affect APC resistance and thrombotic risk (Chan et al. 1998; Franco et al. 1999; Norstrom et al. 2002).

Acquired APC-R can be caused by development of autoantibodies against factor V following exposure to bovine thrombin, or with untreated hematological malignancies, lupus anticoagulants, pregnancy, oral contraceptives, active thrombosis, elevated factor VIII, and mutations in the factor VIII gene (Khor and Van Cott 2009; Margetic 2010).

Laboratory assays for APC-R and FVL mutation include functional assays and genotyping for FVL by DNA analysis. The functional assay for APC-R is based on prolongation of aPTT by degradation of factors Va and VIIIa by exogenously supplied APC. The ratio of aPTT in patient plasma and normal plasma before and after adding APC is calculated. The ratio in normal individuals is 2.0 or higher; heterozygous individuals for FVL mutation will have ratio of 1.5–2.0, and homozygous individuals will have ratio of less than 1.5. Each laboratory should determine its own cutoff for an abnormal result (Khor and Van Cott 2009; Yohe and Olson 2012). Elevated factor VIII, low protein S (less than 20 %), and causes of prolonged baseline aPTT such as heparin, warfarin, DTI, lupus anticoagulant, liver dysfunction, or low factor levels can cause a falsely low APC-R ratio. A second generation assay for functional APC-R uses pre-dilution of patient plasma with factor V-deficient plasma (also containing heparin neutralizer) and provides higher sensitivity and specificity. This assay is less affected by active thrombosis, surgery, inflammatory condition, heparin, or warfarin (Press et al. 2002). Identification of FVL mutation as the cause of APC-R is confirmed by DNA analysis such as polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) or allele-specific PCR (AS-PCR) genotyping. Non-PCR-dependent and simple microtiter plate-based Invader technology using fluorescence resonance energy transfer (FRET) mechanism shows a reliable detection rate for FVL mutation. However, due to the use of specific primers, this test method will only detect specific mutations, i.e., FVL mutation, and will not detect other rare FV mutations related to functional APC-R. In general, a cost-efficient functional assay for APC-R is recommended as an initial screening test, with DNA analysis for FVL mutation in individuals with abnormal APC-R results (Ledford et al. 2000; Murugesan et al. 2012).

## Prothrombin Gene G20210A Mutation

The prothrombin gene mutation is a gain of function mutation which results in elevated functional prothrombin (factor II) level due to increased synthesis. A mutation which changes guanine to adenine at the 20210 position of the PT gene (G20210A) occurs in an intron near the 3′ end of the gene. This alters mRNA formation by affecting 3′ end processing and/or enhancing translation efficiency, resulting in increased plasma protein levels (McGlennen and Key 2002). However, the exact mechanism of how increased prothrombin gene expression causes hypercoagulability remains unclear. The prothrombin gene G20210A mutation is the second most common hereditary risk factor for venous thrombosis (Eby and Olson 2008a; Margetic 2010). Prevalence varies by ethnic group; 2–4 % of Europeans carry the mutation, which is rare in Asians, native Americans, or Africans (Rosendaal et al. 1998; Ballard and Marques 2012; Yohe and Olson 2012). This mutation is present in approximately 1–3 % of the general population, 5–10 % of patients with venous thrombosis, and up to 20 % of patients with venous thrombosis from thrombophilic families. Heterozygous individuals show threefold increased risk of venous thrombosis. However, venous thrombosis risk will be

drastically increased when the patients carry additional inherited or acquired risk factors (Poort et al. 1996; Khor and Van Cott 2009; Margetic 2010).

The prothrombin gene testing to detect the 20210 single nucleotide polymorphism (SNP) can be performed by various PCR-based methods. A commonly utilized method is PCR followed by DNA sequencing by gel electrophoresis, restriction endonuclease digestion, and radioisotope probing. Technologic advances in molecular diagnostic testing have led to automated genotyping analyses based on various PCR methods coupled with fluorescence polarization methods or the Invader assay. DNA microarray technology can detect multiple genetic markers simultaneously using various DNA chip platforms with a relatively low cost as a single test compared to conventional DNA assays (McGlennen et al. 1992; Murugesan et al. 2012). The DNA analysis cannot be affected by other conditions such as active thrombosis, surgery, inflammatory conditions, or anticoagulant therapy. However, these tests require expensive equipment and skilled personnel, have the risk of contamination, and may require reflex confirmatory assays (e.g., sequencing) if there is an ambiguous or atypical pattern by PCR (Murugesan et al. 2012).

## Protein C Deficiency

Protein C is a vitamin K-dependent glycoprotein primarily synthesized as an inactive form by the liver (Khor and Van Cott 2010a). Activation of functional protein C requires interaction with the thrombin–thrombomodulin–endothelial protein C receptor complex and cofactor protein S. Protein C regulates thrombin generation by degradation of activated coagulation factors Va and VIIIa (Eby and Olson 2008a). Protein C deficiency occurs in 0.14–0.50 % of the general population and an estimated 1–3 % of patients with VTE (Khor and Van Cott 2009; Yohe and Olson 2012). It is inherited in an autosomal dominant fashion. Risk for venous thrombosis increases sevenfold in heterozygotes (Ballard and Marques 2012). Such individuals usually shows functional protein C levels of 40–65 % of normal. The first thrombotic event usually presents between the ages of 10 and 50 years (Khor and Van Cott 2009). Protein C deficiency also carries increased risk for warfarin-induced skin necrosis. Patients who are homozygous for the deficiency are very rare and can present with neonatal purpura fulminans or disseminated intravascular coagulation (DIC).

Protein C assays measure either protein C activity (functional) or antigen quantity (immunological). As an initial test, functional protein C assay, which provides a measure of both functional and antigenic levels, is commonly performed. If the result is low, an antigenic protein C assay is required. Type 1 protein C deficiency is quantitative and characterized by reduced functional activity and antigen levels and more common than type 2 deficiency, which is a qualitative defect, resulting in reduced activity and normal antigen level. If only a quantitative antigenic assay is used, type 2 deficiency cannot be detected (Eby and Olson 2008a; Margetic 2010). Functional protein C levels can be measured by clotting time-based or chromogenic assays. Clotting time-based assay can detect both type I and type 2 deficiency. However, it can give falsely increased results with anticoagulant therapy, lupus anticoagulant, and FVL mutation and falsely decreased results with elevated factor VIII levels (particularly greater than 250 %) or low protein S (in acute phase response). The chromogenic assay is less affected by interfering substances than the clotting time-based assay and is more reproducible; however, it can detect only functional protein C related to the peptide-binding site and therefore can miss some type 2 deficiencies (Eby 2008b; Khor and Van Cott 2009; Yohe and Olson 2012).

Acquired protein C deficiency is more common than hereditary protein C deficiency. The acquired causes should be excluded first before making a diagnosis of hereditary protein C deficiency. Because protein C is synthesized in hepatocytes and is vitamin K dependent, both liver dysfunction and vitamin K deficiency (including warfarin therapy) can decrease protein C levels. Protein C has a short half-life (6–8 h), and protein

C levels are more rapidly reduced in liver disease, anticoagulation therapy, and vitamin K deficiency compared to other coagulation proteins such as protein S or antithrombin. Conversely protein C levels rapidly recover into the normal reference range after discontinuation of anticoagulation therapy or correction of vitamin K deficiency. Even so, to ensure the most accurate results, protein C levels should not be measured for at least 10 days after stopping anticoagulants such as warfarin. Protein C levels are lowered in recent or current thrombosis, DIC, l-asparaginase therapy, and nephrotic syndrome and during the intra- or immediately postoperative period; neonates also have relatively low protein C levels (range 17–53 %). Oral contraceptive use and pregnancy can increase protein C level (Van Cott et al. 2002; Eby 2008b; Margetic 2010). Initially low protein C assays should be repeated after any such conditions have resolved.

## Protein S Deficiency

Protein S is a vitamin K-dependent glycoprotein which acts as a cofactor to protein C, accelerating protein C proteolysis of factor Va and VIIIa approximately twofold (Maurrissen et al. 2008). Approximately 60 % of protein S in the plasma is bound non-covalently to C4bBP in plasma with high affinity; the remaining free (unbound) protein S is the predominantly active form. Recent studies have shown that protein S also exerts its own anticoagulant activity by direct binding of factors V, VIII, and X and also appears to act as a cofactor for the tissue factor pathway inhibitor, which results in inhibiting tissue factor-mediated factor X activation (Maurrissen et al. 2008; Rosing et al. 2008). Hereditary protein S deficiency is transmitted in an autosomal dominant fashion. Protein S deficiency occurs in 0.2–0.5 % in general population and 1–3 % of patients with first venous thrombosis (Dykes et al. 2001; Eby and Olson 2008a; Khor and Van Cott 2010a). Functional protein S levels range between 20 and 64 % in heterozygous patients (Aillaud et al. 1996). Homozygous patients typically present as newborns with purpura fulminans and DIC.

There are three types of protein S deficiency. Type I and type III are quantitative deficiencies with both low free protein S antigen and low protein S activity and together account for 95 % of cases. Type I deficiency can be further differentiated from type III deficiency as the former shows low total protein S antigen level while the latter shows normal total protein S levels. Type III deficiency may be related to excess binding of protein S to C4bBP. Type II deficiency is a qualitative defect with low protein S activity with normal antigenic free and total protein S levels (Castodi and Hackeng 2008; Ten Kate et al. 2008; Ballard and Marques 2012).

There are three protein S assays measuring its activity (functional) and antigen levels of free and total protein S (immunological). As with protein C, the functional activity assay, which can detect quantitative and qualitative protein S deficiencies, is the recommended initial test, and the antigenic assay is performed only if functional activity is low. Functional protein S activity measured by clotting time-based assay is sensitive, but not specific. Measurement of antigenic protein S is currently performed by monoclonal antibody-based enzyme immunoassay and immunoturbidimetric assay.

Acquired causes of protein S deficiency should be excluded before making a diagnosis of protein S deficiency. Protein S will be decreased in clinical conditions which decrease protein C (see above). In addition to those conditions, protein S will be also decreased in acute phase response because its binding protein C4bBP is an acute phase reactant; when C4bBP is increased, it lowers both protein S activity and free antigen. Protein S is also decreased with elevated factor VIII (greater than 250 %) and infectious and autoimmune conditions such as HIV infection, Crohn's disease, or ulcerative colitis (Dykes et al. 2001; Khor and Van Cott 2009; Yohe and Olson 2012). Protein S levels are usually lower in women, especially during hormone replacement therapy, oral contraceptive use, and the second or third trimester of pregnancy (Eby and Olson 2008a). Protein S level should be repeated after any such conditions causing acquired protein S deficiency are resolved.

## Antithrombin Deficiency

Antithrombin is a glycoprotein of the serine protease inhibitor (serpin) family which primarily inactivates activated thrombin (factor IIa) and factor Xa and, to a lesser extent, factors IXa, XIa, and XIIa. Antithrombin acts as a so-called suicide inhibitor by forming a 1:1 covalent complex between antithrombin and serine proteases. This inhibitor activity is greatly accelerated by interaction with heparin. Although antithrombin is synthesized in the liver parenchyma, it is not vitamin K dependent and has a half-life of 2–3 days (Vossen et al. 2004; Rogers and Kottke-Marchant 2012).

Antithrombin deficiency is inherited in autosomal dominant fashion. The prevalence rates are approximately 0.05–0.1 % in the general population. Estimated annual incidence of a first episode of VTE in carriers of antithrombin deficiency is 1.0–2.9 % per year in retrospective studies (Yohe and Olson 2012; Khor and Van Cott 2010b). Antithrombin deficiency is associated almost exclusively with venous thrombosis. In general, the risk of thrombosis appears to be higher in antithrombin deficiency than for protein C or protein S deficiency, APC-R, or prothrombin gene G20210A mutation and thus has the highest risk for VTE among the known hereditary thrombophilias. Most cases are heterozygous because homozygosity for antithrombin deficiency is almost universally fatal in utero. Functional antithrombin levels in heterozygous individual range from 35 to 70 % (Kottke-Marchant and Duncan 2002; Picard et al. 2000; Khor and Van Cott 2009; Middeldorp 2011). VTE in antithrombin deficiency usually occurs as DVT of the extremities and PE but can also occur in unusual sites, such as cerebral sinuses, mesenteric, portal, and renal veins. It usually occurs at a young age (<50 years), but it is uncommon during the first two decades of life and may or may not follow a provocative event. Approximately 58 % of these episodes occur spontaneously, while 42 % show an association with a transient risk factor, which can be potentially preventable. Patients with concurrent defects such as factor V Leiden mutation are associated with higher risk of VTE and at younger ages with a median age of 16 years (Dykes et al. 2001; Khor and Van Cott 2010b).

Both antithrombin functional activity and antigen quantity can be measured. Assays of antithrombin function are predominantly chromogenic assays. If an initial functional level is normal or elevated, antithrombin deficiency is unlikely. If it is low, then a confirmatory functional test should be done on the patient using a repeat specimen. On the repeat specimen, both functional and antigenic levels are tested to determine whether the patient has a type 1 or type 2 antithrombin deficiency. Antigen levels can be measured by enzyme immunoassays and immunoturbidimetric methods (Khor and Van Cott 2009; Yohe and Olson 2012).

There are two major types of inherited antithrombin deficiency. Type 1 antithrombin deficiency is most commonly caused by a lack of the antithrombin gene product showing proportionately reduced functional and antigenic levels. It is frequently observed in the heterozygote state, resulting in approximately 50 % activity and antigen levels. Type 2 antithrombin deficiency is a qualitative deficiency, resulting in lower functional activity than antigen levels. Type 2 antithrombin deficiencies are further classified by antithrombin mutation site and performance on different antithrombin assays: (1) type 2a mutations affect the antithrombin reactive site, (2) type 2b mutations cause abnormalities in the heparin-binding site, and (3) type 2c mutations have a pleiotropic effect affected on both sites. Type 2c pleiotropic defects are associated with a moderate decrease in both antithrombin function and antigen levels (typically function levels are lower than antigen levels). However, subclassification is generally not clinically necessary because anticoagulant therapy does not differ between types (Dykes et al. 2001; Vossen et al. 2004; Picard et al. 2000; Rogers and Kottke-Marchant 2012).

Acquired antithrombin deficiency must be excluded before making a diagnosis of hereditary antithrombin deficiency and can be caused by drugs such as heparin or L-asparaginase. Other causes of low antithrombin levels include reduced synthesis in liver disease and increased anti-

thrombin loss in nephrotic syndrome, protein-losing enteropathy, DIC, sepsis, burn, trauma, hepatic veno-occlusive disease, thrombotic microangiopathies, cardiopulmonary bypass surgery, hematomas, or metastatic tumors. Antithrombin activity can be reduced by up to 30 % during full-dose unfractionated heparin therapy, but will not be reduced during LMW heparin therapy, and levels will normalize when heparin is discontinued. Antithrombin levels can be low in premenopausal women, oral contraceptive use, and pregnancy. As with low protein C and low protein S, confirmatory testing should be repeated on a new specimen after any potential confounding conditions have resolved (Rao et al. 1981; Kottke-Marchant and Duncan 2002; Eby 2008b; Khor and Van Cott 2009).

## Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is the most common cause of acquired thrombophilia. The presence of antiphospholipid antibody (APA) is associated with an increased risk of both arterial and venous thrombosis and recurrent pregnancy loss. APAs are acquired autoantibodies directed against phospholipid–protein complexes and are present in 3–5 % of the general population (Van Cott and Eby 2008; Miyakis et al. 2006). There are both primary and secondary forms of APAs arising spontaneously or in association with another condition. These antibodies, also known as lupus anticoagulants (LA) due to their prevalence in patients with systemic lupus erythematosus (SLE), are extremely heterogeneous and are directed against a wide variety of anionic phospholipids, including cardiolipin, beta2 glycoprotein 1 (B2GP1), or cell-membrane phosphatidylserine (Kottke-Marchant 1994; Pengo et al. 2009).

Diagnosis of APS is made by clinicopathologic evaluation. In addition to clinical criteria such as vascular thrombosis or pregnancy morbidity, repeated laboratory testing of APA is required for diagnosis because transient low level increases in APA can be detected in a variety of clinical conditions including acute phase response. Laboratory diagnostic criteria include positive testing for one of the following on two or more occasions, at least 12 weeks apart: (1) lupus anticoagulant, (2) anticardiolipin antibodies (IgG or IgM) in medium or high titer, and (3) B2GP1 antibodies (IgG or IgM) in medium or high titer (Finazzi et al. 1996; Miyakis et al. 2006; Heit 2007; Pengo et al. 2009). Based upon consensus criteria from the International Society for Thrombosis and Haemostasis (ISTH), confirmation of LA requires that the following four criteria should be met (Brandt et al. 1995; Pengo et al. 2009). First, there should be prolongation of at least one phospholipid-dependent clotting test (e.g., aPTT, dilute Russell's viper venom test [DRVVT] screen, or hexagonal phospholipid neutralization screen). DRVVT is considered as the screening test, and the second test should be a sensitive aPTT with low phospholipids and silica as an activator. Second, there is an evidence of inhibitory activity in the patient plasma demonstrated by mixing patient plasma with pooled normal plasma (e.g., immediate and incubated mixing study or DRVVT mixing study). Third, phospholipid dependence of the inhibitor should be demonstrated on a confirmatory test which demonstrates shortening of clotting time with the addition of more phospholipid (e.g., DRVVT confirmatory ratio, hexagonal phospholipid neutralization ratio, platelet neutralization). Fourth, the presence of a specific factor inhibitor, particularly factor VIII, and anticoagulant drugs such as heparin or direct thrombin inhibitor should be excluded (Brandt et al. 1995; Reber and de Moerloose 2004; Miyakis et al. 2006; Pengo et al. 2009; Nichols et al. 2012).

Paradoxically, LAs prolong clot-based assays in vitro while predisposing to thrombosis in vivo. In fact, approximately 30 % of LA patients will experience thrombosis. In approximately 15 % of patients with DVT, clotting is attributable to the presence of LA (Margetic 2010; Yohe and Olson 2012). Because no single test is available to detect LA, laboratory testing for LA consists of a panel of assays following a diagnostic algorithm (Fig. 1.4). To maximize diagnostic potential, at least two assays based on different principles should be performed to fulfill each of the four criteria, and assays are usually performed with low

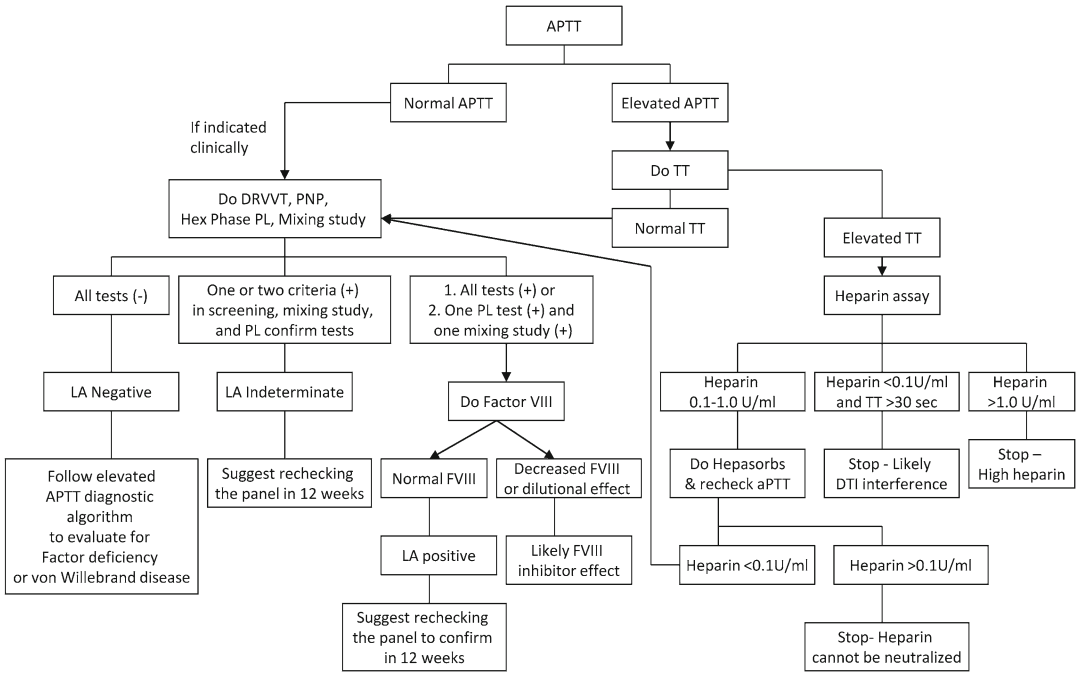


**Fig. 1.4** Diagnostic algorithm for detection of lupus anticoagulant (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013. All Rights Reserved). *aPTT* activated partial thromboplastin time, *DRVVT* dilute Russell's viper venom test, *FVIII* factor VIII, *Hex* hexagonal, *LA* lupus anticoagulant, *PL* phospholipid, *PNP* platelet neutralization procedure, *TT* thrombin time

concentrations of phospholipid to improve sensitivity. Tests for LA are interpreted as positive if the panel demonstrates one positive screening test, one positive mixing test, one positive confirmatory test, and no evidence for a factor inhibitor or anticoagulant drug effect. If fewer than four diagnostic criteria are met and if clinical suspicion for LA exists, the test panel is interpreted as indeterminate and the patient should be retested at a later date (Miyakis et al. 2006; Van Cott and Eby 2008; Moffat et al. 2009; Pengo et al. 2009).

Updated guidelines for LA detection emphasize patient selection to minimize inappropriate requests of LA testing and avoid the risk of obtaining false-positive results. Level of appropriateness of LA testing is divided into three grades according to clinical characteristics. The low grade includes venous or arterial thromboembolism in elderly patients. Moderate grade includes prolonged aPTT in asymptomatic patients, recurrent spontaneous early pregnancy loss, and provoked VTE in young patients. High grade includes unprovoked VTE and arterial thrombosis in young patients (<50 years old), thrombosis at unusual sites, late pregnancy loss, and any thrombosis or pregnancy morbidity in patients with autoimmune disease. General searches for LA in asymptomatic individuals or patients other than those described here are highly discouraged (Pengo et al. 2009).

Specific antibodies against cardiolipin and B2GP1 (IgG or IgM) are measured by commercially available solid-phase enzyme-linked immunosorbent assay (ELISA). Anticardiolipin antibodies recognize a complex of cardiolipin, a naturally occurring phospholipid, bound to B2GP1 protein. Complexes of anionic phospholipids and endogenous plasma proteins provide more than one epitope recognized by natural autoantibodies. Detection of anticardiolipin antibodies is generally considered to be a sensitive test. However, because the antigen target of anticardiolipin antibodies is a B2GP1-cardiolipin complex, B2GP1 antibody assays are considered to be more specific than

anticardiolipin antibody assays (Triplett 2002; Galli et al. 2003; Marai et al. 2003). False-positive results for anticardiolipin antibodies can be associated with high level of rheumatoid factor and cryoglobulins (Finazzi et al. 1996; Miyakis et al. 2006).

Both anticardiolipin antibodies and B2GP1 APA assays are recommended because using just a B2GP1 antibody assay can miss some cases of APA. If a test result for APA diagnosis is positive, repeating the test in a new specimen after an interval of at least 12 weeks should be performed to confirm APA because transient occurrence of APAs can be caused by infection or drugs and is not associated with thrombotic risk (Margetic 2010; Van Cott and Eby 2008). In individuals with high-titer IgG anticardiolipin antibodies (>40 IgG phospholipid units [GPL]), a prospective study found a rate of thrombosis of 6.1 % per year, compared with 0.95 % per year in individuals with no history of thrombosis, 4.3 % in patients with SLE, and 5.5 % in patients with a history of thrombosis (Finazzi et al. 1996; Van Cott and Eby 2008; Khor and Van Cott 2009).

Because APAs have heterogeneous patterns of antigen recognition and different reagents vary in phospholipid composition, there are significant issues of preanalytic interference and interlaboratory variability which need to be considered for selection of APA assays and interpretation (Miyakis et al. 2006; Van Cott and Eby 2008; Pengo et al. 2009; Nichols et al. 2012). Frozen and thawed platelets, which can cause false-negative screening or mixing study results, should not be used. Pooled normal plasma should have a residual platelet count of less than 10,000/μL. Acute thrombotic events or acute phase responses with elevated factor VIII can cause false-negative results. Anticoagulant therapy, such as heparin or direct thrombin inhibitors, or presence of specific coagulation factor inhibitors can cause false-positive results with prolonged aPTT. Thrombin time can help to identify anticoagulant effect or specific inhibitors. Commercial reagents for LA testing include heparin neutralizers which can quench heparin concentrations up to 1.0 U/mL. However, similar reagents are not available for direct thrombin inhibitors; therefore, LA testing should not be performed on individuals taking these drugs. Individuals on long-term vitamin K antagonists should be tested 1–2 weeks after discontinuation of therapy after the INR has normalized to less than 1.5.

## Hyperhomocysteinemia

Homocysteine is an intermediate amino acid produced by demethylation of methionine via methylenetetrahydrofolate reductase (MTHFR) in the folate cycle. The metabolism of homocysteine requires vitamin B6, vitamin B12, and folate. Hyperhomocysteinemia is associated with increased risk of arterial and venous thrombophilia and atherosclerosis. Acquired hyperhomocysteinemia can be caused by deficiency of vitamin B6, vitamin B12 or folate, renal failure, hypothyroidism, psoriasis, inflammatory bowel disease, rheumatoid arthritis, and therapy with certain drugs such as methotrexate, niacin, anticonvulsants, theophylline, l-dopa, thiazide, cyclosporine A or phenytoin (Guba et al. 1999; Eldibany and Caprini 2007; Khor and Van Cott 2009).

Hereditary hyperhomocysteinemia is caused by a mutation in an enzyme in homocysteine conversion pathways. Homozygous mutations of the MTHFR gene are present in 10–13 % of the population, while heterozygous mutations are found in 30–40 % (Guba et al. 1999). A common mutation in the MTHFR gene is C677T, which is a polymorphism with a C to T substitution at nucleotide 677. This mutation has been known to be related to thrombosis risk; however, meta-analyses have found only a weak association (Ray et al. 2002; Deb Heijer et al. 2005; Eby 2008b). Homozygosity for MTHFR C677T is associated with approximately 25 % increase in total plasma homocysteine level. Hyperhomocysteinemia results in a three- to fivefold increase in the risk of coronary artery disease. Lowering homocysteine levels by therapy with vitamin B6, vitamin B12, or folate has not been proven to reduce thrombotic risk (Van Cott et al. 2002; Khor and Van Cott 2009). Given the modest risk of homocysteine on thrombophilic risk, variable prevalence in different ethnic groups (higher prevalence in Caucasian and lower prevalence in African-Americans), and lack of evidence of therapeutic benefit, screening

homocysteine levels in healthy individuals and testing for MTHFR mutations are not currently suggested.

### Elevated Factor VIII

Several thrombophilia studies have demonstrated an association between elevated factor VIII and increased risk of thrombophilia, due at least in part to factor VIII-mediated enhancement of thrombin generation (O'Donnell et al. 2000; Oger et al. 2003; Ota et al. 2011; Jenkins et al. 2012). To date no genetic variations in the factor VIII gene have been identified. Factor VIII appears to be higher in African-Americans and lower in blood group O. The prevalence of elevated factor VIII among patients with venous thrombosis is 20–25 % (Kraaijenhagen et al. 2000; O'Donnell et al. 2000; Jenkins et al. 2012). Elevation of factor VIII appears to be persistent over months to years and is independent of acute phase response. It is not clear yet if factor VIII elevation directly contributes to increased thrombophilic risk. However, studies show that persistent factor VIII level greater than 150 %, or greater than the 90th percentile in the absence of acute phase reaction, elevated estrogen levels and recent exercise is an independent risk factor for thrombophilia (Kraaijenhagen et al. 2000; Kyrle et al. 2000; Benjaber et al. 2003).

Functional factor VIII activity can be measured by aPTT-based clotting assay or chromogenic assay, and antigen quantitation can be accomplished using ELISA. Factor VIII level can be elevated in acute phase reaction, elevated estrogen states, pregnancy, or after aerobic exercise. Factor VIII measurement should be postponed until at least 6 months after an acute thrombotic event and 6 weeks after giving birth and should be repeated after 3–6 months to confirm persistent elevation (Benjaber et al. 2003; Margetic 2010).

### Fibrinogen Defects

Dysfibrinogenemia is a heterogeneous group of disorders resulting in structurally and functionally altered fibrinogen. It can cause bleeding, venous or arterial thrombosis, or both. The prevalence of dysfibrinogenemia in patients with venous thrombosis is approximately 0.8 % (Haverkate and Samama 1995; Cunningham et al. 2002). Although the mechanism of thrombosis is unknown, increased fibrin formation or impaired fibrinolysis may be associated with thrombosis. Dysfibrinogenemia patients can have prolonged prothrombin time, thrombin time and reptilase time, decreased functional fibrinogen, and normal to elevated immunologic fibrinogen. The ratio of functional fibrinogen activity to immunologic fibrinogen antigen will be decreased in dysfibrinogenemia (Hayes 2002; Eby 2008b; Verhovsek et al. 2008; Margetic 2010).

The most commonly used functional assay is the Clauss method. Acquired deficiency of fibrinogen can be caused by liver disease, consumptive states such as placental abruption or DIC, or fibrinolytic therapy. As fibrinogen is an acute phase reactant, the test should be delayed at least 6 months after acute thrombosis (Verhovsek et al. 2008; Yohe and Olson 2012).

## Laboratory Assays for Evaluation of Platelet Function

### Platelet Structure, Activation, and Clot Formation

Platelets are small (2 μm) anucleate cells produced in the bone marrow from the cytoplasm of megakaryocytes. They circulate in the peripheral blood for 7–10 days at a normal concentration between 150,000 and 400,000/μL (George 2000). Platelet cytoplasm is filled with alpha and dense granules, each of which contains specific factors necessary for platelet function. Dense granules contain adenosine triphosphate (ATP) and adenosine diphosphate (ADP), 5-hydroxytryptamine (5-HT), histamine, and cations ($Ca^{2+}$, $Mg^{2+}$). Alpha granules are more complex and contain proteoglycans (e.g., platelet factor 4), adhesive glycoproteins (e.g., von Willebrand factor [vWF]), coagulation factors (e.g., fibrinogen; factors V, VII, XI, XIII; protein S), cellular

mitogens (e.g., platelet-derived growth factor, vascular endothelial growth factor), protease inhibitors (e.g., plasminogen activator inhibitor-I), and other miscellaneous molecules (e.g., immunoglobulins, albumin) (Rendu and Brohard-Bohn 2001). Also vital to platelet function is the platelet cytoskeleton, which forms the internal architecture of the cell and is responsible for platelet conformational changes, and the cell membrane, upon which specific receptors, adhesion molecules, and antigens are anchored (Hartwig 2006).

Platelets respond to endothelial injury with formation of a hemostatic platelet plug. This occurs through a process of adhesion to the site of injury followed by activation, which includes degranulation, and potentiates the platelet for the next step, aggregation (platelet–platelet binding) (Jackson 2007). There are several redundant pathways and positive feedback loops in this process, which provide multiple targets for antiplatelet agents. Platelet activation and aggregation also has multiple synergistic interactions with the coagulation cascade. For example, the platelet alpha granules contain procoagulant molecules (fibrinogen, vWF, factor V) and molecules exposed on the surface of activated platelets provide sites for phospholipid-dependent coagulation complexes. When a platelet encounters the exposed subendothelium of a damaged blood vessel, extracellular matrix proteins interact with receptors on the platelet surface. These interactions lead to rapid platelet adhesion to the site of vascular injury. This initial platelet adhesion is via a weak bond ("rolling") between GPIb/IX/V on the platelet membrane and vWF secreted by endothelial cells. This binding also triggers the release of $Ca^{2+}$ from internal platelet stores which function in platelet activation. Further binding occurs between exposed collagen and collagen receptors on the platelet surface. Binding of collagen to the GPVI/FCRγ complex stimulates platelet activation, while binding to the GPIa/IIa collagen receptor creates stable adhesion ("tethering") (Jackson 2007). In addition to vWF and collagen, there are multiple other triggers, or agonists, of platelet activation including thrombin, epinephrine, and ADP. Binding of ADP to the high-affinity $P2Y_1$ receptor is responsible for inducing platelet shape-changing, release of cytoplasmic $Ca^{2+}$, and the initial wave of aggregation. However, full aggregation cannot be triggered without binding to the low-affinity ADP receptor $P2Y_{12}$. Binding to this receptor also potentiates platelet secretion and thrombus stabilization. Prevention of binding to the $P2Y_{12}$ is the mechanism of action of thienopyridine drugs such as clopidogrel and prasugrel (Geiger et al. 1999).

Once the platelet is activated, it undergoes a change in shape, flattening out and increasing surface area. Platelet activation also leads to changes in cell surface molecules, including the fibrinogen receptor GPIIb/IIIa which is normally maintained in an inactive conformation on the cell surface. Once the platelet is activated, GPIIb/IIIa undergoes a conformational change which allows it to bind fibrinogen which in turn facilitates platelet aggregation via formation of interplatelet fibrinogen bridges (Jackson 2007). Thromboxane $A_2$ ($TxA_2$), a by-product of the platelet cyclooxygenase pathway, stimulates activation of adjacent platelets. Aggregation and platelet recruitment continues with eventual formation of the platelet plug. Fibrinogen binding to activated GPIIb/IIIa on the platelet surface also strengthens platelet adhesion to the site of endothelial injury. Because GPIIb/IIIa is so integral to platelet aggregation, antagonists of this molecule serve as potent antiplatelet agents (Harrington 1999), such as abciximab. Conversely aspirin exerts its antiplatelet function through irreversible inhibition of cyclooxygenase, preventing $TxA_2$ synthesis (Burch et al. 1978).

## Preanalytic and Analytic Variables and Other Consideration in Platelet Testing

There are multiple preanalytic and analytic variables which can complicate laboratory assessment of platelets. Timing and specimen handling is of critical importance in platelet functional testing because platelets spontaneously activate in vitro. Blood should be drawn in a standardized fashion to minimize activation and aggregation. Blood should be collected in a tube with appropriate anticoagulant such as sodium citrate for platelet function testing and ethylenediaminetetraacetic acid (EDTA) for

platelet count. Hemolyzed, lipemic, and short draw specimens are not suitable for platelet function testing. Tests that assay platelet functional activity should be performed as soon as possible and certainly performed within 4 hours of collection. Specimens for function assays should be kept at room temperature; they cannot be chilled and rewarmed as this may cause loss of platelet function (Schmitz et al. 1998; Harrison 2004). Additionally specimens should not be sent via pneumatic tube (Dyszkiewicz-Korpanty et al. 2004; Hubner et al. 2010). These considerations necessitate careful coordination between the clinician and pathologist. Other measurements, such as platelet count, are more stable, and specimens can be tested up to 24 hours after phlebotomy (Gulati et al. 2002).

Drugs with antiplatelet activity are another common confounding variable in platelet testing and can lead to apparently abnormal platelet function, simulating an intrinsic platelet disorder. Drugs with antiplatelet activity include aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), thienopyridines (clopidogrel, prasugrel, ticlopidine), dipyridamole, and glycoprotein (GP) IIb/IIIa inhibitors (abciximab, eptifibatide, tirofiban). As with any evaluation of bleeding or clotting issues, a thorough history, including all medications, is essential to proper platelet testing (George and Shattil 1991). If possible such drugs should be discontinued several days prior to when the specimen is drawn, and a listing of the drugs the patient is taking should be included with requests for platelet testing.

Laboratories should monitor assay performance by internal and external quality control programs to ensure consistent high levels of performance and accuracy (Dybiaer 1994; Hayward and Eilekboom 2007). However, complex platelet assays, such as aggregometry and flow cytometry, are not well standardized between laboratories compared to simple screening tests. Each laboratory may use different reagents, instrumentation, and standards (Moffat et al. 2005). Because of the evanescent nature of platelet function, controls for these tests cannot be stored or shipped and only a few tests have widely available clinical standards (Favaloro 2009). As such, it is important to realize that test results are often not directly comparable between laboratories.

## Platelet Morphologic Assessment

Modern automated hematology instruments measure platelet number and size via impedance and/or modified flow cytometry. Additional platelet indices can be measured, analogous to those reported for erythrocytes, including mean platelet volume (MPV) and platelet distribution width (PDW). An increased MPV may be indicative of higher turnover as larger platelets are released from the bone marrow, and PDW may be elevated in myeloproliferative neoplasms (MPN) due to a mixture of giant and small platelets. The platelet count may be underestimated by automated systems in conditions with very large platelets, such as in congenital macrothrombocytopenias (e.g., Bernard–Soulier, MYH9) in which the platelets are artifactually counted as erythrocytes or lymphocytes. Some automated hematology analyzers are now able to measure immature platelet fractions, which can be used as a measure of platelet production (Ault et al. 1992).

Light microscopic examination of Wright- or Giemsa-stained peripheral blood smears can also be used to estimate platelet number and size. While visual examination is less precise than automated methods, microscopy can identify artifacts including artificially low platelet count due to platelet clumping (a common artifact in specimens collected in EDTA), satellitosis, or misidentification of giant platelets (Moreno and Menke 2002; Froom and Barak 2011). In addition, certain platelet disorders have characteristic morphologic anomalies; decreased or absent granularity is seen in alpha granule disorders such as gray platelet syndrome and occasionally MPN. True congenital macrothrombocytopenias can have uniformly giant platelets with very high MPV and normal PDW (Moreno and Menke, 2002; Mhawech and Saleem, 2000). Bone marrow examination can be useful in diagnosis of myelodysplastic syndromes, MPN, or other disease processes involving the marrow space.

Electron microscopy can be used to assess platelet shape and various properties of alpha and dense granules (Clauser and Cramer-Bordé 2009). However, EM is technically difficult, labor intensive, and costly and, as such, has mostly been supplanted by technologies such as flow cytometry.

## Platelet Function Analysis

The classical screen of platelet function is the bleeding time (BT), determined by making a cut in the skin in the forearm, and observing time until cessation of bleeding (Harker and Slichter 1972). Results are influenced by multiple patient and operator variables including length, depth and site of incision, platelet number and function, skin temperature, fibrinogen concentration, and vascular function. The BT is poorly reproducible, unpopular with patients, and has not been shown to correlate with intraoperative bleeding (Lind 1991; Peterson et al. 1998). For these reasons, BT is no longer performed at most medical centers.

### Platelet Function Screening

The platelet function analyzer (PFA)-100 system (Siemens Healthcare Diagnostics) is used by many centers as an in vitro alternative to BT to screen for global platelet function. The test assesses platelet function for both adhesion and aggregation at high shear rates, which mimics the properties of the human vasculature. Citrated blood is drawn through an aperture in a membrane coated with agonists (collagen/ADP and collagen/epinephrine). The time until occlusion of the aperture is recorded as the closure time (CT) up to 300 s; after that time CT is reported as >300 s (Kundu et al. 1995). The closure time is reflective of interactions of vWF with platelet membrane surface glycoproteins as well as platelet granularity and secretion and thus is prolonged in many cases of von Willebrand disease (vWD). Therefore, it can be used both as a screen of platelet function and vWD, but cannot differentiate between the two. The test may also have some utility in monitoring moderate vWD (Favaloro 2006). CT is sensitive to severe intrinsic platelet defects such as Glanzmann thrombasthenia and Bernard–Soulier syndrome but is less sensitive to disorders such as secretion defects and storage-pool disorders (Hayward et al. 2006). CT is not dependent on coagulation factor concentrations or heparin, but is affected by platelet count, hematocrit, and citrate concentrations. It is sensitive to GPIIb/IIIa inhibitors, and especially aspirin and NSAIDs, but cannot be used to monitor thienopyridines (Harrison 2005; Hayward et al. 2006). The aspirin effect is dose dependent and the test can be used to assay aspirin resistance (Crescente et al. 2008). Isolated abnormal patterns found using the collagen/epinephrine cartridge are often observed in specimens with aspirin-like drug effect or storage-pool disorders (Nurden and Nurden 2009; Kottke-Marchant et al. 1999). When used in conjunction with an adequate bleeding history, CT has proven to be predictive for bleeding risk. The PFA-100 is relatively simple and rapid (results in 5–8 min) and uses a small blood volume (800 μL) on a near-point-of-care platform. However, results are nonspecific and not sensitive for mild platelet dysfunction or vWD (Hayward et al. 2006).

### Platelet Aggregation

Platelet aggregometry, which is considered the gold standard for platelet function testing, measures aggregation of platelets in a stirred sample in reaction to a variety of agonists. Different platelet disorders have different patterns of agonist response. Aggregometry can detect abnormalities in surface glycoproteins, signal transduction, and platelet granularity (Hayward et al. 2009). Optical platelet aggregometry is most commonly performed and is considered to be reflective of in vivo aggregation function of platelets. It uses platelet-rich plasma (PRP), processed by centrifugation of citrated blood, and measures changes (%) in light transmittance through the specimen with a modified spectrophotometer (turbidimetry). Aggregometry can also be performed on whole blood, in which case testing is based on changes in impedance (ohms) between two submerged probes as platelet aggregates form (Dyszkiewicz-Korpanty et al. 2005). The panel and concentration of agonists used varies by laboratories but usually includes ADP, collagen, arachidonic acid (AA), epinephrine, and occasionally thrombin receptor-activation peptide (TRAP) over a range of concentrations (Hayward et al. 2010). Classical platelet responses to agonists including lag, shape change, and primary and secondary aggregation are monitored and measured by maximal amplitude or percentage of aggregation after a fixed period of time (Nurden and Nurden 2009). Another important reagent used for platelet func-

tion is the antibiotic ristocetin which acts as a platelet agonist by facilitating binding of vWF to GPIb/IX/V by inducing the same activating conformational change in vWF as does high shear stress in vivo (Berndt et al. 1992).

Different platelet disorders show different patterns of aggregation in response to each of these agonists (Kottke-Marchant and Corcoran 2002). Characteristic aggregometric findings in different platelet disorders are summarized in Table 1.3. Ristocetin-induced platelet aggregation (RIPA) is measured at low and high concentrations and is sensitive to defects in some types of von Willebrand disease or GPIb/IX/V (Bernard–Soulier) (Jenkins et al. 1976). Patients with severe type 1 or type 3 vWD with markedly reduced vWF and type 2A with dysfunctional vWF can have a reduced response to ristocetin; however, patients with type 2B will have heightened response to lower concentration of ristocetin. Aggregometry alone is not very sensitive to storage-pool disorders, but sensitivity can be increased by the use of lumiaggregometry. This specialized test allows simult aneous measurement of platelet aggregation and ATP secretion measured as luminescence using the firefly luciferin–luciferase reaction (McGlasson and Fritsma 2009). Figure 1.5 is a diagram of normal lumiaggregation results showing simultaneous measurement of platelet aggregation and stimulated ATP release by dense granules during platelet aggregation. Aggregometry is a powerful tool for evaluating platelet disorders but is generally performed only in specialized centers. Aggregometry requires a large sample volume (~20 mL of whole blood) and takes several hours. Results are influenced by platelet count, which can be standardized somewhat with the use of PRP (Hayward et al. 2010). Centrifugation technique may alter test results and lipemic, hemolyzed, or icteric samples cannot be used for turbidimetric testing (Dyszkiewicz-Korpanty et al. 2005).

## Platelet Flow Cytometry

Platelet flow cytometry is another powerful tool which can be used to evaluate multiple aspects of platelet structure and function. Flow cytometry simultaneously assesses multiple parameters of cells including size (forward scatter), granularity (side scatter), and presence of various molecules on the cell surface through the use of fluorescently labeled antibodies. Flow cytometry can be utilized to identify absence, decreased expression, or abnormalities of cell surface receptors; similarly, activation can be measured using antibodies specific for active conformations of cell surface molecules (Schmitz et al. 1998). Flow cytometry can also be used for the detection of platelet-reacting antibodies in patients with immune thrombocytopenic purpura or drug-induced thrombocytopenia, which is sensitive but nonspecific (Romero-Guzman et al. 2000). Mepacrine, which is taken up in dense granules, can be used to measure number of dense granules, platelet signaling, and granule release function (Wall et al. 1995). P-selectin, which is newly expressed on the platelet surface after activation, can be used to measure platelet alpha granule release (Fig. 1.6). Flow cytometry has the benefit of requiring a relatively small amount of blood compared to aggregation studies. However, platelet flow cytometry, like aggregometry, is generally performed only in specialized medical centers as it requires specialized instruments and skilled technologists. This can make performing these studies logistically very difficult because platelet function studies by flow cytometry (such as alpha or dense granule release studies) should be performed within 1 hour of venipuncture because of progressive activation of platelets during in vitro storage. However, interrogation of surface glycoproteins, such as is used for making a diagnosis of Glanzmann thrombasthenia, can be measured in specimens up to 24 hours post-collection (Michelson 1996; Michelson 2006; Kottke-Marchant 2008).

## Thromboelastography

Thromboelastography and thromboelastometry are similar techniques used to monitor the viscosity and elasticity of blood clots and can simultaneously measure coagulation, platelet function, and fibrinolysis through analysis of the viscoelastic properties of clotting blood. There are two commercially available platforms available for clinical use: thromboelastography (TEG; Haemoscope/Haemonetics Corp, Niles, IL, USA) and rotational thromboelastometry (ROTEM; TEM International, Munich, Germany) (Bolliger

**Table 1.3** Summary of platelet disorders with characteristic laboratory findings

| | Defect | Platelet count | Morphology | Characteristic aggregometric findings | Other platelet assay results | Comments |
|---|---|---|---|---|---|---|
| **Surface glycoprotein disorders** | | | | | | |
| Glanzmann thrombasthenia | Deficient or defective GPIIb/IIIa receptor (AR) | Normal | Normal | Absent with all agonists except ristocetin | Prolonged CT<br>Decreased GPIIb/IIIa receptor by flow cytometry | Similar picture with afibrinogenemia |
| Bernard–Soulier syndrome | GPIb/V/IX receptor (incompletely recessive AR) | Decreased (40,000–1,000,000/μL) | Giant platelets high MPV | Normal with all except ristocetin | Prolonged CT<br>Decreased expression of GPIb/V/IX receptor by flow cytometry | Mucocutaneous bleeding out of proportion to thrombocytopenia. Surgical bleeding risk |
| Velocardiofacial syndrome | Deletion of 22q11.2 (GPIb beta gene) | May be decreased | Giant platelets high MPV | Absent with all agonists except high dose ristocetin | | Bleeding uncommon |
| Collagen receptor deficiency | Defect in collagen receptors (GPIa/IIa, GPVI) | Normal | Normal | Absent with collagen and low dose ristocetin | | Very rare |
| Platelet-type/pseudo-von Willebrand disease | Gain-of-function mutation in GPIb receptor → decreased HMW vWF multimers | Decreased | Normal (rarely giant platelets) | Increased with low dose ristocetin. Normal with all others | Prolonged CT | Similar picture in Type 2B vWD; differentiate by *VWF* gene sequencing, mixing studies, and platelet aggregation by adding cryoprecipitate to PRP |
| **Storage-pool disorders** | | | | | | |
| Gray platelet syndrome | Deficiency of alpha granules | Often decreased | Large, gray-colored (agranular) platelets | May be decreased with thrombin and collagen | Decreased alpha granules in EM | Marrow and pulmonary fibrosis, splenomegaly<br>Mild lifelong bleeding |
| Wiskott–Aldrich syndrome and X-linked thrombocytopenia | Defect in Wiskott–Aldrich syndrome protein gene (XR) | Decreased | Uniformly small, low MPV | Absent with ADP, epinephrine and collagen | | Immune deficiency, recurrent infection, often decreased dense granules, increased bleeding risk |

(continued)

**Table 1.3** (continued)

| | Defect | Platelet count | Morphology | Characteristic aggregometric findings | Other platelet assay results | Comments |
|---|---|---|---|---|---|---|
| Dense granule deficiency | Defective dense granules. Can be isolated or as part of hereditary syndromes | Normal | Usually normal | May have absent second wave with ADP, epinephrine, and decreased response to AA and collagen. Normal in 25 % of patients | Decreased mepacrine uptake by flow cytometry | Hermansky–Pudlak, Chediak–Higashi, thrombocytopenia with absent radii (TAR) syndromes |
| **Activation disorders** | | | | | | |
| Signaling defects | Defects of cyclooxegenase, phospholipase C pathways | Normal | Normal | Decreased primary aggregation with epinephrine and collagen, absent secondary with ADP | Normal mepacrine uptake | Decreased granule release without granule deficiency |
| Secretion defects | No release in dense granules | Normal | Usually normal | Decreased primary aggregation and absent secondary aggregation | | |
| Scott syndrome | Defective "flip" of membrane phospholipids to outer surface during activation | Normal | Normal | Normal | Abnormal platelet procoagulant activity | Decreased binding of activated coagulation factors, decreased microparticle formation |
| **Drug-induced disorders** | | | | | | |
| ASA/NSAIDs | Cyclooxegenase inhibition: ASA-reversible; NSAIDs-irreversible | Normal | Normal | Absent second wave with ADP; decreased or absent with others except ristocetin | Prolonged CT (often collagen/ epinephrine cartridge) | Similar findings with COX-1 deficiency or functional abnormality → defective $TxA_2$ |
| Thienopyridines (e.g., clopidogrel) | Inhibition of ADP receptor ($P2Y_{12}$) | Normal | Normal | Absent second wave with ADP | Prolonged/ normal CT | Similar picture in very rare cases of congenital ADP receptor impairment |
| GPIIb/IIIa inhibitors (e.g., abciximab, eptifibatide, tirofiban) | Inhibition of GPIIb/IIIa receptor | | | Decreased or absent for all except ristocetin | Prolonged CT | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Others** | | | | | | |
| Myeloproliferative neoplasms | Clonal neoplasm | Increased, often >1,000,000/μL; sometimes decreased | Variable size and morphology; occasional hypogranularity | Variable. Decreased with epinephrine in ET | May show reduced GPIIb/IIIa expression by flow | Bleeding or thrombosis |
| *MYH9* disorders | Mutations in non-muscle myosin heavy chain IIA protein | Decreased (60,000–100,000/μL) | Giant platelets, high MPV, neutrophilic inclusions | Normal | | Includes May–Hegglin anomaly, Sebastian, Fechtner, and Epstein syndromes. Mild bleeding |

*CT* closure time, *HMW* high molecular weight, *vWF* von Willebrand factor, *GP* glycoprotein, *AR* autosomal recessive, *XR* X-linked recessive, *ET* essential thrombocythemia, *MPV* mean platelet volume

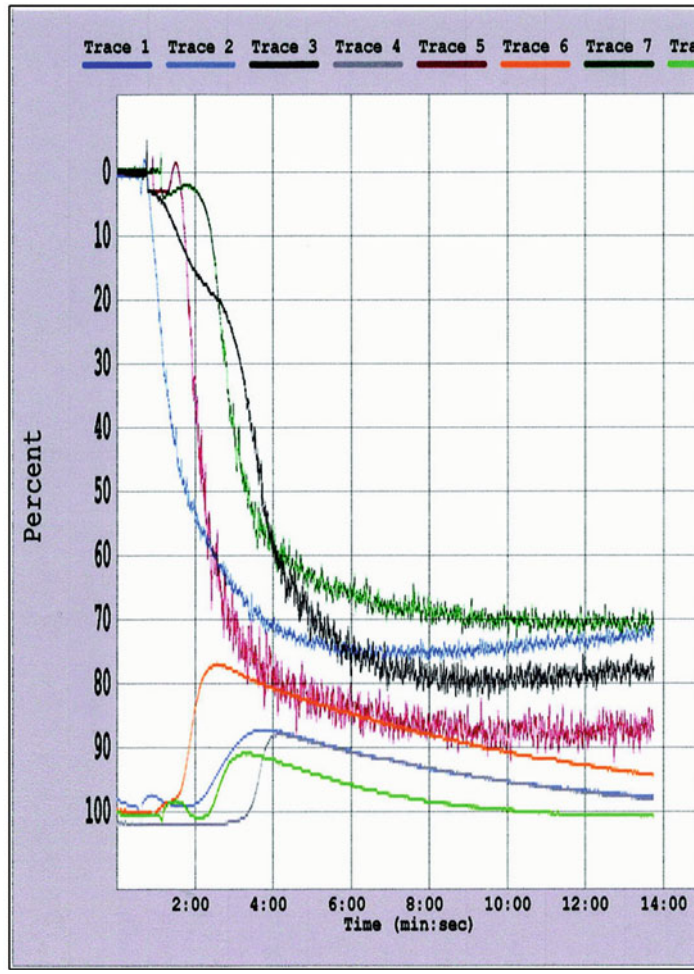


**Fig. 1.5** Example of normal lumiaggregation with simultaneous measurement of platelet aggregation and stimulated ATP release by dense granules during platelet aggregation. Platelet aggregation, which is shown at the *top*, is measured by maximal percentage of aggregation, and agonists in this diagram include ADP (5 μM; *blue*), collagen (2 μg/mL; *red*), arachidonic acid (500 μg/mL; *green*), and epinephrine (10 μM; *black*). The corresponding ATP aggregation to ADP (*light blue*), collagen (*orange*), arachidonic acid (*light green*), and epinephrine (*gray*) is shown at the *bottom*

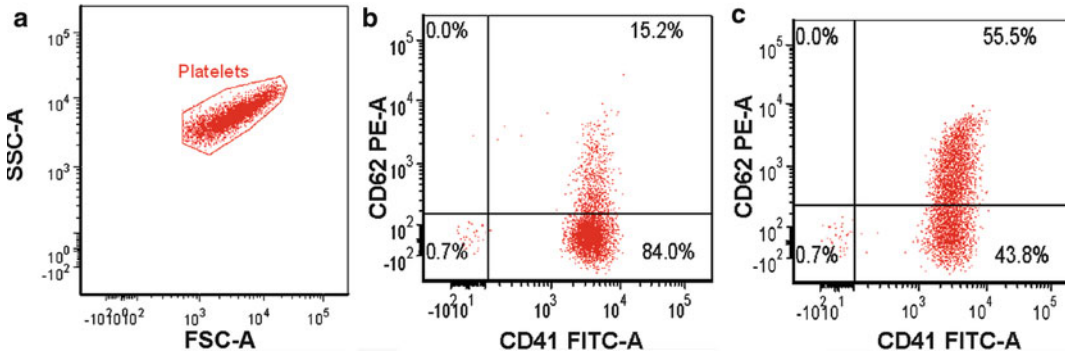


**Fig. 1.6** Example of normal platelet flow cytometry. (**a**) Platelet population is gated by the platelet size (forward scatter: FSC) and granularity (side scatter: SSC) in whole blood. (**b**) Basal P-selectin (CD62): resting platelets express surface glycoproteins GPIIb/IIIa (CD41), but do not express high levels of P-selectin (CD62). (**c**) Stimulated P-selectin (CD62): upon stimulation with ADP, most platelets release granules from alpha granules and express high levels of P-selectin (CD62) on the platelet membrane surface

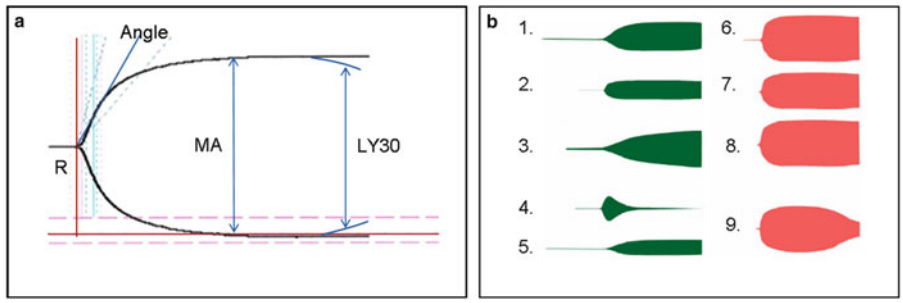


**Fig. 1.7** Thromboelastography (TEG). (**a**) Diagram of TEG tracing. R, reaction time until initiation of clotting; angle, degree of the strength of clot growth; MA, maximum amplitude indicative of maximum strength or stiffness of developed clot; LY30, percent lysis 30 min after maximum amplitude. (**b**) Examples of abnormal tracing pattern: *1*. low clotting factors, *2*. low platelet function, *3*. low fibrinogen level, *4*. primary fibrinolysis, *5*. hypocoagulable state, *6*. platelet hypercoagulability, *7*. enzymatic hypercoagulability, *8*. platelet and enzymatic hypercoagulability, and *9*. secondary fibrinolysis (Image of the TEG® Thromboelastograph® Hemostasis tracings is used by permission of Haemonetics Corporation. TEG® and Thromboelastograph® are registered trademarks of Haemonetics Corporation in the USA, other countries, or both)

et al. 2012). The test involves measuring tensile force generated between a plastic cup of recalcified citrated whole blood and a metal pin immersed within it which increases as blood clots. In TEG, the cup rotates around the pin, while the pin rotates in a stationary cup in ROTEM. Clot activator is added to the specimen, and as clot forms the torque of the rotating component is transmitted to the stationary component and is plotted as a kinetic curve. The instrument measures time until initiation of clotting (*R*), time until a fixed level of clot firmness (*K*), degrees of the strength of clot growth (angle), maximum amplitude (MA) indicative of maximum strength or stiffness of developed clot, and percent lysis 30 min after MA (LY30) (Bolliger et al. 2012). Figure 1.7 shows a diagram of thromboelastography tracing and examples of abnormal tracing pattern such as factor deficiency, anticoagulant therapy, fibrinolysis, hypercoagulability, and consumptive coagulopathy status. The basic test utilizes a contact activator to initiate clotting (kaolin in TEG, ellagic acid/phospholipids for ROTEM), but others, such as tissue factor, are available. The test can be modified through the use of various activators and inhibitors to interrogate different components of both hemostasis and clot lysis (Chen and Teruya 2009). Although the basic test is insensitive to aspirin or thienopyridines, modifications have been developed to monitor such antiplatelet therapy or heparin reversal (Swallow et al. 2006). This near-point-of-care test can be used intra- and perioperatively (results in 20–30 min) to guide transfusion therapy, and transfusion protocols which incorporate TEG have been shown to decrease bleeding in patients with massive transfusion (Afshari et al. 2011). TEG is sensitive to hematocrit and platelet count, and thus far methods are not well standardized, but efforts are ongoing to improve this area (MacDonald and Luddington 2010; Kitchen et al. 2010).

## Point-of-Care Tests

Currently there are a number of commercially available point-of-care (POC) tests for platelet function which provide rapid results, use small sample volumes, and require little to no specimen preparation (Table 1.4). POC tests also have the advantage of bypassing some of the logistical issues in platelet function testing such as timing and specimen transport. However, these tests are problematic in a variety of ways including a lack of standardization of methods, and performance by untrained personnel, in addition to having expensive reagents, quality control materials, and consumables, resulting in a relatively high cost-per-test (Gardiner et al. 2009). POC tests are generally used to predict intraoperative bleeding or monitor antiplatelet therapy. Some are designed

**Table 1.4** Summary of commercially available point-of-care (POC) tests for platelet function

| | Analysis method | Use | Specimen volume, anticoagulant | Advantages | Disadvantages |
|---|---|---|---|---|---|
| Plateletworks® (Helena Laboratories) | Comparison of free platelet count in blood with and without agonist | Monitor antiplatelet therapy<br>Predict bleeding | 1 mL, EDTA or special tubes | Results in 5 min<br>No sample preparation | Test must be done within 10 min |
| Verify Now® (formerly Ultegra, Accumetrics) | Turbidimetric (light transmittance) aggregometry | Monitor antiplatelet therapy | 2 mL, citrate or heparin, depending on test | Results in 5–10 min<br>No sample preparation | Test must be done within 4 h |
| Multiplate® (Roche) | Impedance aggregometry | Monitor antiplatelet therapy<br>Identify patients at risk for bleeding | 0.3 mL, hirudin | Results in 10 min | Not currently available in US |
| Sonoclot® (Sienco) | Impedance of ultrasonic vibration as clot forms | Cardiac and transplantation surgery | 400 µL, whole blood | Global view of coagulation, platelet function, and fibrinolysis | May take up to 45–60 min |
| Impact™ Cone and Plate (Matis) | Image analysis of adhesion and aggregation under shear stress | Diagnosis of platelet defects<br>Monitor antiplatelet therapy | 130 µL, citrate | Results in 6 min<br>High shear stress | Clinical point-of-care assay not currently available in USA |

to assess the effect of specific antiplatelet therapies using reagent cartridges based on the drug class of interest. Others such as the Sonoclot and Impact Cone and Plate(let) Analyzer provide a more global view of coagulation, platelet function, and fibrinolysis, similar to TEG (Pakala and Waksman 2011; Zeidan et al. 2007).

### Platelet Disorders

Specific platelet disorders will be discussed in detail in other chapters of this book. The following table summarizes characteristic laboratory findings in different platelet disorders (Table 1.3).

## Role of the Pathologist in the Hemostasis Laboratory and Clinical Hemostasis Consultation

### Role of the Pathologist in the Hemostasis Laboratory

In the United States, laboratories are regulated under the Clinical Laboratory Improvement Amendments (CLIA), passed by Congress in 1988. This act established quality standards for laboratory testing to ensure the accuracy, reliability, and timeliness of patient test results (CLIA Regulations). Laboratories are accredited according to CLIA standards by deemed organizations, such as the College of American Pathologists (CAP).

The CLIA act established the role of the Laboratory Director, which is often performed by a pathologist. According to CLIA regulations, the Director of laboratories, such as the hemostasis laboratory, is responsible for the overall operation of the laboratory and ensures compliance with applicable regulations (CLIA Regulations). Laboratory Directors ensure that the testing systems employed provide quality laboratory service in the preanalytic, analytic, and postanalytic phases of testing. Laboratory Directors review and sign off on all new and substantially changed policies and procedures prior to implementation and whenever there is a change in Laboratory Director. The Laboratory Director establishes a quality management process in the laboratory and monitors key performance indicators, such as quality control and quality assurance, with corrective action, as necessary. For accreditation, laboratories must also perform proficiency testing, where laboratories test unknown specimens, with their results compared against peer groups. Laboratory Directors must make provisions for proficiency testing and review the laboratory's results. In addition, the Laboratory Director must ensure that there are appropriate numbers of trained individuals to perform testing and that the physical facility is adequate.

### Role of the Pathologist in the Clinical Hemostasis Consultation

In hemostasis testing, there are many unique issues which may make a pathologist's consultation helpful. Apart from the rapidly expanding knowledge of both bleeding and thrombotic disorders and a wide test menu, hemostasis testing is very sensitive to preanalytic issues (hemolysis, fill volume, time, temperature) and the interference of many commonly prescribed drugs.

The pathologist can serve an important role in the evaluation of a patient for a bleeding or thrombotic disorder. Several hemostasis laboratories in major medical centers have established consultative hemostasis testing services where clinicians are offered a panel or battery of initial hemostasis tests (Marques et al. 2011; Hayward et al. 2012). At the Cleveland Clinic, our consultative hemostasis testing service offers several interpretive panels, such as an elevated aPTT panel, an elevated PT/aPTT panel, a lupus anticoagulant panel, a hypercoagulation panel, and a von Willebrand disease panel.

When such a panel is ordered on a patient, the specimen is collected and processed and multiple aliquots are prepared and stored. A specialized physician, such as a hematopathologist, evaluates the initial results and then adds additional tests from the stored specimen, based on the patient's clinical scenario, medication history, and a laboratory-defined algorithm. When all testing is complete, the pathologist interprets the testing

results and issues a personalized written diagnostic report, indicating the cause and significance of the hemostasis abnormality.

This consultative hemostasis testing service may improve the efficiency and quality of patient care as it is designed to streamline the testing type and cost, where only the needed tests are performed. It can improve patient experience by decreasing the need for multiple phlebotomies and potentially decreasing the sample volume needed for diagnosis. It may also improve the patient outcome by preventing misdiagnosis due to the effect of interfering drugs; for example, where the effect of a direct thrombin inhibitor can be detected before erroneously diagnosing a lupus anticoagulant. In one such consultative hemostasis testing service, 77 % of surveyed ordering physicians felt that the service saved time to diagnosis, 78 % felt that it impacted the differential diagnosis, 72 % felt that it prevented misdiagnosis, while 72 % felt that it reduced the number of tests performed (Laposata et al. 2004).

## References


Adcock DM, Kressin DC, Marlar RA. Minimum specimen volume requirements for routine coagulation testing: dependence on citrate concentration. Am J Clin Pathol. 1998;109(5):595–9.

Adcock DM, Gosselin R, Kitchen S, Dwyre DM. The effect of dabigatran on select specialty coagulation assays. Am J Clin Pathol. 2013;139(1):102–9.

Afshari A, Wikkelso A, Brok J, Moller AM, Wetterslev J. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemotherapy versus usual care in patients with massive transfusion. Cochrane Database Syst Rev. 2011;16(3), CD007871.

Ageno W, Gallus AS, Wittkowsky A, Crowther M, Hylek EM, Palareti G, American college of chest physicians. Oral anticoagulant therapy: antithrombotic therapy and prevention of thrombosis, 9th ed: American college of chest physicians evidence-based clinical practice guidelines. Chest. 2012;141(2 Suppl):e44S–88.

Aillaud MF, Pouymayou K, Brunet D, Parrot G, Alessi MC, Amiral J, et al. New direct assay of free protein S antigen applied to diagnosis of protein S deficiency. Thromb Haemost. 1996;75(2):283–5.

Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CP, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. Am J Clin Pathol. 1992;98(6):637–46.

Baglin T, Gray E, Greaves M, Hunt BJ, Keeling D, Machin S, et al. Clinical guidelines for testing for hereditary thrombophilia. Br J Haematol. 2010;149(2):209–20.

Bailey EL, Harper TA, Pinkerton PH. The "therapeutic range" of the one-stage prothrombin time in the control of anticoagulant therapy: the effect of different thromboplastin preparations. Can Med Assoc J. 1971; 105(10):1041–3.

Ballard RB, Marques MB. Pathology consultation on the laboratory evaluation of thrombophilia: when, how, and why. Am J Clin Pathol. 2012;137(4):553–60.

Benjaber K, Constans J, Cougnard A, Salmi LR. High levels of factor VIIIc and risk of venous thrombosis: critical analysis of case control studies. Rev Med Interne. 2003;24:366–71.

Berndt MC, Ward CM, Booth WJ, Castaldi PA, Mazurov AV, Andrews RK. Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. Biochemistry (NY). 1992;31(45):11144–51.

Bolliger D, Seeberger MD, Tanaka KA. Principles and practice of thromboelastography in clinical coagulation management and transfusion practice. Transfus Med Rev. 2012;26(1):1–13.

Bowyer A, Kitchen S, Makris M. The responsiveness of different APTT reagents to mild factor VIII, IX and XI deficiencies. Int J Lab Hematol. 2011;33(2): 154–8.

Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/ Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Thromb Haemost. 1995;74(4):1185–90.

Breitenstein A, Camici GG, Tanner FC. Tissue factor: beyond coagulation in the cardiovascular system. Clin Sci (Lond). 2009;118(3):159–72.

Burch JW, Stanford N, Majerus PW. Inhibition of platelet prostaglandin synthetase by oral aspirin. J Clin Invest. 1978;61(2):314–9.

Castodi E, Hackeng TM. Regulation of coagulation by protein S. Curr Opin Hematol. 2008;15(5):529–36.

Chan WP, Lee CK, Kwong YL, Lam CK, Liang R. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. Blood. 1998;91(4):1135–9.

Chen A, Teruya J. Global hemostasis testing thromboelastography: old technology, new applications. Clin Lab Med. 2009;29(2):391–407.

Clauser S, Cramer-Bordé E. Role of platelet electron microscopy in the diagnosis of platelet disorders. Semin Thromb Hemost. 2009;35(2):213–23.

CLIA (Clinical Laboratory Improvement Amendments) Regulations 42 CFR 493, Centers for Disease Control and Prevention; 2004.

Colucci M, Semeraro N. Thrombin activatable fibrinolysis inhibitor: at the nexus of fibrinolysis and inflammation. Thromb Res. 2012;129(3):314–9.

Crescente M, Di Castelnuovo A, Iacoviello L, de Gaetano G, Cerletti C. PFA-100 closure time to predict cardiovascular events in aspirin-treated cardiovascular patients: A meta-analysis of 19 studies comprising 3,003 patients. Thromb Haemost. 2008;99(6):1129–31.

Cunningham MT, Brandt JT, Laposata M, Olson JD. Laboratory diagnosis of dysfibrinogenemia. Arch Pathol Lab Med. 2002;126(4):499–505.

Cunningham MT. Abnormal thrombin time. In: Kottke-Marchant K, Van Cott EM, editors. An algorithmic approach to hemostasis testing. Northfield, IL: Collage of American Pathologists (CAP); 2008.

Dager WE, Gosselin RC, Kitchen S, Dwyre D. Dabigatran effects on the international normalized ratio, activated partial thromboplastin time, thrombin time, and fibrinogen: a multicenter, in vitro study. Ann Pharmacother. 2012;46(12):1627–36.

Deb Heijer M, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiologic studies. J Thromb Haemost. 2005;3(2):292–9.

Dybiaer R. Quality assurance, accreditation, and certification; needs and possibilities. Clin Chem. 1994;40: 1416–20.

Dykes AC, Walker ID, McMahon AD, Islam SI, Tail RC. A study of protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113(3):636–41.

Dyszkiewicz-Korpanty A, Quinton R, Yassine J, Sarode R. The effect of a pneumatic tube transport system on PFA-100 trade mark closure time and whole blood platelet aggregation. J Thromb Haemost. 2004;2(2):354–6.

Dyszkiewicz-Korpanty AM, Frenkel EP, Sarode R. Approach to the assessment of platelet function: comparison between optical-based platelet-rich plasma and impedance-based whole blood platelet aggregation methods. Clin Appl Thromb Hemost. 2005; 11(1):25–35.

Eby C, Olson JD. Arterial and venous thrombosis in adults. In: Kottke-Marchant K, editor. Algorithmic approach to hemostasis testing. 1st ed. Northfield, IL: College of American Pathologists; 2008a. p. 239–66.

Eby C. Laboratory diagnosis of inherited thrombophilia. In: Kottke-Marchant K, editor. Algorithmic approach to hemostasis testing. 1st ed. Northfield, IL: College of American Pathologists; 2008b. p. 267–77.

Eldibany MM, Caprini JA. Hyperhomocysteinemia and thrombosis. Arch Pathol Lab Med. 2007;131(6):872–84.

Favaloro EJ. The utility of the PFA-100 in the identification of von willebrand disease: a concise review. Semin Thromb Hemost. 2006;32(5):537–45.

Favaloro EJ. Internal quality control and external quality assurance of platelet function tests. Semin Thromb Hemost. 2009;35(2):139–49.

Favaloro EJ, Lippi G, Koutts J. Laboratory testing of anticoagulants: the present and the future. Pathology. 2011;43(7):682–92.

Finazzi G, Brancaccio V, Moia M, Ciaverella N, Mazzucconi MG, Schinco PC, et al. Natural history and risk factors for thrombosis in 360 patients with antiphospholipid antibodies: a four-year prospective study from the Italian Registry. Am J Med. 1996;100(5):530–6.

Franco RF, Elion J, Tavella MH, Santos SE, Zago MA. The prevalence of factor V Arg306 → Thr (factor V Cambridge) and factor V Arg306 → Gly mutations in different human populations. Thromb Haemost. 1999;81(2):312–3.

Fritsma GA, Dembitzer FR, Randhawa A, Marques MB, Van Cott EM, Adcock-Funk D, Peerschke EI. Recommendations for appropriate activated partial thromboplastin time reagent selection and utilization. Am J Clin Pathol. 2012;137(6):904–8.

Froom P, Barak M. Prevalence and course of pseudothrombocytopenia in outpatients. Clin Chem Lab Med. 2011;49(1):111–4.

Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta-2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. Blood. 2003;102(8):2717–23.

Gallimore MJ, Harris SL, Jones DW, Winter M. Plasma levels of factor XII, prekallikrein and high molecular weight kininogen in normal blood donors and patients having suffered venous thrombosis. Thromb Res. 2004;114(2):91–6.

Garcia DA, Baglin TP, Weitz JI, Samama MM. American college of chest physicians parenteral anticoagulants: antithrombotic therapy and prevention of thrombosis, 9th ed: American college of chest physicians evidence-based clinical practice guidelines. Chest. 2012;141(2 Suppl):e24S–43.

Gardiner C, Machin S, Mackie I. Point-of-care testing in hemostasis. In: Kitchen S, Olson JD, Preston EF, editors. Quality in laboratory hemostasis and thrombosis. West Sussex, UK: Wiley-Blackwell; 2009. p. 72–80.

Geiger J, Brich J, Honig-Liedl P, Eigenthaler M, Schanzenbacher P, Herbert JM, et al. Specific impairment of human platelet P2Y(AC) ADP receptor-mediated signaling by the antiplatelet drug clopidogrel. Arterioscler Thromb Vasc Biol. 1999;19(8):2007–11.

George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. N Engl J Med. 1991;324(1):27–39.

George JN. Platelets. Lancet. 2000;355(9214):1531–9.

Girolami A, Candeo N, De Marinis GB, Bonamigo E, Girolami B. Comparative incidence of thrombosis in reported cases of deficiencies of factors of the contact phase of blood coagulation. J Thromb Thrombolysis. 2011;31(1):57–63.

Glassman A, Abram M, Baxter G, Swett A. Euglobulin lysis times: an update. Ann Clin Lab Sci. 1993; 23(5):329–32.

Gouin-Thibaut I, Martin-Toutain I, Peynaud-Debayle E, Marion S, Napol P, Alhenc-Gelas M, AGEPS Hemostasis Group. Monitoring unfractionated heparin with APTT: a French collaborative study comparing sensitivity to heparin of 15 APTT reagents. Thromb Res. 2012;129(5):666–7.

Greengard JS, Sun X, Xu X, et al. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet. 1994;343(8909):1361–2.

Griffin JH, Zlokovic BV, Mosnier LO. Protein C anticoagulant and cytoprotective pathways. Int J Hematol. 2012;95(4):333–45.

Guba S, Fonseca V, Link L. Hyperhomocysteinemia and thrombosis. Semin Thromb Hemost. 1999;25(3): 291–309.

Gulati GL, Hyland LJ, Kocher W, Schwarting R. Changes in automated complete blood count and differential leukocyte count results in induced by storage of blood at room temperature. Arch Pathol Lab Med. 2002; 126(3):336–42.

Halbmayer WM, Weigel G, Quehenberger P, Tomasits J, Haushofer AC, Aspoeck G, Loacker L, Schnapka-Koepf M, Goebel G, Griesmacher A. Interference of the new oral anticoagulant dabigatran with frequently used coagulation tests. Clin Chem Lab Med. 2012;50(9):1601–5.

Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. N Engl J Med. 1972;287(4):155–9.

Harrington RA. Overview of clinical trials of glycoprotein IIb-IIIa inhibitors in acute coronary syndromes. Am Heart J. 1999;138(4 Pt 2):276–86.

Harrison P. Measuring platelet function? Hematol J. 2004;5 Suppl 3:S164–9.

Harrison P. Platelet function analysis. Blood Rev. 2005;19:111–23.

Hartwig JH. The platelet: form and function. Semin Hematol. 2006;43(1 Suppl 1):S94–100.

Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia. Report on a study of the SSC subcommittee on fibrinogen. Thromb Haemost. 1995;73(1): 151–61.

Hayes T. Dysfibrinogenemia and thrombosis. Arch Pathol Lab Med. 2002;126(11):1387–90.

Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK, Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost. 2006;4(2):312–9.

Hayward CP, Moffat KA, Plumhoff E, Van Cott EM. Approaches to investigating common bleeding disorders: an evaluation of North American coagulation laboratory practices. Am J Hematol. 2012;87 Suppl 1:S45–50.

Hayward CPM, Eilekboom J. Platelet function testing: quality assurance. Semin Thromb Hemost. 2007;33(3):273–82.

Hayward CP, Pai M, Liu Y, Moffat KA, Seecharan J, Webert JE, et al. Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. J Thromb Haemost. 2009;7(4):676–84.

Hayward CP, Moffat KA, Raby A, Israel S, Plumhoff E, Flynn G, et al. Development of north American consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. Am J Clin Pathol. 2010;134(6):955–63.

Heit JA. Thrombophilia: common questions on laboratory assessment and management. Hematology Am Soc Hematol Educ Program. 2007;2007:127–35.

Hoffman M, Monroe III DM. A cell-based model of hemostasis. Thromb Haemost. 2001;85(6):958–65.

Hoffman M, Monroe DM. Coagulation 2006: a modern view of hemostasis. Hematol Oncol Clin North Am. 2007;21(1):1–11.

Hubner U, Bockel-Frohnhofer N, Hummel B, Geisel J. The effect of a pneumatic tube transport system on platelet aggregation using optical aggregometry and the PFA-100. Clin Lab. 2010;56(1-2):59–64.

Jackson SP. The growing complexity of platelet aggregation. Blood. 2007;109(12):5087–95.

Jenkins CS, Phillips DR, Clemetson KJ, Meyer D, Larrieu MJ, Luscher EF. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation. Studies of the proteins on platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. J Clin Invest. 1976;57(1):112–24.

Jenkins PV, Rawley O, Smith OP, O'Donnell JS. Elevated factor VIII levels and risk of venous thrombosis. Br J Haematol. 2012;157(6):653–63.

Kasthuri RS, Roubey RA. Warfarin and the antiphospholipid syndrome: does one size fit all? Arthritis Rheum. 2007;57(8):1346–7.

Kershaw G, Favaloro EJ. Laboratory identification of factor inhibitors: an update. Pathology. 2012;44(4): 293–302.

Khor B, Van Cott EM. Laboratory tests for protein C deficiency. Am J Hematol. 2010a;85(6):440–2.

Khor B, Van Cott EM. Laboratory tests for antithrombin deficiency. Am J Hematol. 2010b;85(12):947–50.

Khor B, Van Cott EM. Laboratory evaluation of hypercoagulability. Clin Lab Med. 2009;29(2):339–66.

Kitchen DP, Kitchen S, Jennings I, Woods T, Walker I. Quality assurance and quality control of thromboelastography and rotational thromboelastometry: the UK NEQAS for blood coagulation experience. Semin Thromb Hemost. 2010;36(7):757–63.

Kottke-Marchant K. Laboratory diagnosis of hemorrhagic and thrombotic disorders. Hematol Oncol Clin North Am. 1994;8(4):809–53.

Kottke-Marchant K, Powers JB, Brooks L, Kundu S, Christie DJ. The effect of antiplatelet drugs, heparin, and preanalytical variables on platelet function detected by the platelet function analyzer (PFA-100). Clin Appl Thromb Hemost. 1999;5(2):122–30.

Kottke-Marchant K, Corcoran G. The laboratory diagnosis of platelet disorders. Arch Pathol Lab Med. 2002;126(2):133–46.

Kottke-Marchant K, Duncan A. Antithrombin deficiency: issues in laboratory diagnosis. Arch Pathol Lab Med. 2002;126(11):1326–36.

Kottke-Marchant K. Platelet testing. In: Kottke-Marchant K, editor. Algorithmic approach to hemostasis testing. 1st ed. Northfield, IL: College of American Pathologists; 2008. p. 93–112.

Kraaijenhagen RA, in't Anker PS, Koopman MM, Reitsma PH, Prins MH, van den Ende A, et al. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism. Thromb Haemost. 2000;83(1):5–9.

Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer—PFA-100. Semin Thromb Hemost. 1995;21 Suppl 2:106–12.

Kyrle PA, Minar E, Hirschl M, Bialonczyk C, Stain M, Schneider B, et al. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. N Engl J Med. 2000;343(7):457–62.

Laga AC, Cheves TA, Sweeney JD. The effect of specimen hemolysis on coagulation test results. Am J Clin Pathol. 2006;126(5):748–55.

Laposata ME, Laposata M, Van Cott EM, Buchner DS, Kashalo MS, Dighe AS. Physician survey of a laboratory medicine interpretive service and evaluation of the influence of interpretations on laboratory test ordering. Arch Pathol Lab Med. 2004;128:1424–7.

Ledford M, Friedman KD, Hessner MJ, Moehlenkamp CM, Williams TM, Larson RS. A multi-site study for detection of the factor V (Leiden) mutation from genomic DNA using a homogeneous invader microtiter plate fluorescence resonance energy transfer (FRET) assay. J Mol Diagn. 2000;2:97–104.

Leung LLK. Overview of hemostasis. In: Mannucci PM, Tirnauer JS, editors. UpToDate [Internet]. Waltham, MA; 2013 [cited 2012 Dec 21].

Lind SE. The bleeding time does not predict surgical bleeding. Blood. 1991;77(12):2547–52.

Loeliger EA, Poller L, Samama M, Thomson JM, Van den Besselaar AM, Vermylen J, Verstraete M. Questions and answers on prothrombin time standardisation in oral anticoagulant control. Thromb Haemost. 1985;54(2):515–7.

MacDonald SG, Luddington RJ. Critical factors contributing to the thromboelastography trace. Semin Thromb Hemost. 2010;36(7):712–22.

Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laffan M, British Committee for Standards in Haematology. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol. 2013;35(1):1–13.

Mann KG. Thrombin formation. Chest. 2003;124(3 Suppl):4S–10.

Marai I, Gillburd B, Blank M, Shoenfeld Y. Anticardiolipin and anti-beta2-glycoprotein I (beta2GP-I) antibody assays as screening for anti-phospholipid syndrome. Hum Antibodies. 2003;12(3):57–62.

Marques MB, Anastasi J, Ashwood E, Baron B, Fitzgerald R, Fung M, Krasowski M, Laposata M, Nester T, Rinder HM. The clinical pathologist as consultant. Am J Clin Pathol. 2011;135(1):11–2.

Margetic S. Diagnostic algorithm for thrombophilia screening. Clin Chem Lab Med. 2010;48 Suppl 1:S27–39.

Marlar RA, Potts RM, Marlar AA. Effect on routine and special coagulation testing values of citrate anticoagulant adjustment in patients with high hematocrit values. Am J Clin Pathol. 2006;126(3):400–5.

Mazodier K, Arnaud L, Mathian A, Costedoat-Chalumeau N, Haroche J, Frances C, Harlé JR, Pernod G, Lespessailles E, Gaudin P, Charlanne H, Hachulla E, Niaudet P, Piette JC, Amoura Z. Lupus anticoagulant-hypoprothrombinemia syndrome: report of 8 cases and review of the literature. Medicine (Baltimore). 2012;91(5):251–60.

Maurrissen LF, Thomassen MC, Nicolaes GA, Dahlback B, Tans G, Rosing J, et al. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. Blood. 2008;111(6):3034–41.

McCraw A, Hillarp A, Echenagucia M. Considerations in the laboratory assessment of haemostasis. Haemophilia. 2010;16 Suppl 5:74–8.

McGlasson DL, Fritsma GA. Whole blood platelet aggregometry and platelet function testing. Semin Thromb Hemost. 2009;35(2):168–80.

McGlennen RC, Key NS. Clinical and laboratory management of the prothrombin G20210A mutation. Arch Pathol Lab Med. 2002;126(11):1319–25.

Mhawech P, Saleem A. Inherited giant platelet disorders. Classification and literature review. Am J Clin Pathol. 2000;113(2):176–90.

Michelson AD. Flow cytometry: a clinical test of platelet function. Blood. 1996;87(12):4925–36.

Michelson AD. Evaluation of platelet function by flow cytometry. Pathophysiol Haemost Thromb. 2006;35(1–2):67–82.

Middeldorp S. Evidence-based approach to thrombophilia testing. J Thromb Thrombolysis. 2011;31(3):275–81.

Milos M, Herak DC, Zadro R. Discrepancies between APTT results determined with different evaluation modes on automated coagulation analyzers. Int J Lab Hematol. 2010;32(1 Pt 2):33–9.

Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(2):295–306.

Moffat KA, Ledford-Kraemer MR, Nichols WL, Hayward CP, North American Specialized Coagulation Laboratory Association. Variability in clinical laboratory practice in testing for disorders of platelet function: results of two surveys of the North American specialized coagulation laboratory association. Thromb Haemost. 2005;93(3):549–53.

Moffat KA, Ledford-Kraemer MR, Plumhoff EA, McKay H, Nichols WL, Meijer P, et al. Are laboratories following published recommendations for lupus anticoagulant testing? An international evaluation of practices. Thromb Haemost. 2009;101(1):178–84.

Moll S, Ortel TL. Monitoring warfarin therapy in patients with lupus anticoagulants. Ann Intern Med. 1997;127(3):177–85.

Moore GW, Savidge GF. The dilution effect of equal volume mixing studies compromises confirmation of inhibition by lupus anticoagulants even when mixture

specific reference ranges are applied. Thromb Res. 2006;118(4):523–8.
Moreno A, Menke D. Assessment of platelet numbers and morphology in the peripheral blood smear. Clin Lab Med. 2002;22(1):193–213.
Murugesan G, Jans S, Han J-Y. Single nucleotide polymorphisms in molecular diagnostics. In: Kottke-Marchant K, Davis BH, editors. Laboratory hematology practice. International Society for Laboratory Hematology. West Sussex, UK: Wiley-Blackwell; 2012. p. 168–80.
Nichols WL, Kottke-Marchant K, Ledford-Kraemer MR, Homburger HA, Cardel LK. Lupus anticoagulants, antiphospholipid antibodies, and antiphospholipid syndrome. In: Kottke-Marchant K, Davis BH, editors. Laboratory hematology practice. International Society for Laboratory Hematology. West Sussex, UK: Wiley-Blackwell; 2012. p. 509–25.
Norstrom E, Thorelli E, Dahlback B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. Blood. 2002;100(2):524–30.
Nurden P, Nurden A. Diagnostic assessment of platelet function. In: Kitchen S, Olson JD, Preston FE, editors. Quality in laboratory hemostasis and thrombosis. West Sussex, UK: Wiley-Blackwell; 2009. p. 110–24.
O'Donnell J, Mumford AD, Manning RA, Laffan M. Elevation of FVIII: C in venous thromboembolism is persistent and independent of the acute phase response. Thromb Haemost. 2000;83(1):10–3.
Oger E, Lacut K, Van Dreden P, Bressollette L, Abgrall JF, Blouch MT, et al. High plasma concentration of factor VIII coagulant is also a risk factor for venous thromboembolism in the elderly. Haematologica. 2003;88(4):465–9.
Ota S, Yamada N, Ogihara Y, Tsuji A, Ishikura K, Nakamura M, et al. High plasma level of factor VIII: an important risk factor for venous thromboembolism. Circ J. 2011;75(6):1472–5.
Pakala R, Waksman R. Currently available methods for platelet function analysis: advantages and disadvantages. Cardiovasc Revasc Med. 2011;12(5):312–22.
Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M, et al. Update of the guidelines for lupus anticoagulant detection. J Thromb Haemost. 2009;7(10):1737–40.
Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock Jr WA, et al. The preoperative bleeding time test lacks clinical benefit: college of American pathologists' and American society of clinical pathologists' position article. Arch Surg. 1998;133(2):134–9.
Picard V, Nowak-Gottl U, Biron-Andreani C, Fouassier M, Frere C, Goualt-Heilman M, et al. Molecular bases of antithrombin deficiency: twenty-two novel mutations in the antithrombin gene. Hum Mutat. 2000;27(6):600.
Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88(1):3698–703.
Press RD, Bauer KA, Kujovich JL, Heit JA. Clinical utility of factor V Leiden (R506Q) testing for the diagnosis and management of thromboembolic disorders. Arch Pathol Lab Med. 2002;126(11):1304–18.
Preston FE, Lippi G, Favaloro EJ, Jayandharan GR, Edison ES, Srivastava A. Quality issues in laboratory haemostasis. Haemophilia. 2010;16 Suppl 5:93–9.
Rao AK, Niewiarowski S, Guzzo J, Day HJ. Antithrombin III levels during heparin therapy. Thromb Res. 1981;24(1–2):181–6.
Rapaport SI, Rao LV. The tissue factor pathway: how it has become a "prima ballerina". Thromb Haemost. 1995;74:7.
Ray JG, Shmorgun D, Chan WS. Common C677T polymorphism of the methylenetetrahydrofolate reductase gene and the risk of venous thromboembolism: meta-analysis of 31 studies. Pathophysiol Haemost Thromb. 2002;32(2):51–8.
Reber G, de Moerloose P. Anti-beta-2-glycoprotein I antibodies: when and how should they be measured? Thromb Res. 2004;114(5–6):527–31.
Rendu F, Brohard-Bohn B. The platelet release reaction: Granules' constituents, secretion and functions. Platelets. 2001;12(5):261–73.
Ricker PM, Miletich JP, Hennekens CH, Burling JE. Ethnic distribution of factor V Leiden in 4047 men and women. Implications of venous thromboembolism screening. JAMA. 1997;277(16):1305–7.
Roberts HR, Monroe DM, Oliver JA, Chang JY, Hoffman M. Newer concepts of blood coagulation. Haemophilia. 1998;4(4):331–4.
Rogers HJ, Kottke-Marchant K. Antithrombin deficiency. In: Gulati G, Filicko-O'Hara J, Krause JR, editors. Case studies in hematology and coagulation. Chicago, IL: American Society for Clinical Pathology Press; 2012. p. 416–8.
Romero-Guzman LT, Lopez-Karpovitch X, Paredes R, Barrales-Benitez O, Piedras J. Detection of platelet-associated immunoglobulins by flow cytometry for the diagnosis of immune thrombocytopenia: a prospective study and critical review. Haematologica. 2000;85(6):627–31.
Rosendaal FR, Doggen CJM, Zivelin A, Arruda VR, Aiach M, Siscovick DS, et al. Geographic distribution of the 20210 G to A prothrombin variant. Thromb Haemost. 1998;79(4):706–8.
Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood. 1995;85(6):1504–8.
Rosing J, Maurissen LF, Tchaikovski SN, Tans G, Hackeng TM. Protein S is a cofactor for tissue factor pathway inhibitor. Thromb Res. 2008;1 Suppl 122:S60–3.
Schmaier AH, Miller JL. Coagulation and fibrinolysis. In: McPherson RA, Pincus MR, editors. Henry's Clinical diagnosis and management by laboratory methods. 22nd ed. Philadelphia: Saunders; 2011.

Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, et al. European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. Thromb Haemost. 1998;79(5):885–96.

Sermon AM, Smith JM, Maclean R, Kitchen S. An International Sensitivity Index (ISI) derived from patients with abnormal liver function improves agreement between INRs determined with different reagents. Thromb Haemost. 2010;103(4):757–65.

Shovlin CL, Angus G, Manning RA, Okoli GN, Govani FS, Elderfield K, Birdsey GM, Mollet IG, Laffan MA, Mauri FA. Endothelial cell processing and alternatively spliced transcripts of factor VIII: potential implications for coagulation cascades and pulmonary hypertension. PLoS One. 2010;5(2):e9154.

Swallow RA, Agarwala RA, Dawkins KD, Curzen NP. Thromboelastography: potential bedside tool to assess the effects of antiplatelet therapy? Platelets. 2006;17(6):385–92.

Ten Kate MK, Platteel M, Mulder R, Terpstra P, Nixolaes GA, Reitsma PH, et al. PROS1 analysis in 87 pedigrees with hereditary protein S deficiency demonstrates striking genotype-phenotype associations. Hum Mutat. 2008;29(7):939–47.

Triplett DA. Antiphospholipid antibodies. Arch Pathol Lab Med. 2002;126(11):1424–9.

Tripodi A, Chantarangkul V, Clerici M, Negri B, Galli M, Mannucci PM. Laboratory control of oral anticoagulant treatment by the INR system in patients with the antiphospholipid syndrome and lupus anticoagulant. Results of a collaborative study involving nine commercial thromboplastins. Br J Haematol. 2001;115(3):672–8.

Tripodi A, Chantarangkul V, Martinelli I, Bucciarelli P, Mannucci PM. A shortened activated partial thromboplastin time is associated with the risk of venous thromboembolism. Blood. 2004;104(12):3631–4.

Tripodi A, Anstee QM, Sogaard KK, Primignani M, Valla DC. Hypercoagulability in cirrhosis: causes and consequences. J Thromb Haemost. 2011;9(9):1713–23.

Van Cott EM, Eby C. Antiphospholipid antibodies. In: Kottke-Marchant K, editor. An algorithmic approach to hemostasis testing. Northfield, IL: College of American Pathologists Press; 2008. p. 295–304.

Van Cott EM, Laposata M, Prins MH. Laboratory evaluation of hypercoagulability with venous or arterial thrombosis: venous thromboembolism, myocardial infarction, stroke and other conditions. Arch Pathol Lab Med. 2002;126(11):1281–95.

Verbruggen B. Diagnosis and quantification of factor VIII inhibitors. Haemophilia. 2010;16(102):20–4.

Verbruggen B, van Heerde WL, Laros-van Gorkom BA. Improvements in factor VIII inhibitor detection: from Bethesda to Nijmegen. Semin Thromb Hemost. 2009;35(8):752–9.

Verhovsek M, Moffat KA, Hayward CP. Laboratory testing for fibrinogen abnormalities. Am J Hematol. 2008;83(12):928–31.

Vossen CY, Conard J, Fontcuberta J, Makris M, Van Der Meer FJ, Pabinger I, et al. Familial thrombophilia and lifetime risk of venous thrombosis. J Thromb Haemost. 2004;2(9):1526–32.

Wall JE, Buijs-Wilts M, Arnold JT, Wang W, White MM, Jennings LK, et al. A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. Br J Haematol. 1995;89(2):380–5.

Yohe S, Olson J. Thrombophilia: assays and interpretation. In: Kottke-Marchant K, Davis BH, editors. Laboratory hematology practice. International Society for Laboratory Hematology. West Sussex, UK: Wiley-Blackwell Publishing Ltd; 2012. p. 492–508.

Zehnder JL. Clinical use of coagulation tests. In: Leung LLK, Tirnauer JS, editors. UpToDate [Internet]. Waltham, MA; 2013 [cited 2013 Jan 11].

Zeidan AM, Kouides PA, Tara MA, Fricke WA. Platelet function testing: state of the art. Expert Rev Cardiovasc Ther. 2007;5(5):955–67.

Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, et al. A single genetic origin for a common Caucasian risk factor for venous thrombosis. Blood. 1997;89(2):397–402.