Testing for Inherited and Acquired Thrombotic Disorders

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Virchow's triad, the major risk factors that predispose to thrombosis, include vascular injury, stasis, and hypercoagulability. Arterial and venous thrombosis have different pathogenic mechanisms. For example, arterial thrombosis primarily involves vascular injury (atherosclerosis) and platelet deposition. Venous thrombosis primarily involves stasis of blood flow and hypercoagulability, which is defined as altered blood composition associated with a thrombotic tendency. Fibrin deposition is the normal consequence of venous thrombosis.

The laboratory evaluation of inherited thrombosis is based on identifying abnormalities in regulatory proteins of coagulation; also known as natural anticoagulants, as well as mutations of other coagulation proteins. Over the past 20 years, major inherited etiologies of thrombosis including the factor V Leiden and prothrombin gene mutations have been identified. Acquired etiologies of thrombosis, including cancer, antiphospholipid antibodies, and hyperlipidemia are more common than inherited etiologies. This chapter will summarize the pathologic basis for thrombosis as related to laboratory testing for inherited and acquired etiologies. Methods for laboratory evaluation of thrombosis will be presented, and the utility of laboratory thrombosis testing will be discussed. All assays discussed in this chapter have FDA approval.

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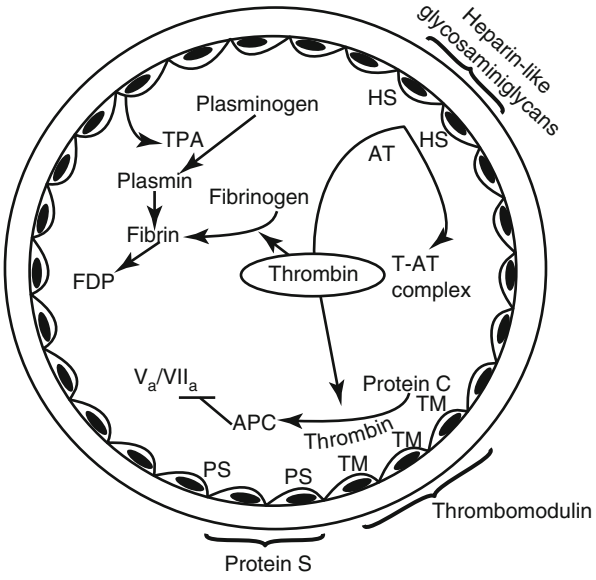

Fig. 9.1 Regulation of thrombin activity by vascular endothelium. The effects of major antithrombotic properties of the blood vessel wall are shown. Regulation of thrombin activity is important because thrombin is a major factor in thrombosis (platelet activation, fibrin formation). Abnormalities in regulation of thrombin activity may lead to hypercoagulability and an increased risk of thrombosis. Major antithrombotic properties include: heparin-like glycosaminoglycans (heparan sulfate, *HS*) on the luminal surface that catalyze antithrombin (*AT*) inhibition of thrombin, generating inactive thrombin-antithrombin (*T-AT*) complexes; thrombomodulin (*TM*), an endothelial cell receptor for thrombin. The thrombin-TM complex converts protein C to APC; protein S (*PS*) functions as a cofactor binding protein for APC, permitting inactivation of factors $V_a/VIII_a$, resulting in inactivation of coagulation; and endothelial cell secretion of tissue-plasminogen activator (*TPA*) that initiates fibrinolysis. Most of the coagulation components shown in this figure can be assayed by the laboratory to identify thrombosis risk (From Rodgers [2], with permission)

9.1 Pathophysiology of Thrombosis

Vascular endothelium plays a pivotal role in maintaining thromboresistance of the blood. The coagulation mechanism is modulated by several endothelial cell regulatory mechanisms [1]. Figure 9.1 summarizes the antithrombotic properties of endothelium that can be evaluated in the laboratory. A key regulatory mechanism is the protein C pathway, which consists of two vitamin K–dependent plasma proteins—protein C and protein S. Protein C is converted to an active form, activated protein C (APC); this activation is mediated by an endothelial cell receptor, thrombomodulin. Thrombomodulin forms a complex with thrombin; this complex activates protein C generating APC, which in turn inactivates factors Va and VIIIa. Protein S binds to the endothelial cell surface, providing a receptor for APC [1]. A factor V gene mutation called factor V Leiden results in thrombosis due to the inability of APC to

degrade the abnormal factor V_a molecule. This phenomenon is termed APC resistance and can be measured by the laboratory [3].

Antithrombin is a natural anticoagulant that irreversibly binds to and inactivates activated clotting factors such as factor Xa and thrombin. This inactivation is catalyzed by heparin-like glycosaminoglycans on the endothelial cell surface (see Fig. 9.1) or by therapeutic heparin [1].

The fibrinolytic mechanism components include plasminogen, tissue-plasminogen activator (TPA), plasmin, α_2 – antiplasmin, and plasminogen activator inhibitor (PAI-1). Fibrinolysis is initiated when vascular thrombosis triggers endothelial cell secretion of TPA. In the presence of TPA, plasminogen is converted to plasmin that degrades fibrin clots. Plasmin activity is regulated by α_2 – antiplasmin, while TPA activity is regulated by PAI-1. Either deficiency or excess of these components may occur leading to hypofibrinolysis and a thrombotic risk or hyperfibrinolysis and a bleeding risk [1].

9.2 The Inherited Thrombotic Disorders

Table 9.1 summarizes the inherited thrombotic disorders and describes their prevalence, inheritance patterns, and clinical features. Abnormalities of the protein C pathway (protein C, protein S, factor V Leiden, thrombomodulin) constitute most cases of inherited thrombosis [3]. Most inherited disorders are transmitted in an autosomal dominant manner, and venous thromboembolism is the usual clinical feature. The importance of inherited fibrinolytic disorders (TPA deficiency or excess PAI-1 activity) is uncertain.

Another common inherited thrombotic disorder is the prothrombin gene mutation. This mutation is associated with elevated plasma prothrombin levels, which may explain the predisposition to thrombosis [5].

Homocysteinemia is a metabolic disorder associated with thrombosis. Although pediatric patients present clinically with homozygous homocysteinemia (homocystinuria), adult patients heterozygous for homocysteinemia have primarily premature arterial disease (myocardial infarction, stroke, peripheral vascular disease). Heterozygous homocysteinemia may account for a significant number of patients with arterial vascular disease in the absence of traditional risk factors (e.g., smoking, hypertension, hyperlipidemia). Between 1 and 2 % of the general population have heterozygous homocysteinemia. Homocysteinemia is also associated with venous thromboembolism [6].

A recently described inherited risk factor for thrombosis is elevated levels of factor VIII activity. Although factor VIII is an acute-phase response protein, as many as 10–20 % of patients with recurrent venous thrombosis have elevated factor VIII levels as their only risk factor [3].

Increased levels of other coagulation factors, including fibrinogen, factor IX, and factor XI have also been associated with thrombosis. However, routine laboratory testing of these factors is not recommended by the College of American Pathologists Consensus Conference on Thrombophilia (see Table 9.2).

Table 9.1 Summary of the inherited thrombotic disorders

Classification and disorders	Inheritance	Estimated prevalence[a]	Clinical features
Deficiency or qualitative abnormalities of inhibitors to activated coagulation factors			
AT deficiency	AD	1 %	Venous thromboembolism (usual and unusual sites), heparin resistance
TM deficiency	AD	1–5 %	Venous thrombosis
Protein C deficiency	AD	1–5 %	Venous thromboembolism
Protein S deficiency	AD	1–5 %	Venous and arterial thromboembolism
APC resistance due to Factor V Leiden	AD	20–50 %	Venous thromboembolism
Abnormality of coagulation zymogen or cofactor			
Prothrombin mutation	AD	5–10 %	Venous thromboembolism
Elevated factor VIII	Unknown	20–25 %	Venous thromboembolism
Elevated factor IX	Unknown	~10 %	Venous thromboembolism
Elevated factor XI	Unknown	~10 %	Venous thromboembolism
Impaired clot lysis			
Dysfibrinogenemia	AD	1–2 %	Venous thrombosis >arterial thrombosis
Plasminogen deficiency	AD, AR	1–2 %	Venous thromboembolism
TPA deficiency	AD	?	Venous thromboembolism
Excess PAI-1 activity	AD	?	Venous thromboembolism and arterial thrombosis
Metabolic defect			
Homocysteinemia	AR	1 in 300,000 live births;	Arterial and venous thrombosis (homozygous patients);
		10–25 % of patients with recurrent thrombosis	Premature development of coronary and cerebral coronary and cerebral arterial thrombotic disease (heterozygous patients)

Source: Robetorye and Rodgers [4]
Abbreviations: *AT* antithrombin, *APC* activated protein C, *TPA* tissue plasminogen activator, *PAI-1* plasminogen activator inhibitor-1, *TM* thrombomodulin, *AD* autosomal dominant, *AR* autosomal recessive, *?* uncertain prevalence of abnormal fibrinolysis
[a]Prevalence data are estimated by pooling information from studies in which large groups of patients with thrombosis were screened for these disorders. Results are expressed in terms of a percentage that each disorder might constitute of the total patient population with inherited thrombosis (Assays for TM mutations are not widely available)

9.3 General Principles of Thrombosis Testing

1. Laboratory evaluation should be postponed until 2–3 months after the acute thrombotic event when the patient is clinically well and has not received anticoagulant therapy for 2 weeks. Thrombosis induces an acute-phase response that makes

Table 9.2 Summary of the College of American Pathologists' recommendations on laboratory testing for inherited thrombosis

Thrombotic disorder	Who should be tested?	Test method(s)	Comments
Factor V Leiden	First VTE at age <50 years Recurrent VTE First unprovoked VTE First VTE, unusual site First VTE, positive family history First VTE related to pregnancy or hormonal therapy Unexplained second or third trimester pregnancy loss	APC resistance assay using factor-V deficient plasma or DNA-based assay	Patients with relatives who are known to have FVL should be tested directly with DNA-based assays. Patients with positive APC resistance assays should have confirmatory DNA tests.
Prothrombin gene mutation	As above	DNA-based assay	Prothrombin activity assays should not be used
Homocysteinemia	Arterial vascular disease Controversial for VTE	HPLC or immunoassays	Genotyping for MTHFR mutations is not recommended. Fasting may not be necessary. Proper sample processing is necessary. Testing in VTE patients may be appropriate to identify and treat affected patients with vitamins.
Protein C deficiency	Infants with neonatal purpura fulminans VTE patient from a family with known PC deficiency	Chromogenic substrate assays are preferred	Avoid testing during acute thrombosis or anticoagulant therapy. Exclude causes of acquired PC deficiency. Consider age-dependent reference ranges.
	Asymptomatic female from a known PC-deficient family prior to hormonal therapy	Functional assays are useful Immunologic assays are discouraged	

(continued)

Table 9.2 (continued)

Thrombotic disorder	Who should be tested?	Test method(s)	Comments
Protein S deficiency	Patient with VTE from a family with known PS deficiency	Functional assay or Immunoassay for free PS Total PS antigen assays not recommended	Abnormal functional assay results should be confirmed with an immunoassay for free PS. Exclude acquired causes of PS deficiency. Avoid testing during acute thrombosis, anticoagulant therapy, and pregnancy. Consider age- and gender-dependent reference ranges.
Antithrombin deficiency	Patient with VTE from a family with known AT deficiency Asymptomatic female from a known AT-deficient family prior to hormonal therapy	Chromogenic substrate assays are preferred AT antigen assays not recommended	Exclude acquired causes of AT deficiency. Avoid testing during acute thrombosis or anticoagulant therapy.
Elevated factor VIII levels	Controversial	Factor VIII activity assay	Test 6 months after thrombosis. Avoid anticoagulant therapy.
Dysfibrinogenemia	Not recommended		
Heparin cofactor II	Not recommended		
Factor XIII polymorphisms	Not recommended		
Plasminogen activator inhibitor-1	Not recommended		
Plasminogen deficiency	Test in non-DVT patients with ligneous conjunctivitis		

Source: Greer et al. [7], with permission
From the College of American Pathologists' Consensus Conference on Thrombophilia [8], with permission
FVL factor V Leiden, *VTE* venous thromboembolism, *APC* activated protein C, *HPLC* high performance liquid chromatography, *MTHFR* methylenetetrahydrofolate reductase, *PC* protein C, *PS* protein S, *AT* antithrombin

interpretation of coagulation-based tests difficult. Reliable data for assays such as antithrombin, protein C, and protein S activities are best obtained in the absence of anticoagulant therapy. If anticoagulants cannot be discontinued in the affected patient, then surrogate testing of symptomatic family members who are not receiving anticoagulants can be done. However, if DNA-based assays (for the factor V Leiden or the prothrombin gene mutations) are performed, these results will not be affected by acute-phase changes of thrombosis or anticoagulant therapy. Similarly, homocysteine testing will not be affected by acute-phase changes or anticoagulant therapy. If factor VIII activity testing is to be done, it should be deferred until 6 months after the thrombotic event [3].

2. The probability of obtaining positive thrombosis testing results is increased if the patient population being investigated is restricted to young patients (<50 years of age) with recurrent thrombosis or patients with a single event and a positive family history for thrombosis [3].
3. Functional coagulation assays are recommended over immunologic assays for evaluation of antithrombin, protein C, or protein S deficiencies. Functional assays detect both quantitative deficiency and qualitative abnormality of the protein. On the other hand, functional assays are affected by anticoagulant therapy, and interpretation of abnormal functional assay results must take into account whether the patient is receiving anticoagulants [3].
4. Assay for common etiologies first (factor V Leiden/APC resistance, prothrombin gene mutation, homocysteinemia) [3].
5. Heterozygous homocysteinemia should be considered as a cause for thrombosis in middle-aged patients with premature vascular disease as well as a cause of venous thrombosis [3].

9.4 Laboratory Testing Strategy for Inherited Thrombosis

There are two testing strategies for inherited thrombosis-arterial and venous etiologies. Most cases of arterial thrombosis are not inherited, but acquired, including disorders such as diabetes, hyperlipidemia, and other causes of atherosclerosis, plus other etiologies such as vasculitis, myeloproliferative disorders, etc. Inherited etiologies for arterial thrombosis include elevated levels of PAI-1, homocysteinemia, and some patients with protein C or S deficiencies.

In contrast to the limited etiologies for arterial thrombosis, inherited venous thrombosis testing is more detailed. Figure 9.2 depicts one suggested strategy for testing in the venous thrombosis setting [10]. Assay of the most common etiologies is done initially (factor V Leiden/APC resistance, prothrombin gene mutation, homocysteinemia). If these tests are normal, then evaluation of uncommon causes of venous thrombosis are done (antithrombin, protein C, protein S). If the patient is to be evaluated for elevated factor VIII levels, this should be deferred for at least 6 months.

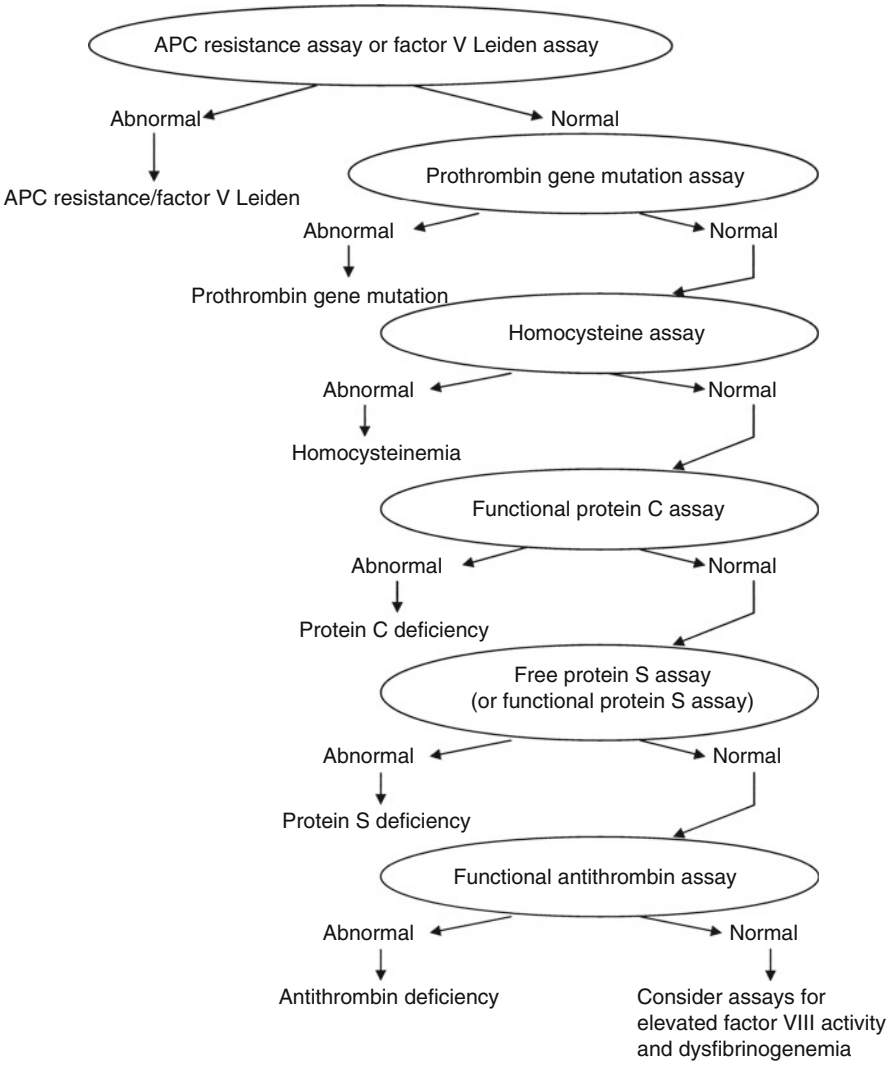

Fig. 9.2 An algorithm approach for the evaluation of patients with inherited venous thrombosis. It is assumed that patients have been appropriately selected and that certain assays (antithrombin, protein C, protein S) will not be performed until anticoagulation has been completed. The CAP Consensus Panel recommends confirmation of positive results for APC resistance assays with the factor V Leiden DNA test. Testing for elevated factor VIII levels is controversial, but if this is to be done, testing should be deferred until 6 months after the thrombotic event when anticoagulation has been discontinued (From Rodgers and Chandler [9], with permission)

9.5 Utility of Inherited Thrombosis Testing

The two major reasons to evaluate patients for inherited thrombosis are: (1) to screen family members for whom an inherited diagnosis may change management, and (2) to identify an inherited thrombotic disorder in the patient that may affect

their management (e.g., intensity or duration of anticoagulation) [11]. There is justification for the first reason to test, because for example, if a patient has female siblings or children who might also inherit a thrombotic disorder, this would potentially affect their treatment with oral contraceptives, or change counseling regarding pregnancy. In contrast, there is limited justification to test patients for the second reason (a thrombosis test result affects patient management). Several clinical trials in the past few years have produced results suggesting that positive test results for inherited thrombotic disorders do not predict recurrence of thrombosis; therefore, a positive result would not change patient treatment. Also, there is no data that patients with an inherited thrombotic disorder require a different intensity of anticoagulation than thrombosis patients without an inherited disorder [11]. One conclusion from this data is that testing should primarily be done if there are potentially affected female family members. It should also be noted that comprehensive testing can be expensive – between $1,000 and $2,000 for a single patient evaluation.

9.6 Laboratory Tests for Inherited Thrombosis

This section will briefly describe the principles of interpretation of common assays used to evaluate etiologies of inherited thrombosis. Most assays are available as commercial kits with standard methodologies that can be automated.

9.7 Activated Protein C Resistance Assay

APC resistance due to the factor V Leiden mutation is a very common cause of inherited venous thrombosis, especially in Caucasian populations. Test options include a clotting-based assay or a DNA-based test [12, 13].

APC is an anticoagulant that will prolong the clotting time of normal plasma, but patients with the factor V Leiden (or certain other) mutations will exhibit clotting times that are less prolonged (i.e., these plasmas exhibit APC resistance). In the APC resistance assay, two determinations are made, one with and one without the addition of APC. A ratio is obtained [e.g., Partial Thromboplastin Time with APC/Partial Thromboplastin Time without APC] and compared with that of a normal population. The ideal APC resistance assay uses either prothrombin time (PT) or partial thromboplastin time (PTT)-based assays in which patient plasma is diluted in factor V-deficient plasma. Using factor V-deficient plasma makes the test useful in patients who are receiving heparin or warfarin therapy, or who have lupus anticoagulants. If a normalized assay is done (the APC ratio of the patient is divided by the APC ratio of a control pooled plasma sample from the same test run), this method can distinguish normal vs. heterozygotes vs. homozygotes with the factor V Leiden mutation. If the laboratory has test samples from large numbers of patients receiving heparin or warfarin, and the local assay method does not use factor V-deficient plasma dilutions, then uninterpretable results may occur. In this situation, it may be preferable to perform factor V Leiden DNA testing.

9.8 Factor V Leiden DNA Test

With the discovery of the factor V Leiden mutation and the prothrombin gene mutation, each due to highly conserved point mutations, the utility of molecular diagnostic testing for thrombosis was enhanced.

For diagnosis of factor V Leiden, the original method used the restriction enzyme *Mnl* to digest a 267-bp amplified fragment of patient DNA. Digestion of normal patient DNA results in three fragments. A variety of molecular methods are available (summarized in the CAP Consensus Conference report) [13], including fluorescent detection of real-time PCR products, as well as non-PCR based methods.

Although DNA-based assays will be more expensive than the APC resistance assays, there will be no interferences, and the result should unequivocally be either normal, or heterozygous or homozygous for the factor V Leiden mutation. Negative test results for the factor V Leiden mutation will not exclude APC resistance due to other genetic defects (~5 % of patients with APC resistance).

9.9 Prothrombin Gene Mutation Assay

As with the factor V Leiden mutation, the prothrombin gene mutation is mostly seen in Caucasian populations. Although many patients with this mutation will have elevated levels of prothrombin activity, it is recommended that patients be tested for the specific DNA abnormality [5].

Multiple molecular diagnostic methods are available for assay, including restriction endonuclease digestion, automated fluorescence detection, or real-time fluorescence detection. Results are reported as normal, heterozygous, or homozygous for the prothrombin gene mutation [5].

The typical prothrombin gene mutation is G20210A. A variant prothrombin gene mutation has been reported in African-American patients – C20209T. This latter mutation may be under-recognized because standard PCR assays for the G20210A mutation may not detect the variant mutation. Laboratories that evaluate large numbers of African-American patients for thrombosis testing should consider assays for the C20209T mutation.

9.10 Homocysteine Assays

Unlike the other coagulation analytes discussed in this chapter that are linked to thrombosis, homocysteine is an amino acid whose levels can be elevated in inherited or acquired circumstances. It is usually assayed in the chemistry laboratory. Elevated levels of homocysteine may be associated with arterial or venous thrombosis, but consensus opinion suggests focusing on evaluation of patients with arterial thrombosis (Table 9.2) [6].

There are also molecular diagnostic tests available to identify mutations in the methylene tetrahydrofolate reductase (MTHFR) gene; however, consensus opinion is that testing for the MTHFR mutation should not be performed [6].

Earlier data suggested obtaining fasting samples for homocysteine measurement; this is no longer recommended. Either plasma or serum samples can be used, but collected blood samples should be placed on ice and red cells promptly separated. Quantification in the past was done by high-performance liquid chromatography, but the availability of fluorescence-based immunoassays has expanded the use of homocysteine measurement in smaller laboratories.

9.11 Protein C Assay

Unlike the highly conserved point mutations of factor V Leiden or the prothrombin gene mutations, deficiencies of protein C, protein S, and antithrombin are caused by numerous mutations. Consequently, molecular diagnostic tests are not clinically useful in evaluating patients for these disorders.

A variety of antigenic and functional assays are available to measure protein C levels [14]. Antigenic assays include ELISA, electroimmunoassay (Laurell rocket technique), and radioimmunoassay. The antigenic assays measure protein C levels (normal and des-carboxy forms), so these methods are not affected by oral anticoagulant therapy. However, antigenic assays will not measure protein C function and will therefore not detect qualitative protein C abnormalities. Functional protein C assays are preferred since they will detect both quantitative and qualitative protein C deficiency. Functional assay methods include clotting and chromogenic techniques. A common functional assay method uses Protac®, a snake venom activator of protein C. The APC generated is then measured in a PTT-based assay (clotting method) or in an amidolytic assay (chromogenic substrate method). The CAP Consensus Study recommends use of the latter chromogenic assay, primarily because therapeutic heparin levels do not affect assay results. Anticoagulant therapy and elevated factor VIII levels will affect the clot-based methods [14]. However, the Russell's viper venom-based clotting method containing heparin neutralizer eliminates the effects of both elevated factor VIII levels and therapeutic heparin.

One important aspect of interpreting protein C levels is that there is age-dependence for protein C levels; younger patients may not be correctly classified unless pediatric reference intervals for protein C levels are considered. Table 6.4 summarizes pediatric reference intervals for coagulation analytes, including protein C.

Numerous acquired variables impact protein C levels (Table 9.3), and interpretation of abnormal results should be made with caution. It is useful for the laboratory to provide a comment on factors that may result in low protein C levels, to assist clinicians in correct interpretation of the test. Ideally, evaluation of protein C deficiency should be done at a time distant from the acute thrombotic event when anticoagulation has been completed for at least 2 weeks.

9.12 Protein S Assay

As with protein C assays, antigenic and functional methods are available to measure protein S levels [16]. Total protein S antigen assays can be performed using ELISA methods, Laurell rocket technique, radioimmunoassay, etc. Functional assays can

Table 9.3 Acquired conditions associated with protein C, protein S, and antithrombin deficiency

Analyte	Causes of deficiency
Protein C	DIC
	Acute thrombosis
	Vitamin K deficiency, including oral anticoagulant therapy
	Newborn infants, children
	Liver disease
	Post-operative state
Protein S	DIC
	Acute thrombosis
	Inflammatory illness of any cause
	Vitamin K deficiency, including oral anticoagulant therapy
	Newborn infants, children
	Liver disease
	Pregnancy
	Nephrotic syndrome
Antithrombin	DIC
	Acute thrombosis
	Liver disease
	Oral contraceptives
	Nephrotic syndrome
	Pregnancy
	Heparin (therapeutic levels)

Source: Kjeldsberg et al. [15], with permission
Conditions, drugs, or diseases listed in this table may result in acquired deficiency of protein C or S, or antithrombin. These causes should be considered before evaluating patients for inherited deficiency
Abbreviation: *DIC* disseminated intravascular coagulation

be either based on PT or PTT clotting assays. The "gold standard" test for protein S has been identified as free protein S antigen levels [16]. Unlike protein C measurement, functional protein S assays are less useful because factor V Leiden (APC resistance) interferes with functional protein S assays results. For example, spuriously low protein S levels may be seen in patients with factor V Leiden when tested with functional assays for protein S.

Free protein S levels can be measured by monoclonal antibody-ELISA. Functional protein S assays are based on PT or PTT assays. Diluted patient plasma is added to protein S-deficient plasma in the presence of APC and factor Va for the PTT format assay. Positive results in the functional assay should be confirmed with another assay method, e.g., a free protein S assay.

Numerous acquired conditions may result in protein S deficiency (Table 9.3), and these must be considered in the interpretation of a low protein S test result. It is useful to comment on these acquired etiologies when reporting the test results to

assist clinician interpretation. Age-dependent reference ranges are necessary to correctly classify patients (Table 6.4). Additionally, males have higher mean plasma levels of protein S, so gender should also be taken into account when reporting protein S levels.

9.13 Antithrombin Assay

Functional and antigenic assays are available to measure antithrombin levels [17]; functional methods are preferred since many antithrombin-deficient patients have qualitatively-abnormal molecules that would be missed if antigenic assays were used. Functional assays for antithrombin measure heparin cofactor activity using a chromogenic substrate method to assay thrombin or factor Xa inhibition. Antigenic methods include Laurell immunoelectrophoresis, radial immunodiffusion, and microlatex particle immunoassay [17].

Plasma antithrombin levels can be markedly decreased by therapeutic heparin, and long-term warfarin treatment may increase antithrombin levels. Therefore, patients should be tested off of anticoagulant therapy. Other acquired conditions that can affect antithrombin results are listed in Table 9.3. Pediatric reference ranges should be used to correctly classify laboratory results of children (see Chap. 6, Table 6.4).

9.14 Laboratory Testing for Other Inherited Thrombotic Disorders

Assays exist to measure numerous other analytes that have been linked to thrombosis, for example, dysfibrinogenemias, heparin cofactor II, and fibrinolytic components (plasminogen, tissue plasminogen activator, and plasminogen activator inhibitor-1). However, the association between these parameters and thrombosis is either weak or unproven, and the CAP Consensus Conference has recommended that they not be tested for (Table 9.2).

Assay for elevated factor VIII levels is controversial. Factor VIII is an acute-phase response protein, so elevated levels would be seen during the acute event. Also, the assay for factor VIII is clot-based, so heparin therapy would affect the measurement. A summary of the CAP recommendations on thrombophilia testing is given in Table 9.2.

9.15 The Acquired Thrombotic Disorders

Table 9.4 summarizes the acquired thrombotic disorders. These include autoimmune disorders such as vasculitis and antiphospholipid antibodies, hematologic disorders such as the myeloproliferative disorders, paroxysmal nocturnal hemoglobinuria, thrombotic thrombocytopenic purpura, and heparin-associated thrombocytopenia with thrombosis, metabolic disorders such as obesity, diabetes

Table 9.4 Acquired thrombotic disorders

Autoimmune disease
Vasculitis
Antiphospholipid antibodies
Inflammatory bowel disease
Hematologic diseases
Myeloproliferative disorders (polycythemia vera, essential thrombocytosis)
Plasma cell disorders (Waldenstrom's macroglobulinemia)
Heparin – associated thrombocytopenia with thrombosis
Paroxysmal nocturnal hemoglobinuria
Metabolic disorders
Diabetes
Hyperlipidemia
Obesity
Miscellaneous disorders
Trauma
Post-surgery
Cancer
Hormone therapy
Nephrotic syndrome

and hyperlipidemia, and miscellaneous disorders such as pregnancy, nephrotic syndrome, hormone therapy, and cancer. Laboratory evaluation of thrombotic thrombocytopenic purpura and heparin-associated thrombocytopenia are discussed in Chaps. 7 and 8. D-dimer assays which are frequently elevated in cancer patients with thrombosis are discussed in Chap. 5. Antiphospholipid antibodies are frequently assayed in the coagulation laboratory and will be discussed in this chapter.

Testing for antiphospholipid antibodies (anticardiolipin antibodies, antibodies to β_2-glycoprotein-1, lupus anticoagulant) is appropriate for thrombosis patients (arterial and venous), especially if they have idiopathic clots without a family history, or if there is autoimmune disease. Testing for antiphospholipid antibodies is also appropriate for evaluating patients with recurrent miscarriage. Criteria for diagnosis of antiphospholipid antibody syndrome require demonstration of antibody persistence for 12 weeks (Table 9.5).

9.16 Anticardiolipin Antibody Assay

A standardized ELISA is recommended using high-sensitivity microtiter plates; these plates permit greater antigenic density and improve detection of antibodies [19]. The buffer system is also important. Reporting of antibody titer is critical in interpretation of an anticardiolipin antibody ELISA result. Moderate or high titer antibody levels are required for diagnosis, and the laboratory should provide clinicians with an interpretive comment that allows correct classification of patients.

Table 9.5 Revised clinical and laboratory criteria for the antiphospholipid antibody syndrome

Clinical criteria	
Vascular thrombosis –	Arterial or venous or small vessel
or	
Pregnancy morbidity –	One or more unexplained deaths of a morphologically-normal fetus at or beyond 10 weeks gestation
	or
	One or more premature births of a morphologically – normal neonate before the 34th week of gestation because of eclampsia/ preeclampsia or placental insufficiency
	or
	Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation (with exclusion of anatomic, hormonal or genetic causes)
Laboratory criteria	
Anticardiolipin antibody:	(I_gG/I_gM), in moderate or high-titer, present on at least 2 occasions 12 weeks apart, measured by standardized ELISA
or	
Lupus anticoagulant:	Present on at least 2 occasions 12 weeks apart measured by ISTH criteria (Table 9.6).
or	
Anti-β_2-glycoprotein-1 antibody:	(I_gG/I_gM), present on at least 2 occasions 12 weeks apart, with levels >99th percentile measured by a standardized ELISA

Source: Miyakis et al. [18]
Definite APS is present if at least one clinical and one laboratory criteria are present

Positive results for IgG antibodies are considered the most important, although positive results for IgM antibodies that persist may also be clinically important. The utility of IgA anticardiolipin antibody testing is uncertain.

A consensus conference on laboratory criteria used to diagnose antiphospholipid antibody syndrome also recommends ELISA testing for antibodies to β_2-glycoprotein-1. Antibody (IgG/IgM) levels >99th percentile that persist for at least 12 weeks are required to meet this diagnostic criterion. Table 9.5 summarizes the clinical and laboratory criteria for diagnosing patients with antiphospholipid antibody syndrome.

9.17 Lupus Anticoagulant (LA) Assay

The LA is an antiphospholipid antibody that affects phospholipid-based coagulation assays; it is clinically associated with thrombosis and miscarriage. The LA antibody actually recognizes a protein in a phospholipid-protein complex; the protein is usually β_2-glycoprotein-1 or prothrombin. Assays to detect the LA are coagulation-based, and therefore can be impacted by numerous variables that affect clot-based tests, including sample collection, processing, and the presence of anticoagulants.

Table 9.6 ISTH Criteria for laboratory diagnosis of the LA

Prolongation of at least one phospholipid-dependent coagulation test with platelet – poor plasma (PTT, dilute PT, DRVVT, kaolin clotting time, etc.)
Failure to correct the prolonged clotting time when patient and normal plasma are mixed
Correction of the originally-prolonged clotting time by addition of excess phospholipid
Exclusion of other inhibitors to coagulation (heparin, factor VIII antibodies)

Source: Brandt et al. [21]
Abbreviations: *ISTH* International Society of Thrombosis and Haemostasis, *LA* lupus anticoagulant

A good quality phlebotomy (non-activated sample) is necessary. Platelet-poor plasma (platelet count <10,000 μl) is critically important; if the sample platelet count is higher, many LAs will not be detected since platelet phospholipid in a frozen-thawed sample will neutralize antibody activity [18–20].

Guidelines have been published recommending laboratory criteria for diagnosis of the LA. It is suggested that more than one test system (phospholipid-dependent coagulation assay) be used to optimize identification of the LA. Test options include the PTT, dilute PT, dilute Russell's Viper venom time (DRVVT), kaolin clotting time, Taipan venom test and Textarin time. International Society of Thrombosis and Haemostasis (ISTH) guidelines for laboratory detection of the LA are summarized in Table 9.6.

If the screening test demonstrates a prolonged clotting time, a mixing study is performed with normal plasma. If the mixing study result fails to correct, then an inhibitor is suspected. When excess phospholipid or hexagonal-phase phospholipids are added to the screening test sample, the prolonged clotting time shortens or corrects, validating that the originally prolonged clotting time was due to antibodies to phospholipid. Lastly, other inhibitors to coagulation should be excluded clinically or by the laboratory (heparin, factor VIII antibodies).

"Integrated" coagulation reagents have been developed that permit testing for the LA using the ISTH criteria. For example, a DRVVT kit is available that uses reagents for screening and confirmation of the LA. Similarly, the Staclot LA® test also uses a methodology to screen and confirm the LA. Some of the integrated LA kits contain a heparin neutralizer that permits performing the test on patients receiving heparin therapy.

9.18 Utility of Laboratory Thrombosis Testing

The availability of hypercoagulability or thrombosis panels and identification of new thrombophilia disorders has led to increased test ordering of these assays. There are two reasons to test patients – to screen other family members (especially females) who may be similarly affected, and to possibly change the management of the patient being tested. The current literature is controversial on this subject, with some investigators insistent that widespread testing is useful, while others state that the information obtained is of limited usefulness. Based on the

literature (summarized in the review article by Rondina et al.), several conclusions can be drawn:

1. Testing for the inherited disorders (factor V Leiden, prothrombin gene mutation, protein C and S deficiencies, antithrombin deficiency) is of limited utility in guiding treatment for the affected patients. Large clinical trials have demonstrated that positive results for these tests do not predict recurrent thrombosis, and therefore, do not assist clinicians in determining duration of anticoagulation.
2. Testing for the above inherited disorders may be useful if the patient being tested has female siblings or children for whom a diagnosis might change management (e.g., hormonal therapy).
3. Testing for homocysteinemia is controversial. While patients who have this diagnosis made can be treated with vitamins, at this time, there is no data that shows a clear-cut clinical benefit from suppressing homocysteine levels.
4. Testing for antiphospholipid antibodies (anticardiolipin antibodies, antibodies to β_2-glycoprotein, lupus anticoagulant) is helpful in patient management, not because it changes the intensity of anticoagulation, but rather because patients should be anticoagulated for the duration that antiphospholipid antibodies persist.

Most clinicians are not aware of these limitations of thrombosis testing. Laboratory directors should educate their clinicians so that they are aware of when thrombosis testing can be helpful (family screening), what tests to order (functional assays for the natural anticoagulants), and when to order them (not acutely; not while the patient is anticoagulated), etc.

9.19 Key Points

- The most common inherited thrombotic disorders are activated protein C resistance (factor V Leiden), the prothrombin gene mutation, and homocysteinemia.
- Antithrombin deficiency, and deficiencies of proteins C and S are very uncommon. Optimal assays for these analytes include functional coagulation methods or free protein S antigen assay.
- If assays for antithrombin, protein C, and protein S are to be done, postpone testing until the patient has completed anticoagulation and the patient is clinically well.
- Be aware that inherited thrombosis testing is most useful for family screening. There is little, if any, data that such results alter management of the patient with thrombosis.
- Pediatric reference intervals are now available for inherited thrombosis analytes (antithrombin, protein C, and protein S deficiencies).
- Appropriate testing for antiphospholipid antibodies includes standardized assays for anticardiolipin antibodies, β_2-glycoprotein-1 antibodies, and the lupus anticoagulant.

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