# Screening for thrombophilia: a laboratory perspective

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**ABSTRACT**

The worldwide annual incidence of venous thrombosis is estimated at 1 in 1000 individuals, and associated pulmonary embolism represents a major cause of morbidity and mortality. Thrombophilia may be an inherited or acquired condition, with the former identified in approximately 25-30% of patients with thromboembolic disease. Recently published guidelines on thrombophilia testing recommend assays for protein C, protein S and antithrombin; a modified activated protein C resistance test (with factor V-deficient plasma); polymerase chain reaction for prothrombin G20201A, together with prothrombin time, activated partial thromboplastin time, thrombin clotting time and assays to detect antiphospholipid antibodies. This review highlights some of the issues that laboratories should consider when employing tests for the diagnosis of thrombophilia.

KEY WORDS: Antithrombins.
Factor V Leiden.
Protein C.
Protein S.
Thrombophilia.

## Introduction

Thromboembolic disease is a common and major medical problem. The worldwide annual incidence of venous thrombosis is estimated at 1 in 1,000 individuals, and associated pulmonary embolism represents a major cause of morbidity and mortality, accounting for 50,000 to 100,000 deaths each year in the USA.[1] Although there is no internationally accepted definition,[2] the term thrombophilia is generally used to describe subjects with a predisposition or tendency to thrombosis. Thrombophilia may be an inherited or acquired condition, with the former being identified in approximately 25-30% of patients with thromboembolic disease.[3]

Screening for inherited (familial) thrombophilia began with the description of a family with a deficiency of the serine protease inhibitor antithrombin in 1965.[4] Subsequently, a wide variety of defects have been associated with inherited thrombophilia, and a similarly wide range of tests have been employed by laboratories to identify individuals and families with these defects (Table 1).

The pattern of tests employed by laboratories has changed as new defects have been discovered and epidemiological studies have either confirmed or disproved links between a candidate defect and thrombophilia. For example, fibrinolytic tests formed a major part of the thrombophilia screen of many departments in the mid 1980s but these have subsequently been shown to have little diagnostic value.[5] The pattern of thrombophilia tests employed by one haemostasis and thrombosis centre (Royal Hallamshire Hospital) over a period of 20 years is shown in Table 2.

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The number of laboratories performing thrombophilia screening and the number of tests performed in the UK is increasing annually. Some authors consider a clinical history of greater relevance than laboratory investigation,[6] although a recent article by Mannucci[7] reaffirmed support for screening for heritable thrombophilia.

This review aims to highlight some of the issues laboratories should consider when employing tests for the diagnosis of thrombophilia.

## Which tests?

Recently published guidelines on thrombophilia testing[2] recommend assays for protein C (PC), protein S (PS) and antithrombin (AT); a modified activated protein C resistance (APCr) test (with factor V-deficient plasma); polymerase chain reaction (PCR) for prothrombin G20201A, together with prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time, and assays to detect antiphospholipid antibodies. This review will deal mainly with the genetic causes of thrombophilia; guidelines for the diagnosis of lupus anticoagulant, which have been published recently,[8] will not be addressed here.

**Table 1.** Assay performed as part of a routine thrombophilia screen (UK NEQAS questionnaire, November 1996)

| | | Centres including this assay in their routine screen (%) | Centres using a local normal range (%) |
|---|---|---|---|
| Protein C | Antigen | 13.5 | 59 |
| | Activity | 99.4 | 39 |
| Protein S | Total antigen | 42.3 | 63 |
| | Free antigen | 47.9 | 60 |
| | Activity | 58.3 | 30 |
| Antithrombin | Antigen | 11.0 | 50 |
| | Activity | 98.8 | 37 |
| APC resistance | | 89.6 | 68 |
| Factor V Leiden | | 80.4 | - |
| Plasminogen | | 20.2 | 56 |
| Prothrombin time/INR | | 93.9 | 88 |
| APTT | | 93.9 | 89 |
| Fibrinogen | | 82.8 | 71 |
| Thrombin time | | 39.3 | 86 |
| Lupus anticoagulant | | 56.4 | - |
| Anticardiolipin antibodies | | 28.8 | - |

Other assays performed as part of a routine screen by fewer than 10 centres included full blood count, FII or FVII, FVIII:C, FXII:C, reptilase time, euglobulin clot lysis time (ECLT), fibrin plate lysis assay, tissue plasminogen activator (TPA), plasminogen activator inhibitor (PAI), D-dimer, C-reactive protein, vWF:Ag, liver function tests, autoimmune profile, antinuclear antibodies, spontaneous aggregation, antiplatelet antibodies, plasma viscosity, antiplasmin, lipoprotein A, ESR, blood group, homocysteine, methyl tetrahydrofolate reductase and heparin co-factor II. Since distribution of this questionnaire, many centres now screen for the prothrombin G20210A mutation.

**Table 2.** Pattern of thrombophilia testing in one hospital laboratory (1980-2002)

| | 1980 | 1985 | 1995 | 2002 |
|---|---|---|---|---|
| Coagulation screen | + | + | + | + |
| Antithrombin | + | + | + | + |
| Protein C | | + | + | + |
| Protein S (total/free) | | | + | + |
| APC resistance | | | + | + |
| Plasminogen | + | + | + | No |
| ECLT | + | + | No | No |
| TPA, PAI | | + | No | No |
| Factor V Leiden | | | + | + |
| Prothrombin G20210A | | | | + |
| DRVVT, anticardiolipin | | | + | + |
| Anti-β2 glycoprotein 1 | | | | + |
| Global protein C screen* | | | | No |
| Functional protein S* | | | No | No |
| Homocysteine | | | | +/- |
| Factor VIII:C | | | | +/- |

* Tests evaluated but not recruited for routine use

## Protein C

Protein C is a vitamin K-dependent glycoprotein with a molecular weight (MW) of approximately 62000, synthesised in the liver and circulating in plasma as a two-chain serine protease zymogen. Protein C is activated by thrombin, cleaving an Arg169-Leu170 bond on the heavy chain, releasing a small 12aa peptide (PC activation peptide).[9] The activation rate is accelerated by formation of a complex between thrombin and the endothelial cell membrane protein thrombomodulin,[10] and further enhanced by endothelial cell PC receptor.[11]

Activated PC acts by inactivating factors VIIIa and Va by selective proteolytic cleavage, reducing the procoagulant activity of plasma. This inactivation requires a cofactor, protein S, together with calcium ions and a phospholipid membrane surface. Activated PC is neutralised by PC inhibitor, α2-antiplasmin and α2-macroglobulin.[12]

**Table 3.** Effect of APC resistance on functional assays for PC. Median PC results from participants in UK NEQAS thrombophilia exercises

| | | | Defect | | |
|---|---|---|---|---|---|
| Assay | units | *n* | Heterozygous FV Leiden | Homozygous FV Leiden | FV Leiden absent |
| Protein C antigen | u/dL | 41 | 97.0 | 79.0 | 102.0 |
| Protein C clotting activity | u/dL | 63 | 82.5** | 50.5** | 113.0* |
| Protein C chromogenic activity | u/dL | 107 | 99.0 | 82.0 | 109.0 |

* significantly different from chromogenic assay ($P<0.001$)

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The first case of PC deficiency was described by Griffin *et al.* in 1981,[13] in a family with a history of recurrent thromboembolic disease, which displayed reduced levels of PC to around 50% of normal.

Heterozygous PC deficiency, like antithrombin deficiency, is associated with deep vein thrombosis, with or without pulmonary embolism at a young age (<40 years),[14] although there is a higher association of PC deficiency with superficial thrombophlebitis and arterial disease.[15] Warfarin-induced skin necrosis, although rare, is also a feature of this deficiency.

Homozygous PC deficiency is associated with massive and potentially fatal thromboembolism shortly after birth, with central nervous system thrombosis, ophthalmic thrombosis and characteristic purpuric skin lesions.[16]

The prevalence of PC deficiency is a matter of some debate and is determined, in part, by the source of the study population.[17-21] It is now suggested that two cohorts of PC-deficient patients exist – those with a clinically dominant disorder, which may be linked to co-segregation of additional risk factors, and those with a clinically recessive picture. However, mutations in those subjects with asymptomatic PC deficiency are equally as 'bad' as those in symptomatic cases.[22]

Protein C deficiency can be subdivided into type I and II deficiency, based on results of functional and immunological assays. In type I deficiency, a concordant reduction in PC antigen and activity is seen. The majority of cases of PC deficiency fall into this category and up to 60% of the mutations that cause type I deficiency are due to missense mutations.[9]

In type II deficiency, an abnormal PC molecule is implied by reduced activity in the presence of normal quantities of protein. Acquired PC deficiency is seen in patients with liver disease, disseminated intravascular coagulation, insulin-dependent diabetes, essential hypertension and sickle cell disease.[12] Discovery of a low PC level should be followed up by assay of at least two other vitamin K-dependent clotting factors to allow confirmation of a specific deficiency.

## Laboratory measurement of protein C

### *Protein C activity*

Initial assays to measure PC function were complicated by the need to separate PC from other plasma coagulation factors, in particular fibrinogen, prior to its activation by thrombin. (Francis and Patch,[23] Sala *et al.*[24] and Comp *et al.*[25]). Discovery of a snake venom from *Agkistrodon contortrix contortrix,* which directly activates PC, allowed development of simplified assays for PC activity. Endpoint detection may be by clotting[26] or chromogenic assay,[27] the latter being associated with greater specificity.

A number of variables may interfere with clot-based assays, such as the presence of heparin, raised FVIII levels, lupus anticoagulant and factor V Leiden (FVL).[28,29] Table 3 shows data from UK NEQAS thrombophilia surveys in which plasma samples from patients with and without FVL were distributed for PC assay. Significant differences were seen in results between clot-based and chromogenic assays for both heterozygous and homozygous FVL subjects. Subsequent study confirmed the differences were due to the presence of FVL, and demonstrated that a 1 in 4 dilution of test plasma in PC-deficient plasma would correct the effect of APCr.[30]

However, in one UK NEQAS thrombophilia screening exercise, many centres failed to identify APCr as the cause of the reduced PC level in their assay. With chromogenic assays, autohydrolysis of the substrate may occur and assays should include a blank to avoid overestimation of PC activity,[31,32] particularly for samples where clotting factor activation is suspected. Only 29% of centres that responded to a NEQAS questionnaire indicated that they use a blank in their PC assay protocol.

In addition, some type II (dysfunctional) defects may be missed without a clot-based assay – functional defects which impair PC binding to substrate, PS, and calcium are included in this group.[33] Early studies suggested the frequency of such defects may be 40% of type II PC deficiency,[34] but overestimation due to APC resistance in subjects with FVL may have distorted these figures.

More recent estimates suggest around 5% of type II defects will only be detected by a clot-based assay;[35] however, type I PC deficiency is more prevalent than type II PC deficiency, and the proportion of PC defects missed by a chromogenic assay may be offset by the proportion of misdiagnoses that may occur with a clot-based assay.

### *Protein C antigen*

Early diagnosis of PC deficiency was performed using immunoelectrophoretic methods. However, immuno-precipitation with rocket electrophoresis is compromised by the low plasma concentration of PC; a combination of radio-labelled anti-PC antibodies and autoradiography has been used to visualise the immunoprecipitin rockets.[13] Laurell rocket electrophoresis is further complicated by the fact in that PC levels will be overestimated in patients receiving

coumarin therapy unless EDTA is present in the gel and buffer to ensure equal migration of carboxylated and non-carboxylated forms of PC.[36]

Other, more sensitive immunological assays have been developed subsequently, including enzyme-linked immunosorbent assay (ELISA)[37,38] and radioimmunoassays.[39] Differences have been described in results obtained by the different assay methods and this may relate, in part, to the complete recovery of PC-PC inhibitor complexes by the Laurell method, with only approximately 50% recovery with a polyclonal ELISA.[40]

Although currently there is no evidence that distinguishing between types I and II PC deficiency provides any indication of thrombotic risk, immunological PC assays are still employed by 26% of UK centres performing thrombophilia screens, and can serve as useful additional quality control step by comparison to functional PC levels.

## Protein S

Protein S is a vitamin K-dependent single-chain glycoprotein (MW approximately 70,000). Synthesis occurs in the liver, endothelial cells and megakaryocytes.[41] Some 60% of PS in plasma is bound to C4b-binding protein, which is a complement system component. This binding occurs via a β-subunit of C4b-BP, and all circulating β-chain C4b-BP is bound to PS. Excess PS circulates as a free form, capable of acting as a non-enzymatic cofactor to activated PC.

Protein S is thought to enhance the inactivation of factor Va and VIIIa on phospholipid surfaces, possibly by localising APC at these loci,[42] and also plays a role in the fibrinolytic effect of APC.[43] Recent studies indicate a possible role for bound PS in APC-independent anticoagulant activity.[41]

Protein S is inactivated by thrombin-induced proteolysis, although this occurs at a relatively slow rate. Although C4b-binding protein levels are increased in acute-phase reactions, this increase is mostly of the α-subunit, and ratios of free and bound PS remain relatively constant.[44]

## Deficiency of protein S

The first case of PS deficiency was described by Comp and Esmon in 1984.[45] Deficiency of PS is associated with deep vein thrombosis with or without pulmonary embolism at a young age (<45 years). Purpura fulminans has been described but is a rare occurrence.[46]

The incidence of PS deficiency in the thrombophilic population varies in different reports, and this may be associated with the selection criteria used for the study groups and also assay variability. The probability of a thrombotic event occurring under the age of 45 in subjects heterozygous for PS deficiency in these families is around 50%.[46]

Case control studies have failed to agree on the association between reduced PS levels and thrombotic risk. However, Faioni and colleagues[47] demonstrated a mild increase in risk of venous thromboembolism (VTE; relative risk 2.4) and a prospective cohort study of subjects with PS deficiency showing a significantly higher risk of thromboembolism than in subjects with normal levels of PS would appear to confirm the link between PS deficiency and thrombophilia.[48]

Mutations in the PS gene have been identified in between 41% and 90% of subjects.[46] PS deficiency is subclassified by the levels of total and free antigen and PS activity. Comp *et al*.[49] first described a subject in whom free PS antigen was reduced but total PS antigen was normal. Subsequent studies have identified subjects with type I deficiency (reduced total and free antigen and activity), type II deficiency (reduced PS activity, normal free PS antigen) and type III deficiency (total PS antigen normal or borderline, free PS antigen and activity reduced).[50]

Type II deficiency is rarely encountered and some early descriptions included subjects with the FVL mutation, in whom PS activity is reduced,[51] as an artefact. However, in the PS mutation database published by Gandrille *et al*.,[52] eight out of 126 mutations were associated with a type II phenotype.

Some authors consider that type I and III deficiency are phenotypic variants of the same genetic disorder, which arise because of an age-related increase in total PS antigen levels while free PS antigen remains unchanged.[49]

Acquired PS deficiency has been described in a number of conditions, including pregnancy, oral contraceptive use, liver disease, and following orthotopic liver transplantation.[53,54] However, total PS antigen may be unchanged during disseminated intravascular coagulation.[53]

## Laboratory measurement of protein S

***Protein S antigen***

Protein S antigen may be measured by immuno-electrophoretic methods,[55] ELISA[56,57] and radio-immunoassay.[58] Total PS assays require conditions in which there is dissociation of the PS-C4b-binding protein complex, unless the antibodies used in the assay show equal affinity for bound and free PS.

Problems may arise in Laurell rocket electrophoresis where, under certain conditions, double precipitation peaks may be seen[31] – it is important that both electrophoresis buffer and gel contain EDTA to ensure measurement of fully and partially caboxylated PS, and to prevent complement activation and C4b formation; single precipitation peaks can be obtained with prolonged electrophoresis and higher incubation temperatures.

High dilutions of plasma and long incubation times with primary antibody are required when using ELISA methodology; if the dilution of plasma or the incubation time is inadequate then total PS levels may be underestimated.[31,59] In three subjects with PS deficiency, PS levels measured by centres using a short incubation time (<2 h) were lower than those measured with long incubation times (>18 h);[59] complete dissociation of bound PS can be demonstrated if long incubation times and high plasma dilutions are used.[57]

Measurement of free PS is important to allow diagnosis of type III PS deficiency; also, overlap of total PS levels occurs between normal and PS deficient subjects[60]. Makris *et al*. have shown free PS to be the best indicator (100% specificity) of a PROS1 genetic defect.[61]

Quantitative measurement of free PS can be achieved by removal of the C4b-BP-bound component, which may be achieved by the addition of polyethylene glycol (PEG) to

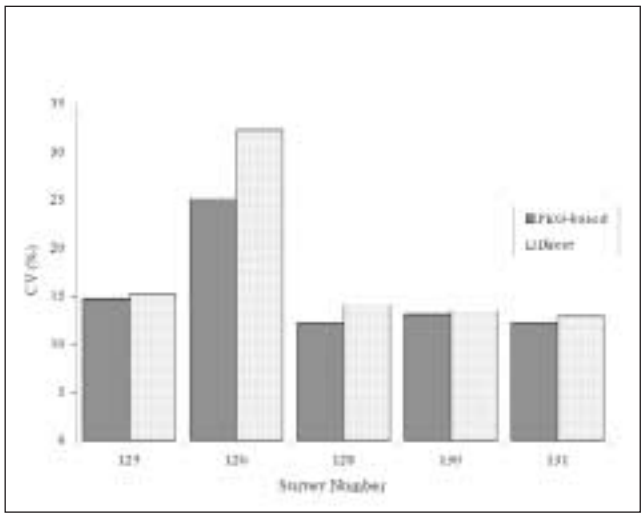

**Fig. 1.** Precision of PEG-based and direct free protein S assays (Data from UK NEQAS exercises, 2000 – 2001).

precipitate bound PS and leave free PS in the supernatant.[49] Concentration of PEG and mixing conditions, however, are critical. Methods for extracting bound PS with different concentrations and MWs of PEG have been described, and participants in the UK NEQAS scheme have also described use of differing incubation times and temperatures. This additional sample-processing step was perceived to contribute to poor precision for this assay and has encouraged the development of methods for direct measurement of free PS.

Monoclonal antibodies with a specificity for free PS have permitted the development of a free PS ELISA that does not require PEG precipitation,[62] and a ligand-based assay using C4b-BP to capture free PS has also been described.[63] Automated turbidometric methods employing these principles are now available.

It is possible that these direct methods will improve between-laboratory agreement for PS assays, although coefficients of variations (CV) for PEG-based and direct PS assays were similar in NEQAS surveys during 2000-2002 (Figure 1).

There also may be pitfalls in the use of direct assays, as it has been noted that the monoclonal assay may overestimate free PS concentration when levels are particularly reduced. Recently published data show that the incubation temperature for direct methods may be critical, and overestimation of free PS in plasma from PS-deficient subjects may occur.[64]

*Protein S activity*

Functional assays for PS activity have been developed and are used widely in UK centres. Methods based on the inhibition of APTT,[65] PT,[66] and FXa and FVa[67] are available. Boyer-Neumann carried out a multicentre comparison of PS functional assays in 1993, identifying misclassifications of functional defects and poor correlation with free PS for one method.[68]

A number of variables may interfere with functional PS assays, including the presence of lupus anticoagulant and FVL.[65] Several authors have reported interference of activated protein C resistance, due to the FVL mutation, causing artificially reduced PS activity.[51,69] Despite this, 24 out of 55 centres in a UK NEQAS exercise misclassified a patient homozygous for the FVL defect as PS-deficient.[70] Predilution of test plasma in PS-deficient plasma will not completely correct the effect of APC resistance for some FVL subjects (unpublished data).

In the authors' experience, overestimation of PS in the presence of a lupus anticoagulant may cause a problem, which cannot always be overcome by the use of increased plasma dilution. Functional PS assays may also be affected by activation of FVII, and repeated thawing of frozen samples should be avoided. Some authors do not recommend the use of PS activity assays,[71] while others recommend confirmation of low PS activity with immunological assays for free PS.[2]

## Antithrombin

Antithrombin (AT), previously known as antithrombin III,[72] is a serine protease inhibitor which primarily inhibits thrombin and FXa but also inhibits many other activated serine proteases including FIX, FXI and FXII.[73] Antithrombin was discovered in 1939 as a substance present in plasma that works with heparin to prevent thrombin formation.[74] The first family diagnosed with familial thrombophilia had thrombosis due to AT deficiency,[75] and the majority of carriers of AT gene mutations have venous thrombosis by middle age.[72]

Antithrombin is a glycoprotein (MW 58 200), the plasma concentration of which is approximately 125 mg/L (2.3 $\mu$mol/L). Half-life of labelled AT in plasma is reported to have three exponential components, and is around 65 hours,[76] although the half-life of infused AT concentrate may be considerably shorter than this.

Basic amino acids on AT bind to specific sulphate groups on the pentasaccharide structure of heparin and the AT becomes activated, with anticoagulant activity increasing by more than a 1000-fold. Enzymes inhibited by AT form stable 1:1 proteolytically inactive complexes with the reactive site of AT, which is termed $P_1$-$P_1'$, and is the peptide bond between arginine 393 and serine 394.[77]

Antithrombin is composed of 432 amino acids and has four glycosylation sites at Asn96, Asn135, Asn155 and Asn192. These may have variable sialic acid content and a minor proportion of AT is not glycosylated at Asn135.[76] Different glycosylation results in normal plasma displaying two populations of AT ($\alpha$ and $\beta$), the minor population (antithrombin $\beta$) with reduced carbohydrate content accounts for some 10-15% of the total and has increased heparin-binding characteristics when assessed by heparin-sepharose chromatography.[78] Antithrombin displays numerous bands when investigated by isoelectric focusing.

Antithrombin deficiency can be congenital or acquired. Causes of acquired deficiency include thrombosis (including disseminated intravascular coagulation), liver disease, sepsis,[78] nephrotic syndrome[79] and treatment with L-asparaginase.[80]

Inherited AT deficiencies are associated with an increased risk of venous thromboembolism. Two major types of deficiency are recognised: quantitative type I is characterised by parallel reduction in activity and antigen levels in plasma, while qualitative type II is defined by discrepant activity and

antigen levels. Three subtypes of qualitative defect are recognised depending on whether the mutation affects the heparin binding site (HBS), reactive site (RS) or has pleiotropic (multiple) effects (PE).

The prothrombotic risk associated with type II HBS deficiency has been questioned, raising the need for a means of discriminating between this type and other AT defects. The Antithrombin Mutation Database (www.med.ic.ac.uk/divisions/7/antithrombin) currently identifies 80 distinct point mutations and 12 partial/whole deletions resulting in type I AT deficiency, while distinct point mutations causing RS, HBS and PE, number 12, 12 and 11, respectively.

## Laboratory measurement of antithrombin

Assays of AT must measure the protein's function, as many deficient patients have a type II deficiency, where AT mass is normal but functional level is significantly reduced. Choice of functional assay must take into account best practice, where precision and sensitivity to functional defects is maximised.

The assay of choice is chromogenic, as this is generally very precise – a necessity for AT assay where the normal range is very narrow and the lower limit of normal is only some 20% lower than the population mean level. The assay must be accurate and sensitive. The former is made possible by the use of standards that have been properly calibrated in international units per mL/dL.

Designing a sensitive assay is not a simple matter, as AT function is measured after it has been activated more than a 1000 times by binding to heparin, which allows efficient cleavage of AT and bonding to the neutralised enzyme. Heparin differentially enhances the activity of AT, reducing the effect of other enzyme inhibitors, with the exception of heparin co-factor II – an additional (confounding) factor in AT assays, especially when human thrombin is used as enzyme and incubation times with plasma are more than 30 seconds.[81]

To significantly reduce this interference, which can cause over-estimation of AT concentration, bovine thrombin can be used in place of human thrombin, or FXa can replace thrombin altogether. Bovine thrombin reacts minimally with heparin co-factor II (and heparin co-factor II does not react with FXa), resulting in more reliable detection of AT deficiency, compared to an assay incorporating human thrombin.[82] Factor Xa-based assays of AT, however, are normal in the presence of antithrombin Cambridge II[7] and antithrombin Denver[83] – both type II RS defects. A short (30 second) incubation of sample dilution with enzyme (with heparin) has been recommended for AT assays, as heparin binding site variants may not be detected with longer incubations.[84, 85]

Immunological assays for AT have been described using Laurell rocket electrophoresis, ELISA by sandwich[86] or competitive assay,[87] and radioimmunoassay.[88] Recently, latex immmunoassay methods have become available.[89] Crossed immunoelectrophoresis, with and without heparin, may be used to identify molecular variants, particularly to distinguish type II HBS and RS variants.[90, 91]

Although immunological assays can assist in the subclassification of AT defects, functional assays are of greater importance in the diagnosis of AT deficiency and in the monitoring of treatment with AT concentrates, where the presence of degraded AT molecules has led to discrepancies between functional and immunological levels of AT.[92] Although AT activity levels are generally measured as a relative concentration (in units/mL or units/dL related to the amount of AT in a pooled normal plasma), immunological levels of AT may be reported as a relative or an absolute concentration (mg/dL or mg/L). However, for comparability and consistency between different thrombophilia screening tests, relative concentrations are preferred.

An approach to the interpretation of AT assay results is given in the *Reference ranges* section of this review.

## Factor V Leiden and activated protein C resistance

Activated protein C resistance is the inability of activated protein C (APC) to anticoagulate plasma, and is associated with an increased tendency to venous thrombosis.[93] In 1994, the APCr mechanism became clearer when a point mutation in the FV gene, G1691A, was identified.[94] The gene product of this mutation is FVL, where arginine 506 is replaced by glutamine at a site required for optimal cleavage of FVa by APC. Subsequently, a second defect caused by the G1691A mutation was identified, namely the inability of FVL to act as co-factor for destruction of FVIIIa by APC.

Factor V is a single-chain glycoprotein (MW 330 000) composed of an amino terminal region containing the A1-A2 domains and a carboxy terminal region containing the A3-C1-C2 domains.[95] Factor V may be activated by thrombin, FXa, meizothrombin and Russell's viper venom. Thrombin activates FV to FVa by cleavage at three specific bonds (Arg709, Arg1018 and Arg1545) and the resultant FVa is composed of the amino terminal region, devoid of most of the B domain, and is linked by a calcium ion bridge to the carboxy terminal region.

Activated protein C destroys FVa activity by cleaving three bonds in the heavy (amino terminal) chain (Arg506, Arg306 and Arg679). Cleavage at Arg506 is required to allow efficient exposure of the other two cleavage sites, and its absence causes APCr.[96]

Factor V Leiden is the cause of APCr in at least 90% of patients with APCr;[97] however, APCr in the absence of FVL is an independent risk factor for venous thrombosis – the greater the degree of APCr, the greater the risk of thrombosis.[98] Subjects without FVL but with APCr may have an increased level FVIII, be pregnant or on the oral contraceptive pill, be on hormone replacement therapy,[99] or have lupus anticoagulant associated with an increased risk of venous thrombosis.[100]

Recently, it has been suggested that high levels of FII, seen, for example, in subjects with the prothrombin G20210A transition, may have increased thrombotic risk through high levels of prothrombin-inhibiting APC and increasing APCr. Prothrombin levels have been shown to correlate inversely with APCr.[101]

Women show a significantly greater number of individuals with APCr in the absence of FVL, and this suggests that they are more likely to suffer from post-operative thrombosis.[102] Conversely, least APCr is seen in blood group O subjects, due to relatively lower levels of FVIII in these subjects.

Factor V Cambridge, a rare mutation where Arg306 is replaced by Thr306,[103] is also associated with APCr. However, a more common mutation, Arg306 replaced by Gly306, found in Chinese subjects is not associated with APCr.[104, 105] To date, no mutations have been found at the third cleavage site in FVa (Arg679). Interestingly, recombinant mutations of FVIII, which lack the Arg336 or Arg562 cleavage site for APC, do not exhibit APCr, as it is only when both mutations are present in the same gene product that APCr becomes apparent.[106]

Factor V Leiden has a frequency of around 4% in the European population and is believed to have arisen from a point mutation in a single individual,[107] some 21 000 to 34 000 years ago. Factor V Leiden is most common in European subjects – generally more so in northern than southern Europe – and is not found in Mongoloid populations of South-East Asia and the Americas.[108]

## Testing for activated protein C resistance and factor V Leiden

The initial diagnosis of APCr was described using addition of APC to plasma to prolong the APTT.[93] Subsequently, Rosen *et al*. showed that although the APC-induced prolongation of the APTT varies considerably between instruments, calculation of the ratio of APTT with APC to APTT without APC will remove much of this varation.[109] An evaluation of the APTT-based APCr test by de Ronde demonstrated a sensitivity of the test to APTT reagent source, calcium chloride concentration and freeze-thawing of plasma.[110] Luddington *et al*. also identified the effect of residual platelets on the APC ratio of frozen plasma.[111]

Lupus anticoagulant and heparin therapy or contamination also affect the APCr. Vasse *et al*.,[112] in an evaluation of three functional assays, demonstrated sensitivity of APTT-based methods to levels of FXII and FVIII, and a reduction in APCr during pregnancy with both APTT- and thromboplastin-based assays. Low APC ratios were also described in immunodepleted PS-deficient plasma. Low levels of FII and FX increase the APCr; thus, it is important that both PT and APTT are normal if a valid APCr is to be reported.

Standardisation of the APTT-based APCr test may be improved by determination of a normalised APC ratio, in which the test APC ratio is divided by the APC ratio of pooled normal plasma in the same test run. Variations between batches of APC and activator may be reduced using this method.[109] However, Tripodi *et al*., in a multicentre study, showed no improvement in reproducibility or discrimination using normalised ratios.[113]

A modification to the APCr test, using a predilution of test plasma in FV-deficient plasma, improves the specificity and sensitivity of the test to FVL by up to 100% in some studies.[114] The test can be performed on subjects receiving coumarin therapy and has also been shown to improve the sensitivity of the APCr test in children.[115] However, careful selection of FV-deficient plasma source and sample dilution is necessary to optimise performance of the modified APCr test.[114]

Some authors advocate the use of the APCr test with and without the FV-deficient plasma modification, to identify individuals carrying the FVL mutation and those with acquired APC resistance. In the authors' laboratory, an abnormal phenotypic screening test for FVL is confirmed by genetic analysis. Phenotypic analysis may suggest homozygous FVL, but confirmation of the homozygous genotype by genetic analysis is essential, as it rules out the presence of a FV-null allele and a true heterozygous genotype.

## Prothrombin G20210A

The prothrombin 20210A allele (PTA allele) was discovered in 1996[116] and is a G to A transition at nucleotide position 20210 of the prothrombin gene. This transition, in a non-translated region of the gene, is associated with increased levels of functionally normal FII, which may be the cause of increased venous thrombotic risk. As with FVL, the G20210A transition is believed to have occurred in a single Caucasian individual and is not the result of multiple mutations.[117]

If a subject is heterozygous for the PTA allele, the risk for venous thrombosis is increased two- to five-fold compared to subjects without the mutation.[116,118,119] As with FVL, the prothrombin G20210A mutation is not a risk factor for myocardial infarction unless other major risk factors for cardiovascular disease are present.[120]

Diagnosis of the prothrombin G20210A mutation is only possible by DNA analysis, as there is no specific phenotypic marker for the allele. Factor II levels are raised, with the mean around 1.3-fold higher in subjects possessing the mutation,[116] but results for individuals often fall within the normal range.

Detection of the PTA allele and the FVL mutation is performed by the polymerase chain reaction (PCR), in which specific primers are used to amplify a sequence of patient's DNA that may contain the point mutation in question. Detection of mutation may be carried out in a number of ways, including restriction enzyme digestion,[94] allele-specific amplification, enzyme-linked immunosorbent assay (ELISA),[121] and using the LightCycler (Roche Diagnostics, Lewes, UK), which utilises fluorescence resonance energy transfer to detect the genotype of the amplified alleles. A multiplex method has also been described for the simultaneous detection of the PTA allele and FVL by a single-strand mutation detection multiplex,[122] and commercial kits are available for multiplex assays.

Detection of mutations by PCR involves the use of probes designed to bind to complementary target sequences in template DNA, and an enzyme – *Thermus aquaticus (Taq)* polymerase – amplifies the DNA between $10^5$ and $10^6$ times.[123] It is clear that contamination of samples with amplified PCR product offers a serious risk for diagnostic errors – spillage of 5 $\mu$L of PCR product contains DNA equivalent to that in 100 mL of blood. Clearly, tests involving the PCR reaction must be carried out meticulously, making use of specialised apparatus and areas of laboratory space, with appropriate decontamination procedures that further eliminate the risk of sample cross-contamination.

The method must also be robust. For example, if a method uses allele-specific amplification then conditions must allow consistent amplification of both alleles. Interpretation of results must be simple and unambiguous so that data can be interpreted correctly, without errors being made due to ambiguity or poor transcription.

It is clear from UK NEQAS returns[124] that mistakes are made frequently by laboratories carrying out these complex

genetic tests; thus, it is sensible to assume that robust assays with meticulous procedures and reporting systems are required to avoid errors. An internal quality control procedure is recommended by Bladbjerg *et al*. for genetic analysis of haemostatic defects.[125] Where a result suggests that a patient has a serious genetic risk of thrombosis due to homozygous FVL, repeat samples should be tested to confirm that no error has occurred in the genotyping due to sampling, analysis or reporting mistakes. It is the practice in the authors' laboratory to check positive FVL screen phenotype against genotype and investigate any discrepancy. The homozygous FVL genotype and samples with prothrombin G20210A transition are always retested to help confirm genotype.

## Global screening tests

Global screening tests for the protein C pathway were developed in the mid- to late 1990s in the hope that they would detect defects analogous to those failures of coagulation detected by APTT, thus simplifying the 'thrombophilia screen'. These tests utilise a non-physiological activator of PC (from the venom of *Agkistrodon contortrix contortrix*) and formed APC interacts with PS and FV to destroy FVa and FVIIIa, thus prolonging a clotting time.[126-128]

Prolongation of clotting time is expressed as a ratio by dividing the APC clotting time by the baseline clotting time. A reference plasma may also be tested and a then a 'normalised ratio' is reported, which minimises reagent, batch and instrument differences. A normally prolonged clotting time might be anticipated to exclude deficiencies of PC and PS, as well as the presence of FVL.

Published trials of these global screening tests generally conclude that sensitivity to FVL is excellent, sensitivity to PC deficiency may be moderate to excellent (rarely 100%) and sensitivity to PS deficiency is poor. Specificity of different techniques to defects in the PC pathway varies between methods. Many subjects with apparently normal PC pathway have an abnormal global screening test result and it has been suggested that this could be due to unexplained PC pathway defects.[126] Lupus anticoagulant often gives rise to abnormal results, as may high levels of FVIII in methods based on APTT.[129]

It has been suggested that the ProC Global test is more likely to be abnormal in subjects with PS deficiency and a history of thrombosis than in PS-deficient subjects without thrombosis, although it is not known whether this is a predisposing factor or a post-thrombotic effect.[130]

A recent evaluation carried out by the Medical Devices Agency concluded that currently available commercial global screening tests for the PC pathway lack sufficient sensitivity to deficiencies of PC and PS.[131]

## Additional tests in screening for thrombophilia

***Homocysteine***

Several publications have linked hyperhomocysteinaemia with thromboembolic disease, and 30 centres in the UK NEQAS (Blood Coagulation) proficiency testing programme now perform homocysteine measurements, either as part of a routine thrombophilia investigation or for selected patients. EDTA or citrated plasma may be used but should be separated from cells within an hour of collection to avoid contamination with red cell homocysteine; however, acidic citrate anticoagulant may confer greater stability than EDTA in this respect.[132]

Appropriate gender-specific reference ranges should be established for the anticoagulant employed. Some authors recommend homocysteine measurement before and after a methionine loading dose. Several different methods are available, apparently giving similar results.[133, 134]

***Dysfibrinogenaemia***

Some cases studies indicate a link between dysfibrinogenaemia and thrombosis. Although the fibrinogen clotting activity:antigen ratio is considered to be the confirmatory test for diagnosis of dysfibrinogenaemia, the majority of inherited abnormalities can be detected by a prolonged thrombin time.[135] The thrombin time also may be abnormal in acquired conditions (liver disease, presence of heparin) and thus serve as a useful screen for interference with other thrombophilia screening tests.

***Lupus anticoagulant/antiphospholipid syndrome screening***

The focus of this review is screening for familial thrombophilia; however, comprehensive guidelines on screening for lupus anticoagulant/antiphospholipid syndrome have been published recently.[8]

***Increased levels of procoagulant factors***

Publications have linked raised levels of FVIII:C, FVII:C, FIX:C and FXI:C with increased incidence of thrombosis.[136] However, it is difficult to identify an inherited component to these increased levels and currently their measurement is likely to remain of value in epidemiological studies only, although a number of laboratories now include FVIII:C as part of their thrombophilia screen. Guidelines on assay design and performance have been published[137,138] and international reference preparations are available.

## Preanalytical variables

There are a number of common principles underlying the preanalytical variables associated with different tests in thrombophilia screening. The importance of good venipuncture technique has been described previously.[139] Samples for most studies can be anticoagulated with trisodium citrate, with 0.105 mol/L citrate anticoagulant now widely adopted for coagulation studies. However, the concentration of citrate may influence results of some tests (e.g. APCr);[110] consequently, it is important that samples from patient and for reference ranges are collected in identical fashion.

Sample processing for homocysteine assay is important. Plasma must be separated from red cells within an hour of collection; however, in the authors' laboratory, levels in separated citrated plasma appear to be stable at room temperature for up to 96 hours.

Although a single centrifugation step (2000 x*g*, 10 min) is sufficient to separate plasma for some studies, double centrifugation is necessary for others (lupus investigations,

**Table 4.** List of some preanalytical variables that may affect thrombophilia screening. Some general variables may affect results to a greater degree than others and are shown in bold type

| Assay | General sources of variation | Specific test variables |
|---|---|---|
| Protein C | **Age**, sex, ABO blood group, circadian variation, **coumarin therapy** | Clot-based assay may also be affected by heparin, FV Leiden, LA, raised FVIII:C |
| Protein S | **Age, sex, oral contraceptive use, HRT, pregnancy, coumarin therapy** | Activity assay may also be affected by FV Leiden, LA, raised FVIII:C, FVIIa, heparin |
| Antithrombin | Age, sex, **oral contraceptive use,** circadian variation, **asparaginase therapy** | Enzyme – thrombin/Xa; thrombin – bovine/human; incubation time with heparin, heparin concentration; lipaemia |
| APCr | **Gender,** blood group, **oral contraceptive/HRT** | Platelet contamination, citrate concentration, frozen/fresh samples, high levels of FVIII, low levels of clotting factors, esp. FII, LA* |

* Several of the variables affecting APCr may be avoiding by the use of FV-depleted plasma.

All levels may be affected by acquired conditions such as liver disease, disseminated intravascular coagulation, malignancy, acute thrombosis

APCr screening). It would seem practical, therefore, to double spin all plasma prior to storage. Cold centrifugation of whole blood may cause activation of platelets and clotting factors; therefore, room temperature centrifugation is recommended.

Although PC, PS and AT levels are stable in plasma stored at -24°C for up to three months, -70°C or below is recommended for long-term storage and for screening tests.[140] However, we have shown that genetic analysis for FVL and PTA can be carried out on citrated blood samples stored for many weeks at room temperature (unpublished data).

Table 4 shows some of the factors known to interfere with thrombophilia screening tests. The list is by no means exhaustive but includes factors that should be taken into consideration when recruiting donors and interpreting results.

Good assay design should be employed for all investigations. Freshly prepared standard curves are recommended, constructed from at least three dilutions of reference plasma. Assay precision should be evaluated to determine whether single or replicate testing is required, and both normal and abnormal quality control material should be tested with each batch of plasma.

Reference plasmas should be obtained either from carefully calibrated commercial sources or from the National Institute for Biological Standards and Controls (NIBSC). It is important to be aware that the assigned potency may only be accurate for the method used to calibrate the plasma.

Quality assurance procedures should include internal quality control to ensure between-run precision of the method, both normal- and abnormal-level controls should be employed, and results plotted to identify trends. Participation in external quality assessment will allow between-laboratory comparison and identify method-related differences.

## Reference ranges

It is important that reference ranges be determined for the method and test system in local use. Ideally, normal donors should be recruited from the local population, and a minimum of 40 is recommended to construct a reference range.[141] However, less than two-thirds of UK NEQAS participants employ locally determined reference ranges for their thrombophilia screening tests (Table 1).

The effect of gender, age and other preanalytical variables (Table 4) should be taken into account, both in terms of specific reference intervals (e.g. gender-specific for functional PS assays) and in interpretation of patient results (e.g. PC levels in children and young adults are relatively low[142]). Interpretation should be made with due regard to analytical and clinical variables to ensure inappropriate diagnoses are avoided.

## A practical approach to testing

As an example of a practical diagnostic approach, the following procedure is employed in the authors' laboratory for AT assays.

The normal range for AT activity is 0.84 iu/mL to 1.16 iu/mL, based on evaluation of levels in 79 normal subjects, and set between the 2.5$^{th}$ and 97.5$^{th}$ percentiles. Distribution of AT levels in the population is non-Gaussian; therefore, a standard deviation-based reference range is not appropriate.[143]

The following guidelines are used for issuing AT results: results greater than 0.90 iu/mL are issued without comment; results less than 0.90 iu/mL are checked by reintroducing the sample to the analyser; AT less than 0.80 iu/mL is checked to confirm that the sample is plasma, not serum; AT less than 0.90 iu/ml is assayed for AT antigen level and the result issued if activity and antigen agree; and if activity and antigen levels are discrepant, both are checked and results issued if in agreement, rechecked if there is an anomaly. This approach of checking and rechecking is adhered to rigidly and has picked up analytical errors on a number of occasions.

Complex analysers make highly accurate analysis of large numbers of samples possible, but they are prone to complex problems. Duplicates should be no more than around 5% different. If excess imprecision is suspected, we assay AT 10 times on plasma with a normal AT level and consider that a lack of precision is present when the CV is greater than

around 2% in this run.

Typically, we observe a CV of 1.3-1.9%, the former typically shows a range of levels varying by only 0.03 iu/mL in a sample with mean AT of 0.98 iu/mL, and the latter shows a maximum difference of 0.06 iu/mL. An unacceptable CV of 2.5% can be associated with results varying from 0.83 iu/mL to 0.90 iu/mL.

It is advantageous to perform runs of at least 20 patient samples, as we consider daily calibration of instrumentation for AT diagnostic assay to be essential. Large batches are economical, and a trend to higher or lower results on any one day can detect errors of accuracy, even when the quality control plasmas are still within 'acceptable' limits.

## Conclusions

There is general consensus in the literature that PC, PS, AT, FVL, prothrombin G20210A mutation and lupus anticoagulant screening should form the panel of tests for thrombophilia. Other tests may be incorporated but their diagnostic utility is a matter of debate. If screening tests for thrombophilia are to be employed by a laboratory, it is important that all variables and possible pitfalls are considered, in order to ensure an accurate diagnosis is made.

Careful selection and validation of test methods should be undertaken by individual laboratories, to ensure both specificity and sensitivity. Appropriate reference ranges should be employed and quality assurance should be maintained through in-house quality control measures and the review of results in external quality assessment programmes. □

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