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Review article
Pregledni članak

LABORATORY INVESTIGATION OF THROMBOPHILIA

LABORATORIJSKO ISPITIVANJE TROMBOFILIJA

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Summary: Laboratory investigation of thrombophilia is aimed at detecting the well-established hereditary and acquired causes of venous thromboembolism, including activated protein C resistance/factor V Leiden mutation, prothrombin G20210A mutation, deficiencies of the physiological anticoagulants antithrombin, protein C and protein S, the presence of antiphospholipid antibodies and increased plasma levels of homocysteine and coagulation factor VIII. In contrast, investigation of dysfibrinogenemia, a very rare thrombophilic risk factor, should only be considered in a patient with evidence of familial or recurrent thrombosis in the absence of all evaluated risk factors mentioned above. At this time, thrombophilia investigation is not recommended for other potential hereditary or acquired risk factors whose association with increased risk for thrombosis has not been proven sufficiently to date. In order to ensure clinical relevance of testing and to avoid any misinterpretation of results, laboratory investigation of thrombophilia should always be performed in accordance with the recommended guidelines on testing regarding the careful selection of patients, time of testing and assays and assay methods used. The aim of this review is to summarize the most important aspects on thrombophilia testing, including whom and when to test, what assays and assay methods to use and all other variables that should be considered when performing laboratory investigation of thrombophilia.

Keywords: acquired risk factors, hereditary risk factors, laboratory investigation of thrombophilia, venous thromboembolism

Sažetak: Cilj laboratorijskog ispitivanja trombofilije je otkrivanje već ustanovljenih naslednih i stečenih uzroka venskog tromboembolizma, među kojima su aktivirana rezistencija na protein C/mutacija faktora V Leiden, mutacija protrombina G20210A, deficijencija fizioloških antikoagulanasa antitrombina, proteina C i proteina S, prisustvo antifosfolipidnih antitela i povišenih nivoa homocisteina i faktora koagulacije VIII u plazmi. Nasuprot tome, ispitivanje disfibrinogenemije, veoma retkog faktora rizika za trombofiliju, treba uzeti u razmatranje samo kod pacijenata kod kojih postoje dokazi o porodičnoj ili rekurentnoj trombozi uz odsustvo svih navedenih faktora rizika. U ovom trenutku, ispitivanje trombofilije se ne preporučuje za ostale potencijalne nasledne ili stečene faktore rizika, čija povezanost sa povećanim rizikom za trombozu još nije nedvosmisleno dokazana. Kako bi se obezbedila klinička relevantnost testiranja i izbeglo pogrešno tumačenje rezultata, laboratorijsko ispitivanje trombofilije trebalo bi uvek vršiti u skladu s preporukama za testiranje koje se odnose na pažljiv odabir pacijenata, vreme testiranja i testove i metode koji se koriste. Cilj ovog preglednog članka je da se ukratko predstave najvažniji aspekti testiranja trombofilije, između ostalog, koga i kada testirati, koje testove i metode upotrebiti i koje sve varijable treba uzeti u obzir prilikom laboratorijskog ispitivanja trombofilije.

Ključne riječi: stečeni faktori rizika, nasledni faktori rizika, laboratorijsko ispitivanje trombofilije, venski tromboembolizam

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List of abbreviations: ACL, anticardiolipin antibodies; anti-β_2GP1, anti-β_2 glycoprotein-1 antibodies; APC, activated protein C; APCR, activated protein C resistance; aPLAs, antiphospholipid antibodies; APLS, antiphospholipid syndrome; APTT, activated partial thromboplastin time; AT, antithrombin; C4B-BP, C4B-binding protein; CRP, C-reactive protein; CBS, cystathionine-b-synthetase; DIC, disseminated intravascular coagulation; dRVVT, dilute Russell's viper venom time; ELISA, enzyme-linked immunosorbent assay; FII, factor II; FIIG20210A, prothrombin G20210A mutation; FVL, factor V Leiden; FVIII, factor VIII; FXa, activated factor X; HC, homocysteine; HHC, hyperhomocysteinemia; HCII, heparin cofactor II; HBS, heparin binding site mutation; HRT, hormone replacement therapy; LA, lupus anticoagulants; MTHFR, methylenetetrahydrofolate reductase; OC, oral contraceptives; PC, protein C; PS, protein S; PT, prothrombin time; RT, reptilase time; TT, thrombin time; VKA, vitamin K antagonists; VTE, venous thromboembolism.

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Introduction

Thrombophilia is defined as a tendency to develop thrombosis due to predisposing hereditary and/or acquired risk factors. Although thrombosis may occur in both veins and arteries, the term thrombophilia is usually considered in the context of venous thromboembolism (VTE), since most of the well-defined thrombophilic risk factors are commonly associated with thrombosis in venous blood vessels. Today, VTE is a serious health problem that affects approximately 1–2 individuals per 1000 in the general population of Western countries each year (1–3). During the last two decades, knowledge on the etiology of thrombophilia has considerably increased and various hereditary and acquired risk factors have been discovered. This has led to widespread laboratory investigation of thrombophilia. Due to the lack of global assays for thrombophilia investigation, testing requires an expensive approach by performing a panel of different assays for each individual patient. However, testing is not always justified because patients are not carefully selected or the appropriate time of testing is often not considered (4–6). The laboratory investigation of thrombophilia should always be performed in accordance with the recommended guidelines on testing, regarding whom and when to test and what assays and assay methods to use. Inappropriate thrombophilia testing outside the recommended guidelines may be more detrimental than helpful for the patient due to possibility of misinterpretation of the test results with simultaneously huge waste of health-care resources.

The aim of this review is to summarize the most important current knowledge in the laboratory diagnosis of thrombophilia including careful patient selection and clinical conditions to be investigated, the recommended assays and assay methods for individual risk factors as well as all other variables that should be considered when employing laboratory investigation of thrombophilia.

Who should be investigated?

The prevalence of any known risk factor for VTE is not sufficient to justify indiscriminate screening of the general population (7–8). The main clinical indication for investigation includes patients with a history of unexplained VTE (7, 9, 10). The clinical usefulness of thrombophilia testing in some other subject populations, such as women with pregnancy complications or failure, women on oral contraceptives (OC) or hormone replacement therapy (HRT) and asymptomatic first degree relatives of VTE patients with known thrombophilia, is still widely debated. According to the current knowledge, thrombophilia testing in these groups of subjects should be critically considered for carefully selected individuals as will be discussed hereinafter.

Patients with venous thromboembolism

Although, as already mentioned, patients with a history of VTE represent the main population suitable for laboratory investigation of thrombophilia, it is important to note that testing is not indicated in unselected patients presenting with a first episode of VTE (10). Instead, the target population of VTE patients that should be considered for testing includes those with a confirmed VTE that fulfill at least one of the following criteria: thrombosis prior to the age of 50 years even in the presence of a transient predisposing risk factor, recurrent venous thrombosis, thrombosis at unusual sites (portal, mesenteric, splenic, hepatic, renal or cerebral veins) and VTE patients with a family history of VTE (9, 11). When considering thrombophilia investigation, it is always important to keep in mind that VTE is a multifactorial disorder which means that a single hereditary or acquired risk factor does not necessarily lead to thrombosis without interaction with other transient predisposing risk factors (12). Although VTE at a young age is an important feature of thrombophilia, the first thrombotic episode may happen later in life. Also, some persons with thrombophilia do not experience a thrombotic event if an additional triggering transient risk factor is not present. Namely, besides those well-defined hereditary and acquired thrombophilic risk factors, which will be discussed below, there are also several transient or environmental risk factors, including trauma, immobilization or prolonged bed rest, surgery and postoperative state, advancing age (>60 years), malignancy, pregnancy and postpartum period, use of estrogen-containing OC or HRT, long distance travel and obesity, that are associated with an increased risk for VTE (12, 13). These risk factors can predispose any individual to thrombosis, but may also stimulate thrombosis in individuals with hereditary or acquired thrombophilia. Interactions between hereditary or acquired thrombophilic defects and transient risk factors further increase the risk of VTE (14, 15). VTE often occurs in subjects with an underlying thrombophilic risk factor in pathophysiological conditions associated with the presence of a transient triggering risk factor, as a result of their synergistic interactions. Currently, VTE patients in whom thrombophilia testing is still debated in the literature include those with unprovoked VTE over 50 years of age. Although age over 50 years is considered to be an exclusion risk factor for testing, the results of some studies have shown that relatively weak hereditary risk factors, such as heterozygosity for factor V Leiden (FVL) and prothrombin G20210A (FIIG20210A) mutations, may result in a first VTE also in subjects older than 50 years (16, 17). Further, thrombophilia testing for patients with a first VTE associated with a known transient risk factor is also debated. According to some experts, neither age nor the presence of pre-disposing transient risk factors at the time of VTE should not be taken as strict criteria to decide on testing because

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VTE may develop later in life and after exposure to high-risk situations (18). However, it is generally accepted that investigation of thrombophilia can be justified if any of the transient risk factors or high-risk situations is present in a VTE patient that fulfill at least one criterion mentioned above (age<50 years, recurrent thrombosis, family history of thrombosis or thrombosis at unusual sites). On the other hand, if only one transient risk factor is present in a patient with VTE without the other aforementioned criteria for testing, laboratory investigation of thrombophilia is considered not to be justified in most clinical situations (7, 9–11).

Patients with vitamin K antagonist-induced skin necrosis

Patients on therapy with vitamin K antagonists (VKA) who develop skin necrosis should be tested for protein C and protein S deficiency after VKA treatment is withdrawn (10). Although rare, VKA-induced skin necrosis is a serious complication of this therapy, associated with heterozygosity of protein C (PC) or protein S (PS) deficiency typically occurring during the first days of therapy.

Patients with arterial thrombosis

Arterial thrombosis is a multifactorial disease whose risk factors mostly do not coincide with those for VTE. The association between most well-defined VTE risk factors and arterial thrombosis, such as acute myocardial infarction and ischemic stroke, is not firmly established to date (19–21). According to the current knowledge, only the presence of antiphospholipid antibodies (aPLAs), hyperhomocysteinemia (HHC) and dysfibrinogenemia can be associated with both venous and arterial thrombosis (22–24). Therefore, laboratory investigation of hereditary thrombophilia is not indicated in most patients with arterial thrombosis (10, 21). Thrombophilia testing is considered to be potentially useful only in a very restricted population, such as children and young patients (<40 years) presenting with arterial thrombosis (25–28).

Children with thrombosis

Thrombosis in children is rare in comparison with adults, but when it occurs two peaks of higher incidence are seen: soon after birth and in the teenage years. According to the Subcommittee for Perinatal and Pediatric Thrombosis and Hemostasis guidelines from 2002, International Society of Thrombosis and Hemostasis recommended laboratory investigation of thrombophilia for a full panel of genetic and acquired prothrombotic traits in all pediatric patients with both venous and arterial thrombosis (29). The rationale for this recommendation was that pediatric patients often have more than one thrombophilic risk factor and even if acquired triggering risk factors are present, testing for genetic thrombophilic defects should also be performed. However, this committee acknowledged that further clinical studies are needed to substantiate this recommendation (29). In recent years, there have been continuous debates related to unselected and uniform thrombophilia testing in all children with thrombosis (30–32). It is obvious that further prospective multicentre clinical studies are needed in order to obtain definitive recommendations regarding thrombophilia testing in children with thrombosis. According to the recent British Committee for Standards in Haematology and the British Society for Haematology guidelines, testing for heritable thrombophilia is not indicated in children with stroke (10). Also, there are other important unresolved issues that should be taken into account when considering thrombophilia investigation in children, such as identification of inherited thrombophilia in an asymptomatic child of probands with known thrombophilia (32, 33). Currently, there is little evidence that supports thrombophilia testing in asymptomatic children. Some experts advise delaying testing until puberty or even until the subject is old enough to make their own decisions regarding testing (34, 35). It seems the most acceptable that the decision to perform testing in asymptomatic children with a positive family history be made on an individual basis after counselling with the family about potential benefits and limitations of testing. Asymptomatic children that are most likely to benefit from thrombophilia testing include teenage females with a positive family history who are intending to use OC.

Pregnancy

Pregnancy and puerperium represent an important transient risk factor for VTE due to hemostatic imbalance towards a prothrombotic state (1, 36). Also, pregnant women with thrombophilic risk factors have further increased risk for VTE (37–40). The incidence of VTE in pregnancy is about 1 per 500 for the FVL mutation, 1 per 200 for the prothrombin mutation and 4.6 in 100 for the combined FVL and prothrombin mutations (41). However, although the relative risk of VTE in pregnancy is increased compared with nonpregnant women of the same age, the absolute risk remains low, with an overall incidence of VTE in pregnancy and the puerperium of 1–2/1000 (1, 42). Therefore, the overall general screening of pregnant women is not indicated since the absolute risk and overall predictive value are relatively low (43, 44). Instead, laboratory investigation of thrombophilia is indicated only in selected pregnant women with previous VTE or a positive family history of VTE in first degree relatives (10, 45, 46) and in case of suspected antiphospholipid syndrome (APLS) as this can

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influence the decision regarding antenatal thrombo-prophylaxis (45). For asymptomatic pregnant women with a family history of VTE, testing is advised if VTE in a first-degree relative was unprovoked, or provoked by pregnancy, OC use or a minor risk factor (10).

Women with pregnancy complications or failure

The results of some studies have shown that both inherited and acquired thrombophilic risk factors are associated with an increased risk of pregnancy complications and failure, including severe pre-eclampsia, placental abruption, intrauterine growth restriction, unexplained consecutive first trimester abortions and second and third trimester unexplained fetal death (47–51), thus suggesting potential clinical importance of thrombophilia investigation in women with a history of adverse outcomes during pregnancy (37, 47, 52–54). However, whether this association is causal remains controversial and unclear to date, since many other factors play a role in the risk of pregnancy complications (55–58). It seems likely that thrombophilia is only one of many factors that may lead to pregnancy complications and is unlikely to be the unique factor that should drive management in subsequent pregnancies (55, 59). To date, there are no uniform recommendations on thrombophilia testing in this population of women, mostly due to the lack of adequate studies to support or exclude causal association of thrombophilia and pregnancy complications. The results of different studies are contradictory and potential benefits of thrombophilia testing in this female population are not well-established at this time. Some expert groups suggest laboratory investigation of thrombophilia in pregnant women with previous obstetric complications including recurrent pregnancy loss, unexplained intrauterine fetal death, preeclampsia, *abruptio placentae*, HELLP syndrome and fetal growth restriction (46). According to the other experts, women with pregnancy loss that is either recurrent or late (second and third trimester) should be evaluated for thrombophilia, while the criteria for screening women with gestational vascular complications other than pregnancy loss are widely debated and vary in different maternal units (9, 34, 56, 57, 60). Among the well-known hereditary and acquired thrombophilic risk factors, only the presence of aPLAs has shown strong association with recurrent pregnancy loss (49) and a recent Cohrane review showed the efficacy and safety of thromboprophylaxis with aspirin and heparin in these women (61), thus suggesting justification of testing for aPLAs in women with recurrent pregnancy loss.

Women in assisted conception procedures

To date, there is no strong evidence for an association between maternal thrombophilia and failure to achieve pregnancy after assisted conception procedures (62, 63). Therefore, laboratory investigation of thrombophilia in asymptomatic women before assisted conception and those with ovarian hyperstimulation syndrome is considered not to be justified (10). Namely, although ovarian hyperstimulation is associated with an increased risk of VTE, the overall risk is small and estimated to be 0.1% per treatment cycle, a similar incidence as in women with pregnancy associated VTE (63, 64).

Women on oral contraceptives or hormone replacement therapy

Laboratory investigation of thrombophilia in women on OC and HRT is continually debated in the literature. It is known that exogenous estrogens used in the combined OC and HRT are associated with an increased relative risk for VTE (65–67). Also, the combination of OC or HRT use with well-defined hereditary or acquired thrombophilic risk factors further increases the risk for VTE (68–70). According to the newer literature data, incidence of VTE in women of reproductive age who are not OC users is about 4–5/10 000 per year, with an approximate doubling of risk into the range of 9–10/10 000 for women who are OC users (71, 72). However, despite of an increased relative risk in women on OC or HRT with and without thrombophilic risk factors, the absolute risk attributable to hormonal therapy remains low, especially in young women on OC (1, 65, 73). In addition, it is generally considered that the benefits of OC use outweigh the risk for VTE for most healthy women of reproductive age (74). For women on HRT therapy, relative risk is similar as in users of OC, while the absolute risk is somewhat higher due to older age, but also remains low. According to the current knowledge, unselected thrombophilia testing of all women on OC or HRT, as well as those prior to the prescription of these drugs is not supported by the majority of investigations and available recommendations (1, 10, 75–78). Rather, thrombophilia investigation is indicated only in selected cases with a previous history of VTE or positive family history (75, 78, 79), in which testing may be helpful in assessing the overall thrombotic risk and balancing benefits and risks for each individual patient. However, it is important to note that the result of thrombophilia testing will not alter the advice that estrogen-containing OC or HRT should be avoided and alternative methods of treatment should be considered in women with a personal history of thrombosis as well as in those with an identified thrombophilic defect in a symptomatic family member (73, 78).

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Asymptomatic first degree relatives of VTE patients with known thrombophilia

There are several valid arguments in support and against thrombophilia testing in asymptomatic individuals who are first degree family members of a VTE patient with a known thrombophilic risk factor (80). The main arguments in support of testing are potential avoidance of transient predisposing risk factors, such as use of combined OC in young females and targeted thromboprophylaxis in high-risk situations, such as immobilization or surgery. However, it is important to note that individual risk for VTE is different even among first degree relatives since it is influenced by the interaction of genetic and environmental factors. A positive test result for a certain thrombophilic risk factor in an asymptomatic subject does not necessarily lead to thrombosis in that subject. Also, individuals with negative test results may be falsely reassured that they are not at an increased risk for VTE. Accordingly, the benefit of identifying a risk factor in asymptomatic relatives is quite uncertain. Further, several other arguments have been made to argue against thrombophilia testing in asymptomatic relatives, such as risk of labelling subjects as carriers of a genetic disease, thus generating possible insurance and employment discrimination and high costs of screening. It has been shown that case finding of asymptomatic relatives of patients with VTE caused by hereditary defects of low risk, such as FVL or prothrombin FIIG20210A mutations, does not reduce the incidence of VTE and that the annual risk of unprovoked VTE in family members who are carriers of one of these two mutations is low (10). In contrast, the risk of VTE in asymptomatic relatives with genetic defects of higher risk, such as deficiencies of natural anticoagulants antithrombin (AT), PC or PS is considered to be significantly higher in comparison with non-affected relatives (81). Therefore, according to the newer available guidelines for heritable thrombophilia testing, case finding of asymptomatic relatives with thrombophilic risk factors of low risk (FVL or FIIG20210A mutations) is not indicated, while case finding of asymptomatic relatives with high risk thrombophilic risk factors, such as deficiency of AT, PC or PS, should only be considered in selected thrombosis prone families with more than two symptomatic family members (10). In general, thrombophilia testing to prevent initial episodes of VTE is not indicated, except possibly in women with a family history of idiopathic VTE who are considering OC therapy (82). It is also important to take into account the possible negative psychological impact of test results on an asymptomatic subject, such as persistent anxiety, fear and depression in case of a positive test result (83, 84). As a general recommendation, if thrombophilia investigation is performed in an asymptomatic subject, the risks, benefits and limitations of testing should always be discussed in order to minimize the negative psychological effects of testing (85, 86).

Surgical and other hospitalized patients

Surgery and postoperative state are well-known transient risk factors for VTE. Short (<30 min) surgical procedures have a low risk for VTE, other non-orthopedic surgical procedures are associated with moderate risk, while orthopedic surgery, such as hip or knee arthroplasty and hip fracture surgery, have a high risk for VTE (87). In patients with moderate and high risk thromboprophylaxis anticoagulant drugs are routinely administered after risk assessment, and a positive test result for a specific thrombophilic defect will not have an impact on the thromboprophylactic treatment (88). Therefore, routine thrombophilia testing before surgical procedures is not recommended and should be considered only in patients with a personal or family history of unexplained VTE (9, 10, 75). Also, laboratory investigation of thrombophilia in other hospitalized patients in order to identify patients at risk of hospital-associated VTE is not indicated (10). All hospitalized patients should be assessed for VTE risk based primarily on a clinical risk assessment (10).

Which tests should be performed?

Laboratory investigation of thrombophilia is aimed at detecting the well-established causes of thrombophilia, including activated protein C resistance (APCR)/FVL, FIIG20210A mutation, deficiencies of physiological anticoagulants AT, PC and PS, the presence of aPLAs, HHC and increased levels of FVIII (89–91). Laboratory investigation of dysfibrinogenemia, as a very rare risk factor for VTE, is not part of routine thrombophilia testing and should be considered in selected patients only, i.e. if all the well-defined risk factors mentioned above are negative in a patient with strong evidence of familial or recurrent thrombosis (10, 79). At this time, thrombophilia investigation is not recommended for other possible hereditary or acquired risk factors, such as abnormalities of the fibrinolytic system (increased levels of plasminogen activator inhibitor-1, deficiencies of plasminogen or tissue plasminogen activator), deficiency of heparin cofactor II (HCII), increased levels of coagulation factors fibrinogen, FVII, FIX, FXI or decreased levels of FXII, since their association with increased risk for thrombosis has not been proven sufficiently to date, or their predictive value is too low to be included in the laboratory investigation of thrombophilia (18, 90, 91).

The general strategy of thrombophilia testing is to investigate individually each of the well-defined risk factors mentioned above, even if one defect has already been identified. The detection of combined defects is clinically relevant due to significantly increased risk for VTE in patients with two or more thrombophilic risk factors in comparison with those with only one risk factor.

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Table I Step-wise approach related to assays and assay methods for individual risk factors included in thrombophilia investigations.

Test	First diagnostic step		Second diagnostic step	Comment
Activated protein C resistance/factor V Leiden (APCR/FVL)	Coagulation-based functional assay with factor V deficient plasma	if positive	DNA analysis for FVL mutation	If the coagulation-based functional APCR test is negative, FVL mutation can be excluded and DNA analysis is not indicated. Positive result of functional APCR test and negative DNA assay result for FVL mutation suggest an acquired APCR.
Prothrombin G20210A mutation (FII G20210A)	DNA analysis	––	––	Measurement of prothrombin (FII) activity in plasma should not be used to screen thrombophilic patients for this mutation due to its inability to clearly distinguish carriers from non-carriers of the mutation.
Antithrombin (AT) deficiency	Functional chromogenic assay	If positive	Measurement of AT antigen by an immunoassay in order to classify the type of deficiency as type I or II	Functional assay in the first diagnostic step identifies both types (I and II) of deficiency. Immunochemical assay should not be performed without functional assay because type II deficiency will not be detected.
Protein C (PC) deficiency	Functional chromogenic assay	If positive	Measurement of PC antigen by an immunoassay in order to classify the type of deficiency as type I or II or III	Functional assay in the first diagnostic step identifies both types (I and II) of deficiency. Immunochemical assay should not be performed without functional assay because type II deficiency will not be detected.
Protein S (PS) deficiency	Functional coagulation-based assay	If positive	Measurement of free or/and total PS antigen by an immunoassay to classify the type of deficiency as type I, II or III	Functional assay in the first diagnostic step is capable of identifying all three types (I, II, III) of deficiency. Be aware of limitations of the functional coagulation assay for PS related to interferences of positive LA, FVIII or APCR. Free PS antigen assay is considered to be the test of choice in the second diagnostic step.
Antiphospholipid antibodies (aPLAs): LA, ACL and anti-β_2-GP1	LA: a panel of screening (two or more) assays and at least one confirmatory assay. ACL and anti-β_2-GP1: enzyme immunosorbent assays for both IgM and IgG isotypes	If positive one or more sub-groups of aPLAs	Repeat testing for a positive test result with at least 12 weeks apart in order to confirm a positive test result	Repeating testing for any positive test result is mandatory in order to exclude the transient occurrence of aPLAs in conjunction with microbial infections and drugs.
Increased factor VIII level (FVIII>150%)	Clotting or chromo-genic functional assay	If positive	Repeat testing 3 to 6 months after initial testing	Repeating testing for a positive test result is mandatory in order to confirm persistent increase in FVIII and to exclude the potential effect of acute phase response.
Hyperhomocysteinemia (HHC)	Plasma level of homocysteine	If positive	Repeat testing in case of a questionable or borderline test result or to confirm a positive test result	Do not use DNA analysis for MTHFR C677T polymorphism as part of thrombophilia investigation.
Dysfibrinogenemia	Screening assays: Thrombin time (TT) and reptilase time (RT). Functional (Clauss) fibrinogen level	If positive	Parallel analysis of functional and immunoreactive fibrinogen as confirmatory assays	Do not perform laboratory investigation of dysfibrinogenemia as part of routine thrombophilia testing. Consider testing only in case of all negative risk factors mentioned above in a patient with familial or recurrent thrombosis.

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Step-wise approach in the laboratory investigation of thrombophilia

A complete personal and family medical history and physical examination are a mandatory first step for each patient who is considering thrombophilia testing. It is important to note that although a family history of thrombosis is an important selection factor for testing since it is suggestive of familial thrombophilia, a negative family history of VTE does not exclude hereditary thrombophilia in a patient provided for testing due to low penetrance of thrombophilic defects and new mutations that may occur.

The initial laboratory investigation should always begin with global coagulation tests, including prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen, in order to exclude anticoagulant therapy that affects the results of many phenotypic assays and also to rule out other clinical conditions associated with acquired coagulation disorders.

Laboratory investigation of thrombophilia should include a step-wise approach regarding the assays and assay methods used. Step-wise approach for an individual assay included in testing is presented in *Table I*. The recommended assays in the first diagnostic step should establish whether the patient has any of the well-established thrombophilic risk factors (91). In case of a positive result for one or more risk factors obtained in the first-step, the second diagnostic step should include recommended assays for the confirmation and/or characterization of the defect (*Table I*). Further, methods that should be used for an individual assay are also important because some methods for a certain assay are better than others, as will be discussed in detail in the text section related to investigation of individual risk factors included in testing. Therefore, it is important to use only the recommended assay methods in order to ensure both sensitivity and specificity of testing.

Laboratory investigation of individual risk factors included in thrombophilia testing

Activated protein C resistance (APCR) and factor V Leiden (FVL)

Inherited APCR is caused by a single point mutation in the factor V (FV) gene (92). This mutation, known as FVL, causes over 90% of positive APCR cases and results in much slower inactivation of activated factor V (FVa) due to more resistance to proteolytic degradation by activated protein C (APC). FVL mutation is the most common inherited risk factor for VTE in whites, with unequal prevalence in different populations and geographical regions (93, 94). The mutation is associated with a 3 to 8-fold increased risk of VTE in heterozygotes, and an 80-fold increased risk in homozygotes (12). However, heterozygotes for FVL mutation show variable penetrance of thrombosis, and some subjects never develop VTE, while others develop thrombosis at an early age.

FVL accounts for most, but not all cases of APCR and an acquired APCR phenotype may be present in the absence of FVL mutation. The known causes of acquired APCR are pregnancy, use of OC, presence of lupus anticoagulant (LA), increased levels of FVIII and thrombosis in myeloma patients (95–98). Since an acquired APCR is also a risk factor for VTE, independent of FVL mutation, laboratory investigation of APCR/FVL includes both a functional coagulation assay and genotyping for FVL. Functional assay identifies both inherited and acquired cases of APCR, while DNA analysis identifies FVL mutation as the cause of APCR. Therefore, the first diagnostic step of APCR/FVL investigation should always include a functional coagulation assay (99). Borderline and positive results obtained by a functional assay require a second diagnostic step, using DNA analysis in order to confirm a positive result and to differentiate between heterozygosity and homozygosity for FVL (*Table I*). It is important to note that genotyping for FVL should not be used alone without a functional coagulation assay, since it would not identify cases of acquired APCR.

The most commonly used functional test for APCR/FVL is a modification of an APTT assay based on prolongation of the APTT by the addition of APC. The result is expressed as the ratio between the APTT measured in the presence and in the absence of added APC, as originally described by Dahlback (100). However, although this method is simple and inexpensive, it is not sufficiently sensitive and specific for the FVL mutation. In the newer second-generation APTT-based assay, patient plasma is prediluted with factor V (FV) deficient plasma, thus significantly improving the sensitivity and specificity for the FVL mutation. Therefore, the modified APCR assay using FV deficient plasma should always be used as a functional assay for the detection of APCR/FVL. Also, in order to improve assay standardization, it is recommended to express the result as a normalized ratio, where the assay ratio is divided by the ratio of normal plasma (pooled or standard human plasma) analyzed in the same test run.

Variables that affect the result of a functional APCR/FVL assay are presented in *Table II*. In contrast to the functional APCR/FVL assay, a DNA-based assay for the FVL mutation is not influenced by anticoagulant therapy or by acute phase of thrombosis.

Prothrombin G20210A mutation (FIIG20210A)

The FIIG20210A mutation is the second most common inherited risk factor for VTE, present in heterozygous form in approximately 2% to 4% of whites, while it is rare in Africans and Asians (101, 102). The

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Table II Variables that affect the results of individual phenotypic assays for thrombophilia.

Thrombophilic risk factor	Variable	Impact on result	Recommendation
APCR functional coagulation-based assay	Plasma contamination with platelets, particularly in frozen and thawed samples	False-positive result	Double centrifugation of the plasma sample is advised if a frozen sample is used.
	Anticoagulant therapy with heparin, hirudin, argatroban, bivalirudin or heparin contamination	False-positive result	Do not perform testing in patients on therapy with listed anticoagulant drugs.
	Interference of positive LA	False-positive result	Suggestions proposed to deal with LA interference include higher plasma dilutions as 1:10 to 1:40 or adding excess phospholipids to neutralize the effect of LA.
Physiological anticoagulants PC, PS, AT	Anticoagulant therapy with vitamin K antagonists (VKA)	False-positive result: VKA cause reduced levels of PC and PS since both are vitamin K-dependent proteins. False-increased level of AT due to its compensatory increased synthesis.	Do not perform testing in patients on therapy with VKA.
	Anticoagulant therapy with heparin, hirudin, argatroban, bivalirudin or heparin contamination	False-positive result: AT activity decreases up to 30% during heparin therapy.	Do not perform testing in patients on anticoagulant therapy with heparin and related drugs except in the following cases: – suspected congenital AT deficiency in a VTE patient from a family with known AT deficiency; – suspected heparin resistance due to markedly reduced levels of AT.
	Acute phase of thrombosis or acute inflammation/infection	False-positive result for AT, PC, PS: decreased AT and PC due to consumption. Falsely lower free PS levels due to increased binding to C4B-BP as an acute phase reactant.	Do not perform testing in the acute phase of thrombosis or acute inflammation/infection with the exceptions for AT measurement in cases listed above: suspected congenital AT deficiency or heparin resistance.
	Pregnancy and puerperium	False-positive result: decreased levels of AT and PS.	Avoid testing in pregnant women until 6 weeks postpartum with the exceptions for AT measurement listed above: suspected congenital AT deficiency or heparin resistance.
	Estrogen therapy with oral contraceptives (OC) or hormone replacement therapy (HRT)	False-positive result: decreased levels of AT, PC, PS.	Avoid testing in women on OC or HRT therapy for three months after discontinuation of therapy with the exceptions for AT listed above: suspected congenital AT deficiency or heparin resistance.
	Age and gender	At birth levels of AT, PC and PS are decreased until the age of 6 months for AT and PS and until adolescence for PC. PS in newborns is almost entirely in the free form due to low C4B-BP levels. Healthy adult women have slightly lower PS levels than adult men and total PS concentrations increase with age in women.	For neonates and other pediatric patients separated reference ranges should be used due to significantly different values of AT, PC and PS. It is recommended to determine separate male and female reference ranges of PS for the adult population.

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	Acquired states of natural anticoagulant deficiency	Antithrombin: liver disease, sepsis, preeclampsia, pregnancy and puerperium, nephrotic syndrome, acute thrombosis Protein C: DIC, sepsis, acute thrombosis, vitamin K deficiency, liver disease, postoperative state Protein S: DIC, sepsis, acute thrombosis, vitamin K deficiency, liver disease, postoperative state, pregnancy and puerperium, nephrotic syndrome	Acquired states of natural anticoagulant deficiencies should be excluded prior to thrombophilia investigation. In case of decreased PS activity, determination of a marker of acute phase (CRP, fibrinogen) or FVIII activity may help in excluding acute inflammation.
	Clot-based functional assays for protein C and protein S	Falsely lower values of PC and PS may be obtained due to positive APCR or FVIII>150%. Falsely increased levels of PC and PS can be obtained due to LA presence or therapy with heparin, hirudin or argatroban.	Chromogenic functional assays are recommended as the test of choice for PC in order to avoid listed interferences. Abnormal result of a functional assay for PS should always be further evaluated with an immunoassay for free PS.
	Contamination of plasma sample with platelets, particularly in frozen and thawed samples	Falsely lower level of PS	Double centrifugation of plasma sample is advised if a frozen sample is used.
Lupus anticoagulant (LA)	Plasma sample with platelet count $>10\times10^9$/L	False-negative LA result due to LA neutralization by platelet phospholipids	Double centrifugation of plasma sample is advised if a frozen sample is used.
	Acute phase of thrombosis	False-negative LA due to possible consumption	Do not perform testing during acute phase of thrombosis.
	Anticoagulant therapy with heparin, hirudin, argatroban or VKA	False-positive result for LA screening tests (APTT, mixing test). VKA: False positive LA result obtained with dRVVT screen and confirm assays	Do not perform testing on any anticoagulant therapy.
	FVIII activity >150%	False-negative results for LA screening tests (APTT, mixing tests)	Measurement of FVIII activity can help to exclude acute phase reaction
ACL and anti-β_2-GPI antibodies	Rheumatoid factor or cryoglobulins	False-positive IgM ACL may be obtained	Do not perform testing at least 6 months after acute phase of thrombosis and at least 1 month after acute phase of inflammatory or infectious diseases.
	Acute phase of thrombosis Acute inflammation or infection	Transient IgM ACL may be found in inflammatory or infectious disorders	
FVIII	Acute phase of thrombosis or acute inflammation/infection	False-positive result since FVIII is an acute phase reactant	Do not perform testing at least 6 months after acute phase of thrombosis or 1 month after acute inflammation/infection. In case of suspected acute phase reaction, CRP and fibrinogen levels can be measured in order to exclude acute inflammation/infection

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	Pregnancy and puerperium	False-positive result due to physiologically increased levels of FVIII	Do not perform testing at least 6 weeks postpartum.
Dysfibrinogenemia	Acute phase of thrombosis or acute inflammation or infection	False-positive result since fibrinogen is an acute phase reactant	Do not perform testing at least 6 months after acute phase of thrombosis or 1 month after acute inflammation/infection.
	Anticoagulant therapy: heparin, hirudin, argatroban or heparin contamination	Prolonged TT	Unlike the TT test which is very sensitive to low amounts of heparin, RT test is not influenced (prolonged) by these drugs.
	Amyloidosis	Prolonged TT and RT tests	TT and RT prolongation are caused by inhibition of fibrinogen conversion to fibrin.
	Liver disease	Can cause acquired dysfibrinogenemia	Liver disease should be excluded as a cause of dysfibrinogenemia.
Hyperhomocysteinemia	Storing of whole blood sample more than 1 hour from blood collection	False-positive result due to contamination of plasma sample with HC from red blood cells	Separation of plasma from cells within an hour from blood collection
	Storing of blood sample at room temperature after blood sampling	False-elevated HC level due to temperature dependent release of HC from blood cells	Blood sample should be placed on ice or refrigerated at 8 °C until separation of plasma from blood cells.
	Diet with supplemented vitamins	False-negative result due to decreased HC plasma level	The diet should be normal and not supplemented with vitamins in the few weeks preceding testing.

VKA, vitamin K antagonists; DIC, disseminated intravascular coagulation; C4B-BP, C4B-binding protein; CRP, C-reactive protein, HC, homocysteine

mutation is found in 6% to 8% of unselected VTE patients and is associated with a 3-fold increased risk for VTE (101, 103). FIIG20210A mutation results in elevated levels of prothrombin in plasma due to its increased synthesis (104). However, the prothrombin activity is often only slightly or moderately raised in carriers of the mutation, with overlapping values between subjects with and without mutation. Therefore, the measurement of prothrombin activity in plasma is not an appropriate assay to investigate FIIG20210A, since it cannot clearly differentiate carriers from non-carriers of the mutation (105). Instead, genotyping for the FIIG20210A mutation should always be performed as part of thrombophilia investigation (*Table I*). In contrast to phenotypic thrombophilia assays, the acute phase of thrombosis and anticoagulant therapy do not affect DNA-based assays.

Deficiencies of physiological anticoagulants

Deficiencies of AT, PC and PS are uncommon, but much stronger hereditary thrombophilic risk factors in comparison with FVL and FIIG20210A mutations. Since hereditary deficiencies of physiological anticoagulants can be caused by a large number of different mutations, molecular diagnostics by DNA analysis is generally not useful for thrombophilia testing. Instead, the laboratory investigation of physiological anticoagulants includes functional and immunochemical assays.

There are two major types of AT and PC deficiencies. Type I is a quantitative defect caused by decreased synthesis of a biologically normal molecule, thus resulting in decreased both activity and concentration of AT or PC. In contrast, type II is a qualitative defect that results in decreased functional activity but normal concentration of AT or PC. The prevalence of both types of AT deficiency is about the same, type I being a more common form of PC deficiency. The laboratory investigation of AT and PC deficiencies should always include a functional assay as the first diagnostic step, since it is capable of detecting both types of deficiencies (*Table I*). In case of a positive test result obtained with a functional assay, immunochemical assays used in the second diagnostic step are useful for the classification of the type of deficiency. An immunochemical assay should not be performed without a previous functional assay because the qualitative type of AT or PC deficiencies will not be detected.

Methodology issues related to the use of different commercially available functional tests for AT and PC are also important. Functional assays for AT are

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chromogenic methods that measure AT activity related to its ability to inhibit thrombin (activated factor II) or activated factor Xa (FXa) (106). Chromogenic methods based on FXa inhibition are recommended as a method of choice, since they are not influenced by other thrombin inhibitors in plasma such as HCII, in contrast to methods based on thrombin as a target enzyme.

Functional assays for PC are either coagulometric or chromogenic methods. Both types of assays are based on the activation of PC in patient plasma using snake venom. The coagulometric assays are based on the ability of a patient's APC to degrade FVa and FVIIIa, thereby prolonging the APTT-based clotting time. Chromogenic methods measure the ability of the APC to cleave a synthetic substrate thus liberating a chromogenic compound that is spectrophotometrically measured, and are generally recommended as a functional assay, since they are not affected by interferences such as the presence of LA, positive APCR or increased FVIII activity, in contrast to coagulometric functional assays (99, 107). Enzyme-linked immunosorbent assays (ELISA) and automated immunoturbidimetric assays are used for immunochemical measurements of AT and PC anticoagulants.

The clinical relevance of characterization of individual types of AT and PC deficiencies is a matter of debate. At present, there is no evidence of clinical relevance for distinguishing between types I and II of PC deficiencies. However, an immunochemical assay for PC is often employed in the second diagnostic step in case of reduced functional activity as a useful additional step by comparison with functional PC levels (108). In contrast, type II deficiency for AT is divided into three subtypes on the basis of the nature of the functional defect that can affect the heparin binding site (HBS mutation), reactive site (RS mutation) or can have pleiotropic effects (PE mutation) (109). Among the three subtypes, only the HBS mutation is considered to have a low risk of thrombosis, thus increasing the clinical relevance of differentiating the subtypes of AT deficiencies (107, 110, 111). In order to identify subtypes, crossed immunoelectrophoresis with and without heparin or a variant of the functional chromogenic assay that measures progressive inhibitory activity of AT may be used (99).

The laboratory investigation of PS deficiency is more complex, mostly due to the lack of well-standardized functional assays. Additionally, there are three types of PS deficiencies based on PS activity and free and total PS antigen levels. Types I and III of PS deficiencies are quantitative defects with both decreased activity and free PS concentration. Total PS antigen level is decreased in type I and normal in type III. Both types I and III of PS deficiencies account for approximately 95% of cases, while type II accounts for approximately 5% of cases, representing a qualitative defect with decreased PS activity and normal concentrations of both free and total PS antigen levels (112). Laboratory investigation of PS deficiency should include both a functional assay for PS activity and an immunochemical assay for the PS antigen. Functional assays for PS are clot-based methods that are often used as the first step of testing since they detect all three types of deficiency. Functional assays measure the ability of PS to serve as a cofactor for APC, augmenting degradation of activated factors V and FVIII and thereby prolonging clotting time. However, it is important to consider the significant limitations of functional assays for PS related to interferences of positive LA and APCR or increased FVIII levels, which can result in falsely decreased PS activity (*Table II*). On the other hand, immunochemical assays for PS free or total antigen levels should not be performed in the first diagnostic step, because type II deficiency will not be detected. Due to methodology limitations of functional PS assays, it is recommended that each abnormal (decreased) result for PS activity obtained with a functional assay in the first diagnostic step be further evaluated using an immunochemical assay for PS antigen levels by ELISA or automated immunoturbidimetric assays (99), as shown in *Table II*.

PS circulates in plasma in two forms: 60% of the entire PS is an inactive form bound to C4B-binding protein (C4B-BP) while 40% of PS is unbound or free PS which is the hemostatically active component of PS. Therefore, immunochemical assays can measure total or free PS concentration, depending on the assay design. Free PS assay measures only the unbound and active fraction of PS, while the total PS antigen measures both bound (inactive) and unbound (active) PS fractions in plasma. It is generally accepted that the measurement of free PS should be the test of choice, since it is closely related to the functional form of PS and thus better discriminates between subjects with and without PS deficiency (113, 114). So, it is not necessary to routinely measure total PS antigen, except in case of decreased free PS in order to further differentiate between types I and III of PS deficiency (113). In the recent years, the newer methods for free PS using specific monoclonal antibodies for the unbound PS fraction have mostly replaced older methods based on precipitation of bound PS with polyethylene glycol followed by measurement of the remaining PS portion in the supernatant. All variables that should be considered when performing thrombophilia investigation of natural anticoagulants are listed in *Table II*.

Antiphospholipid antibodies

Antiphospholipid syndrome (APLS) that is characterized by the presence of circulating aPLAs represents an important acquired thrombophilic risk factor associated with both venous and arterial thromboembolism, recurrent fetal loss and thrombocytopenia

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(115). The aPLAs are a heterogenous group of acquired autoantibodies directed against phospholipid–protein complexes. These autoantibodies are divided into three major subgroups: lupus anticoagulant (LA), anticardiolipin antibodies (ACL) and anti-β_2-glycoprotein-1 (anti-β_2-GP1) antibodies, based primarily on the method of detection. Laboratory investigation of LA includes functional coagulation assays, while ACL and anti-β_2-GP1 antibodies are detected by immunoassays (116).

Laboratory detection of LA comprises a panel of coagulation-based assays including at least two screening tests (since no one single screening test is 100% sensitive) and at least one confirmatory test (116, 117). The most commonly used screening assays for LA include the APTT, the APTT mixing test (APTT determination in a mix of patient plasma and normal plasma) and dilute Russell's viper venom time (dRVVT) test. The dRVVT screening assay is performed with snake venom that directly activates coagulation factor X in the presence of a low concentration of phospholipids. In case of positive LA, the dRVVT screening assay results in prolonged clotting time by interfering with phospholipids. The most commonly used dRVVT confirmation assay is also performed with snake venom, but contains phospholipids in excess that neutralize the effect of LA, thus resulting in correction of the dRVVT clotting time. The result is expressed as a ratio obtained by dividing LA screening and LA confirmation clotting times.

The laboratory evaluation of ACL and anti-β_2-GP1 antibodies is performed using ELISA methods. Both the IgG and IgM isotypes of ACL and anti-β_2-GP1 antibodies should always be measured (115).

Regarding the investigation of aPLAs, it is important to note that laboratory criteria for the diagnosis of APLS should be positive on two separate occasions with at least 12 weeks apart, in order to exclude the possibility of transient presence of aPLAs in conjunction with infections or drugs, that is not associated with an increased risk of thrombosis (115), as shown in *Table I*. Further, laboratory investigation of aPLAs should always include determination of all three groups of antibodies (115, 118). In general, LA and anti-β_2-GP1 antibodies are more specific for APLS, whereas ACL are more sensitive (119–121). Variables that should be considered when performing laboratory investigation of aPLAs are presented in *Table II*.

Hyperhomocysteinemia (HHC)

HHC, characterized by increased concentration of homocysteine (HC) in plasma, can be associated with both venous and arterial thrombosis (122–125). HHC may be a congenital defect due to deficiency of the enzymes involved in homocysteine metabolism, such as cystathionine-b-synthetase (CBS) and methylenetetrahydrofolate reductase (MTHFR), or it may be an acquired disorder due to poor dietary intake of vitamins B12, B6 or folate that all serve as cofactors in HC metabolism (90). Homozygous hereditary deficiency of CBS is a very rare defect that results in HHC, homocysteinuria, atherosclerosis and arterial and venous thrombosis at a young age. Heterozygous CBS deficiency has a frequency of 0.3 to 1.4% in the general population and is associated with mild HHC (111). In contrast, a common variant of the MTHFR gene, i.e. the MTHFR C677T polymorphism, appears in the heterozygous form in 30–40% and in the homozygous form in 10–13% of the general population of whites (126). This polymorphism results in a thermolabile variant of the MTHFR enzyme that has been shown to be associated with slightly or moderately elevated HC plasma concentrations. In the past ten years, genotyping for the MTHFR C677T polymorphism has often been performed as part of routine thrombophilia testing. However, the recent large population-based case control MEGA study has clearly shown that there is no association between the MTHFR C677T polymorphism and risk for VTE (127). It is considered that the MTHFR polymorphism does not predispose to HHC when the folate status is sufficient. Only in combination with vitamin deficiency, heterozygotes for MTHFR have mildly increased HC concentrations. Therefore, most MTHFR C677T heterozygotes do not have HHC and increased risk for thrombosis, if other thrombotic risk factors are not present. As a result of these findings, genotyping for the MTHFR C677T polymorphism is not recommended in the laboratory evaluation of thrombophilia, as it has not been shown to be a risk factor for thrombosis if HC plasma concentrations are normal (90, 127). Instead, the laboratory evaluation of HHC as part of the thrombophilia investigation should be performed exclusively by determining fasting HC plasma concentration (*Table I*).

The methods for HC measurement in plasma include high pressure liquid chromatography and newer and simpler immunoassays, the latter more suited for use in the clinical laboratories and therefore widely applied in the recent years. Variables that affect the results of HC assays are mainly related to sample handling (*Table II*). In contrast to most of the other phenotypic tests for thrombophilia, acute phase of thrombosis and anticoagulant therapy do not affect the results of HC measurement.

Factor VIII

Several studies performed in the recent years have clearly shown persistently increased FVIII activity above 150% to be an independent risk factor for VTE with a 3-fold higher relative risk and high risk of recurrence (128–131). Therefore, determination of FVIII levels should also be included in the laboratory investigation of thrombophilia. It is still not clear whether an inherited disorder leads to a high FVIII

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plasma level, since to date no genetic variation in the FVIII gene has been identified that might account for this phenotype variation. However, the results of some studies have observed a positive correlation of FVIII levels within families (132, 133), thus suggesting that a genetic component may be at least partly responsible for the factor VIII elevations. In addition, the high reported prevalence of approximately 20–25% of persistently elevated FVIII levels among VTE patients suggests that increased FVIII levels are independent of the acute phase response (130). For thrombophilia screening purposes, assays for both the activity and antigen levels of FVIII are suitable. The most widely used methods for measurement of FVIII activity are coagulometric methods using an APTT-based assay with FVIII–deficient plasma while the chromogenic methods are less used as well as the FVIII antigen assays using ELISA-based methods. Variables that should be considered when performing the FVIII assay as part of thrombophilia testing are listed in *Table II*.

Dysfibrinogenemia

Dysfibrinogenemias represent a very rare, but heterogenous group of congenital disorders characterized by a structurally altered fibrinogen molecule that may affect fibrinogen function and result in different hemostatic disorders, such as bleeding, but also venous or arterial thrombosis, dependent of the type of fibrinogen disorder as determined on a molecular basis (134, 135). As it has already been mentioned above, dysfibrinogenemia is a very rare risk factor for VTE, with the prevalence of the disorder in patients with VTE of only 0.8% (135), so laboratory investigation of this risk factor is not part of routine thrombophilia testing. Rather, it can be considered for investigation if the results of all high priority tests described above exclude the more common causes of VTE in a patient with familial or recurrent thrombosis (10, 24). The first step of dysfibrinogenemia investigation includes simple screening assays such as functional fibrinogen levels, thrombin time (TT) and reptilase time (RT). Both TT and RT assays measure the clotting time during the conversion of fibrinogen into fibrin and are typically prolonged in dysfibrinogenemia due to a qualitative defect of fibrinogen. Unlike the TT assay, RT is not prolonged by heparin and related drugs because snake venom (reptilase) is used as reagent instead of thrombin. Positive cases identified with prolonged TT and RT test results should be further evaluated by parallel analysis of functional and antigen (immunoreactive) fibrinogen levels. In dysfibrinogenemia, functional fibrinogen levels are considerably lower than antigen levels that measure fibrinogen quantity, because fibrinogen function is disturbed while fibrinogen quantity is normal. A decreased fibrinogen activity/antigen ratio is a confirmatory test result for dysfibrinogenemia. The most commonly used functional assay for fibrinogen is the Clauss method, while immunoreactive fibrinogen may be determined by ELISA assays or newer automated immunoturbidimetric methods. Variables that affect the results of dysfibrinogenemia investigation are listed in *Table II*.

When should investigation be performed?

When performing laboratory investigation of thrombophilia, it is of particular importance to consider all preanalytical and other variables that may affect the test results (108, 136), as is presented in *Table II*. Appropriate time of testing is one of the most important preanalytical variables that should always be considered when performing thrombophilia testing because the acute phase of thrombosis and anticoagulant therapy considerably affect the results of many phenotypic assays (*Table II*), making interpretation of the results difficult and unreliable. As a general rule, thrombophilia testing should be delayed for six months after the acute phase of thrombosis, and at least four weeks after discontinuation of anticoagulant therapy, because the clinical management of an acute thrombotic event is not influenced by the immediate detection of a specific thrombophilic risk factor (7, 10, 55, 76). The critical importance of appropriate time of testing is often not recognized by clinicians managing patients with thrombosis. The literature data show that up to a half of all requests for thrombophilia testing are ordered in the acute phase of thrombosis or in patients on anticoagulant therapy (4–6). However, there are only a few exceptional cases in which certain thrombophilia tests are immediately indicated, such as testing for aPLAs in patients with catastrophic APLS that may require more aggressive or prolonged anticoagulant therapy (45). Also, in case of suspected hereditary AT deficiency in a VTE patient from a family with known AT deficiency, as well as in case of suspected heparin resistance, immediate testing for AT can be justified, since the use of higher doses of heparin or AT concentrates may be required. Additionally, in children with severe hereditary deficiency of PC or PS, urgent replacement therapy may be required and it can be of clinical value to determine the levels of these natural anticoagulants immediately, rather than waiting until long-term anticoagulant therapy has been discontinued (7, 10). However, it is important to note that any positive test result in the aforementioned cases of immediate testing obtained during anticoagulant therapy and/or acute phase of thrombosis should definitely be verified later in accordance with the recommended rules related to appropriate time of testing.

If oral anticoagulant therapy cannot be discontinued in an affected patient in case of clinical assessment for immediate testing for heritable thrombophilia, then surrogate testing of first degree family members who are not receiving anticoagulant therapy

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can be done (108). Another approach for these rare clinical indications when immediate testing is justified, for example in case of suspected PC and PS deficiencies, is the possibility of replacing VKA treatment with low molecular weight heparin for a period of 10 to 14 days, in order to allow plasma levels of vitamin K-dependent proteins to return to baseline levels.

Other variables that should be considered when employing laboratory investigation of thrombophilia

Besides appropriate time of testing, there are other important variables that should be considered when performing the investigation of thrombophilia, including age, gender, pregnancy and puerperium, acute phase response to inflammatory diseases, liver function and other clinical conditions that may affect the test results of many phenotypic assays (*Table II*). For example, when evaluating pediatric patients for inherited thrombophilic risk factors, such as AT, PC and PS deficiencies, appropriate age-dependent reference ranges should be employed due to significantly decreased values of these parameters in pediatric populations compared with adults (137–139). Further, it is generally advised for all thrombophilia tests to determine reference ranges for the combination of method and test used, with the normal donors recruited from the local population (108, 139). However, very few laboratories are able to employ locally determined reference ranges for thrombophilic tests. Instead, reference ranges are mainly taken over from manufacturers or available literature data.

Furthermore, when considering investigation of hereditary deficiencies of the physiological anticoagulants AT, PC or PS, the possible causes of acquired deficiencies should be excluded first, since certain clinical conditions are associated with reduced plasma levels of anticoagulants (*Table II*).

For every questionable or positive result obtained with phenotypic tests, it is advised to perform a second analysis using a newly collected blood sample, in order to exclude a false-positive result or confirm a positive result for an individual test (10, 108). In case of a positive test result obtained with genotyping tests, it can be definitely demonstrated by testing first degree relatives of the affected patient.

Finally, it is the obligation of the laboratory experts to recognize the limitations of the assays and assay methods they use. In order to avoid misinterpretation of the test results, laboratory experts should report the potential causes of false-positive and false-negative test results using interpretative comments along with the test result reports (140, 141).

Conclusion

Considerably increased knowledge on the etiology of thrombophilia during the last two decades has lead to widespread laboratory investigation of thrombophilia. Today, thrombopilia investigations represent the most frequently performed tests in hemostasis laboratories. The issue is not whether we are able to perform the test, since it is not a problem for our modern laboratories, but there is an obvious need for the currently overutilized ordering practice for thrombophilia testing to be critically reviewed and directed toward those patients in whom the testing can be expected to have real clinical utility. In order to ensure reliable and clinically relevant findings of the results included in testing, laboratory investigation of thrombophilia should always be performed in accordance with the recommended guidelines on testing, regarding whom and when to test, what assays and assay methods to use as well as careful consideration of all other variables that can affect the results of testing. Although several professional societies have published consensus recommendations and guidelines in order to assist clinicians in determining who are appropriate candidates for testing and when to perform it, the practice of inappropriate testing continues today more than ever, thus often resulting in futility of testing and concomitant huge waste of health-care resources. Inappropriate testing outside the recommended guidelines is a poor clinical practice and is likely to be more harmful than beneficial for the patient due to possibly misinterpreted test results. It is obvious that laboratory experts should take a more substantial role in the overall thrombophilia investigation process, in order to help clinicians to direct testing in accordance with recommended guidelines.

At this time, laboratory investigation of thrombophilia allows identification of one or more thrombophilic risk factors in approximately 50–60% of VTE patients. A substantial proportion of patients have no identifiable cause of thrombosis, suggesting that additional hereditary and acquired risk factors remain to be discovered. On the other hand, VTE is a multifactorial disorder that involves a complex interaction between hereditary or acquired and transient predisposing risk factors. Thus, patients with similar hereditary risk factors may or may not experience VTE depending on the presence of other interacting transient risk factors. Future studies should provide new insights into the mechanisms by which individual risk factors interact. More knowledge of these interactions would also enable better estimation of VTE risk for every single patient as an important prerequisite for an individualized approach in the prevention and management of VTE.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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