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Molekulargenetische und zytogenetische Diagnostik

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Diagnosing thrombophilia today with particular reference to genetic factors and the antiphospholipid antibody syndrome[1)]

Paul Cullen[1,*], Susanne Adam[1], Brigitte Kaiser[2] and Michael Neumaier[3] for the working group "Molecular and Chip Diagnostics" of the United German Society of Clinical Chemistry and Laboratory Medicine

[1] MVZ für Laboratoriumsmedizin Dr. Löer, Dr. Treder und Kollegen, Münster

[2] Institut für Molekulare Hämostaseologie, Friedrich-Schiller-Universität Jena, Jena

[3] Institut für Klinische Chemie, Universitätsklinikum Mannheim, Universität Heidelberg, Mannheim

Abstract

Deep vein thrombosis is not a rare medical condition, and the investigation of a clotting disposition or thrombophilia is one of the most common tasks required of the medical laboratory. In this complex approach, the diagnosis is achieved on the basis of the clinical features backed up by the combination of investigations of clotting function, biochemical analyses, and genetic tests. Therefore, the investigation of thrombophilia represents as a good example of the role of molecular genetic testing in laboratory medicine. The aim of the present review is to outline the current state of the art in investigating thrombophilia with particular reference to molecular genetic testing. One cause of thrombophilia that is often overlooked is the presence of antiphospholipid antibodies. Accordingly, they also receive detailed attention.

Keywords: antiphospholipid antibodies; lupus anticoagulant; thrombophilia.

Introduction

In Germany, about one person per 1000 of the general population suffers a deep venous thrombosis each year [1] and up to 40,000 people die each year from pulmonary embolism, its most serious complication [2]. Increased coagulability of the blood – thrombophilia – can be due not only to risk factors such as smoking, overweight, or immobilization, but also to genetic factors which increase the risk of thrombosis during pregnancy or in association with use of oral contraceptives. More than half of all thrombosis patients have a genetic predisposing factor, and it is thought that about 5% of the population exhibit such a genetic predisposition. In addition, several diseases are associated with increased thrombotic risk (Table 1).

Although not all risk factors for thrombosis are known, many have been identified that can be detected by laboratory investigations. This article sets out to describe these factors and to assess their importance. In addition, we present a rational stepwise approach to assessing thrombophilia.

Several classifications of thrombophilia have been proposed. One approach is to divide thromboses according to location into arterial, venous, or mixed. A second is to divide thrombophilias into severe varieties that result from a lack of clotting inhibitors, or mild phenotypes due to an increase in clotting factors. A third divides thrombophilias into inherited and acquired forms. For reasons of simplicity, this article divides known thrombosis risk factors into 1), those that can be detected by biochemical methods; 2), those that can be detected by both biochemical and molecular biological methods; and 3), those that may only be detected by molecular biological methods. It is clear that this distinction is in some respects artificial, as can be seen in the case of APC-resistance. Rather, functional and genetic tests are complementary in the diagnosis of thrombophilia.

Forms of thrombophilia amenable to biochemical or functional testing

A common risk factor for thrombophilia amenable to biochemical testing is a raised homocysteine level, which may be idiopathic, or which may be due to a mutation in the gene for methylene tetrahydrofolate reductase (MTHFR) or to a dietary lack of the vitamins B6 (pyridoxine), B12 (cobalamin) or folic acid. A second fairly common cause of thrombophilia is the presence of

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*Correspondence: Prof. Dr. Paul Cullen, MVZ für Laboratoriumsmedizin Dr. Löer, Dr. Treder und Kollegen, Hafenweg 11, 48155 Muenster, Germany
Tel.: +251-60916 105
Fax: +251-60916 110
E-Mail: p.cullen@labor-muenster.de

Table 1 Prevalence of clotting disorders in continental Europe.

Clotting disorder	Total population (%)	Patients with thrombosis (%)
Factor V_{Leiden} heterozygosity	5–7	23–35
Factor V_{Leiden} homozygosity	0.02	3
Prothrombin 20210 mutation	2–4	5–8
Antithrombin deficiency	0.02–0.20	0.50–1.10
Protein C deficiency	0.2–0.5	2–3
Protein S deficiency	<1	1–2
Increased factor VIII*	5–10	>20
Increased fibrinogen*	1–2	3–5

*Acute phase protein, increased in inflammatory conditions. Derived in modified form from Spannagl M, Mäßmer G. Hämostase in Guder WG, Nolte J. Das Laborbuch für Klinik und Praxis. Elsevier Verlag 2005:59–79.

antiphospholipid antibodies, an acquired risk factor that may appear for example during pregnancy or as a corollary of a rheumatic disorder. The thrombophilia observed in patients with nephritic syndrome or terminal renal failure may at least partly be due to increased excretion and thus lower plasma levels of antithrombin [3, 4]. Other relatively common causes of thrombophilia that may be detected by biochemical means include a prolonged increase in factor VIII, an increased lipoprotein (a) level, and the presence of resistance to activated protein C (APC-resistence). Rarer forms of thrombophilia susceptible to biochemical detection are those caused by a defect in, or lack of, clotting inhibitors. These include defects of fibrinolysis [5] and deficiencies of protein C, protein S or antithrombin. A further cause of thromboembolic disease is paroxysmal nocturnal hemoglobinuria, in which it is the cause of death in 40% of cases [6–8].

In the following, we will particularly concentrate on those analytic methods which may prove difficult in practice. These are the detection of antiphospholipid antibodies or of a lack of protein C, protein S or antithrombin, and the demonstration of an increase in Factor VIII or of resistance to activated protein C.

The anti-phospholipid syndrome (APS)

Anti-phospholipid antibodies (APA) are not actually directed against phospholipids per se but against a complex consisting of the phospholipids and their binding proteins. APA are the commonest acquired inhibitors of the clotting process. They may be of no clinical consequence, but may also cause both venous and arterial clotting (deep venous thrombosis, pulmonary embolus, coronary and cerebrovascular thrombosis). In isolated cases they may also result in a hemorrhagic diathesis.

APA may be grouped into several categories: 1) lupus anticoagulant, which interferes in vitro with one or more phospholipid-dependent steps in the clotting process such as the formation of tenase and – in particular – prothrombinase complexes; 2) anti-cardiolipin antibodies directed against a complex of β_2-glycoprotein I (β_2-GP I) and anionic lipids; and 3) antibodies directed purely against β_2-GP I. These three antibody categories may appear separately or in combination as both IgG and IgM classes. Whereas the anti-cardiolipin antibodies have little impact on the clotting process, lupus anticoagulants increase the clotting times of all phospholipid-dependent tests, in particular the partial thormboplastin time (PTT). Circulating β_2-GP I may be regarded as a natural anticoagulant. It binds negatively charged phospholipids and thus inhibits platelet activation and the clotting process. The mechanism by which β_2-GP I affects platelet function is complex but principally involves an interaction with von Willebrand (vW) factor. β_2-GP I binds the A1 domain of active vW factor thus inhibiting vW-dependent platelet adhesion and aggregation. β_2-GP I antibodies may neutralize this inhibiting effect leading to increased vW factor activity, thus explaining the increased clotting tendency associated with these antibodies [9].

Lupus anticoagulants derive their name from the fact that they were first seen in patients with systemic lupus erythematosus (SLE). High levels of APA are, however, not only seen in SLE or other autoimmune disorders, but also in patients with monoclonal gammopathies or other neoplastic diseases. Low titers of anticardiolipin antibodies and lupus anticoagulants are also found in 2–5% of the general population, and about 30% of children display transient APA antibodies following mild infections.

Despite much research, little is still known of how antiphospholipid antibodies contribute to the pathogenesis of thrombosis or recurrent abortion. Possible mechanisms include not only the previously mentioned increase in the activity of vW factor but also increased resistance to the anticoagulant effect of annexin V, or a Factor V Leiden-independent APC-resistance. Annexin V binds to negatively charged phospholipids on activated cells. This blocks access of these phospholipids to clotting factors, preventing activation of these factors. APA compete with annexin V for these binding sites, thereby promoting the binding and activation of clotting factors [10]. Factor V Leiden-independent APC resistance may also arise from an interaction between APC and the β_2-GP I-antibody complex, which may lead to loss of function of APC and corresponding resistance with regard to the cleavage of clotting factors Va and VIIIa [11]. In addition, APA may inhibit the activation of protein C by the thrombin-thrombomodulin complex either by competing for phosphilipid

binding sites on the platelet surface or by destroying the APC complex after it is formed [10].

Direct activation of the endothelial cell or inhibition of APC may constitute further links between lupus anticoagulant and a thrombotic diathesis [11].

A characteristic of APS is that venous and arterial thrombi and thromboemboli appear in nearly all parts of the vascular tree, including several unusual localizations. In addition, mild thrombocytopenia of unknown origin has been observed in APS. Women with a history of abortion and raised APA levels are at increased risk of further abortions.

There is little correlation between the presence of antiphospholipid or anticardiolipin antibodies in individual measurements and either the risk of thrombosis or the activitiy of an underlying autoimmune disorder. For this reason, the diagnosis of APS should only be made under closely defined clinical and laboratory conditions. In the German-speaking countries, the criteria for diagnosing APS are based on the international consensus originally arrived at in Sapporo in 1999 [12] and updated in 2005 [13].

Thus the diagnosis of APS requires the clinical diagnosis of at least one episode of arterial or deep venous thrombosis, or of a thrombosis of small vessels, verified by imaging procedures, Doppler sonography or histology. APS-associated morbidity during pregnancy manifests itself (i), as the unexplained death of a morphologically intact fetus during or after the 10th week of gestation; (ii), as one or more premature births of a morphologically intact fetus before the 34th week of gestation due to severe pre-eclampsia, eclampsia or placental insufficiency; or (iii), at least three spontaneous abortions before the 10th week of gestation after exclusion of anatomical or hormonal disturbances in the mother and chromosomal anomalies in either parent.

In addition to these clinical criteria, the following laboratory criteria for APS must also be fulfilled: in at least two measurements at least 12 weeks apart and using a standardized enzyme-linked immunosorbent (ELISA) assay [13], IgM or IgG antibodies to cardiolipin or β_2-GP I and/or lupus anticoagulant must be found. It is also recommended that APS should not be diagnosed when an interval of $<$12 weeks or more than 5 years lies between the clinical event and the positive laboratory result [13].

In contrast to the increased thrombotic tendency which dominates the clinical picture, APS is associated with a paradoxical increase of clotting times in in-vitro clotting tests. Demonstration of the presence of lupus anticoagulant therefore takes place in four steps [14]: 1. demonstration of a prolonged phospholipid-dependent clotting time, which 2. is not normalized by admixture of normal plasma, but rather 3. by adding phospholipids, following, 4. the exclusion of other coagulopathies such as the presence of a Factor VIII inhibitor or the administration of anticoagulant [15]. In many cases, the sensitivity of activated partial thromboplastin time (aPTT) is not sufficient to detect a prolonged phospholipid-dependent clotting time. For this reason, more sensitive methods are often used.

One of these methods measures the time to clot formation following addition of diluted venom of the Russell viper (dilute Russell viper venom time, dRVVT), a direct activator of factor X. The dRVV-ratio is derived by dividing the dRVVT in the absence of additional phospholipid by the dRVVT in the presence of additional phospholipid. A ratio below 1.2 excludes lupus anticoagulant, a ratio between 1.2 and 1.5 indicates the presence of weak lupus anticoagulant, while a ratio above 1.5 clearly demonstrates lupus anticoagulant. If the dRVV ratio and/or the clotting time are increased, normal plasma is added to exclude deficiency of a clotting factor.

Kaolin is a porcelain clay that chiefly consists of silica gel. In microcrystalline form, if offers a large negatively-charged surface for contact activation of the intrinsic clotting pathway, thus indirectly activating clotting factor X. The kaolin clotting time (KCT) is regarded as the most sensitive test for detection of lupus anticoagulant. However, clotting factor deficiencies also prolong the KCT, so that false positive results are not uncommon. The addition of normalized plasma allows calculation of the Rösner index and compensation of clotting factor deficiencies. An index above 15 is pathological [16].

If the results of the dRVVT or KCT are unclear, a further screening test, the LCA (lupus circulating anticoagulant) may be performed. This refers to the ratio calculated from the "lupus-sensitive" aPTT measured using a special aPTT-LA reagent. This reagent consists of a mixture of silica gel and phospholipids extracted from rabbit cerebellum. As with kaolin, the silica gel offers a negatively-charged surface for activation of the intrinsic clotting system. The amount of phospholipid is smaller than in conventional aPTT reagent, thus increasing sensitivity for detection of lupus anticoagulant. The reagent is added to patient plasma and the clotting reaction is initiated by addition of calcium chloride. As with the dRVV ratio, a ratio is calculated by dividing the aPTT in the absence of additional phospholipid by the aPTT in the presence of additional phospholipid (La1/La2).

The strict definition of the laboratory criteria, and in particular the requirement of a second measurement after a gap of at least 12 weeks, aim to exclude the false positive diagnosis of antiphospholipid syndrome. Initially positive results which then become negative may indicate the presence of transient antiphospholipid antibodies during an infection. For this reason, it is important to repeat the measurement after the infection has passed.

A common difficulty in practice is that patients have often received anticoagulation therapy at the time when the measurement of lupus anticoagulant is performed. Most of the dRVVT reagents include polybrene, which neutralizes heparin, allowing the measurement to be performed in heparinized patients. In patients receiving warfarin, the test should be deferred if at all feasible. If this is not possible, then the patient sample should be

diluted with an equal volume of normalized plasma providing the INR is <3.5. The test cannot be performed if the INR exceeds 3.5 [13]. The test for lupus anticoagulant may not be performed in patients receiving direct inhibitors of thrombin such as hirudin or argatroban, or of direct inhibitors of activated factor X such as fondoparinux (Arixtra®) or danaparoid (Orgaran®).

In general, lupus anticoagulant is under-diagnosed as the cause of recurrent thromboses or – in particular – recurrent spontaneous abortion. This is important, because lupus anticoagulant detected by clotting tests or antiphospholipid antibodies detected by ELISA are among the most potent of all thrombophilic factors. Both are an indication for life-long anticoagulation. Pre-analytic factors may also cause lupus anticoagulant to be overlooked. For this reason, blood samples should be analyzed immediately after drawing. If this is not possible, the platelets should be removed from the sample either by filtration of by two rounds of high-speed centrifugation. The plasma can then be stored at –20°C until analysis.

The Figure 1 suggests an algortihm for the rational investigation of thrombophilia while paying particular attention to the antiphospholipid syndrome.

Protein S deficiency

Protein S is synthesized primarily in the liver in Vitamin K-dependent fashion. As a cofactor of APC, protein S accelerates the inactivation of factors Va and VIIIa. In the blood, protein S forms a 1:1 complex with complement C4b binding protein, an acute phase reactant. Only the free protein S, which usually comprises about 40% of the total, displays biological activity. Patients who are heterozygous for a deficiency of protein S display a 5 to 12-fold increase in thrombotic risk, depending on the presence of other predisposing factors. Homozygous deficiency of protein S, which is extremely rare, manifests in the newborn period as purpura fulminans and recurrent thromboemboli and is practically incompatible with life. Cases of purpura fulminans are sometimes due to protein S deficiency caused by the presence of a protein

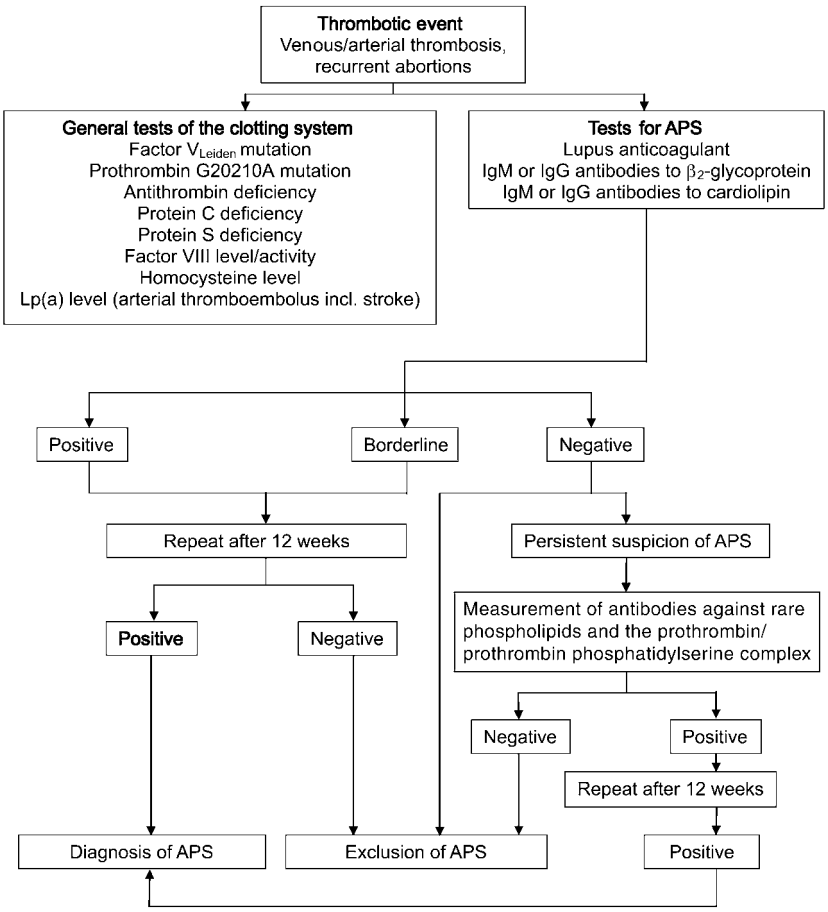

Figure 1 Suggested algorithm for the investigation of thrombophilia paying particular attention to the antiphospholipid antibody syndrome (APS).
Modified from Landenberg P, the antiphospholipid syndrome, internet website of the German Combined Society of Clinical Chemistry and Laboratory Medicine www.dgkl.de/autoimmundiagnostik/aps.html.

S inhibitor. Necrosis has sometimes been observed in the early stages of warfarin treatment in patients with heterozygous protein S deficiency. There are no clear findings that indicate the possible presence of protein S deficiency with the exception of a personal and possibly a family history of recurrent thrombosis.

Congenital protein S deficiency may be classified into three types. Type I is characterized by a reduction in the amount of free and total protein S, accompanied by an equivalent reduction in protein S activity. In type II protein S deficiency, protein S activity is reduced in the presence of a normal or increased concentration of free protein S. In type III protein S deficiency both the concentration of free protein S and protein S activity are reduced in the presence of a normal total protein S concentration.

Protein S activity differs between the sexes, and ranges from 69 to 130% in men and from 58 to 114% in women. In women taking oral contraceptives, the normal range is reduced to 48 to 106%. There is a substantial overlap between the normal and low ranges. In such cases, careful discrimination of congenital and acquired deficiencies is required, since acquired protein S deficiency is usually transient in nature. In addition to pregnancy, the causes of an acquired protein S deficiency include the use of oral contraceptives, lack of vitamin K, disorders of liver synthesis, disseminated intravascular coagulation, cancer, inflammatory bowel disease, HIV infection, systemic lupus erythematosus, treatment with asparaginase and diabetes mellitus. At the time of writing, more than 130 mutations in the protein S gene on chromosome 3 have been described as a cause of inherited protein S deficiency. In addition to the active PROS1 gene, an inactive pseudogene PROS2 exists that exhibits 96.5% homology with exons 2 to 15 of the PROS1 gene. At the present time, there is little clinical use in sequencing the protein S gene, since the complexity of the gene and the size of both the gene and the pseudogene mean that a mutation can be detected only in about a half of patients [17–19]. In addition, the large number of mutations has made it difficult to establish genotype-phenotype correlations [17]. However, it was recently possible to show that some PROS1 gene variants lead to severe defects in the secretion or functioning of protein S, and that these patients have a high risk of thrombosis [17, 18].

The clinical diagnosis of protein S deficiency is usually made based on the determination of protein S activity. Further classification requires the measurement of the circulating levels of free and total protein S. In practice, the determination of protein S activity may present difficulties on account of instability of the measurement reagent. The measurement is also affected by the simultaneous presence of the factor V_{Leiden} mutation. Because of these difficulties, some authors recommend measurement of the concentration of free protein S only, since this will detect about 95% of all patients with a congenital protein S deficiency.

Protein C deficiency

Protein C is synthesized in the liver in a vitamin K-dependent fashion. In its activated form (APC), protein C is a central inhibitor of the clotting process, which it blocks by degrading and inactivating factors Va and VIIIa, thereby preventing the formation of thrombin. APC also promotes fibrinolysis. Protein C has a short half-life and is one of the first factors whose plasma concentration falls in cases of vitamin K deficiency or reduced liver function. For this reason, a rapid fall in protein C may lead to a period of hypercoagulability during the early stages of warfarin therapy. About 0.4% of all Europeans suffer from a congenital lack of protein C [20], which is found in about 3% of all patients with venous thromboembolism. As with other forms of congenital thrombophilia, the thrombotic risk of protein C deficiency is further increased by risk factors such as surgery, sepsis, or the simultaneous presence of other genetic thrombophilias.

Homozygous deficiency of protein C, which is extremely rare, manifests in the newborn period as purpura fulminans and recurrent thromboemboli and is practically incompatible with life. Heterozygous protein C deficiency is associated with a 7 to 10-fold increase in risk of venous thromboembolism [21]. The gene for protein C is located on chromosome 2 and consists of nine exons and eight introns. At the time of writing, more than 330 mutations in the protein C gene have been described, of which more than 160 are associated with protein C deficiency. About 65% of all patients with reduced protein C activity display a mutation in the protein C gene [22]. Because of the large number of mutations and the lack of information on genotype-phenotype correlations, routine sequencing or mutation analysis of the protein C gene is not recommended [17]. Exceptions to this rule are the determination of carrier status in families with known mutations in the protein C gene and the completion of diagnostic analysis in patients with congenital protein C deficiency.

In most cases, protein C activity rather than protein C concentration is measured, since this also detects cases in which activity is reduced even though the concentration of the protein is normal (type II). The normal range is 70 to 140% of the activity of reference plasma, with substantial overlap between normal and low levels. It is important to differentiate carefully between congenital and acquired deficiencies of protein C since the latter is common in transient fashion, particularly in hospital inpatients, and may occur during pregnancy, in association with the use of oral contraceptives, in nephrotic syndrome or diabetes mellitus, or as a result of a lack of vitamin K.

Antithrombin deficiency

Antithrombin (AT) is synthesized in the liver. The glycoprotein contains two binding sites, one for thrombin and

one for anionic molecules such as heparin. AT inhibits not just thrombin but all other active enzymes of the clotting cascade, in particular the clotting factors Xa and IXa. In the absence of co-factors, AT is a slow inhibitor. However, its activity is significantly increased by sulfated glycosaminoglycans such as heparin sulfate or by heparin [23]. Deficiency of AT is associated with the greatest increase in thrombotic risk of all the congenital thrombophilias. The risk of thrombosis in heterozygotes is increased 20-fold, a group whose lifetime risk of thrombosis approaches 50%. Congenital AT deficiency is divided into two types: type I with a reduction of activity and normal AT concentration, and type II with reduced AT activity and reduced protein concentration. In type II AT deficiency, either the binding sites for thrombin (type IIRS) or heparin (type IIHBS) may be selectively affected. A combined disorder of both binding sites is referred to as type IIPE [17]. Heterozygotes for type I deficiency and heterozygotes for the type II thrombin-binding defect display an AT activity of 40 to 70% of normal and have a large increase in thrombotic risk. Heterozygotes for the type II heparin-binding defect display a relatively mild (5 to 10-fold) increase in thrombotic risk. Homozygous type I defects are not compatible with life. Isolated reports exist of patients with homozygous type II deficiencies as a result of mutations in the heparin-binding region [23]. In patients with congenital AT deficiency, the risk of thrombosis is increased further by additional factors such as pregnancy, immobilization or trauma. In Germany, the prevalence of AT deficiency is about 0.2%.

The AT gene lies on chromosome 1q23-25 and consists of seven exons and six introns. At the time of writing, more than 180 mutations causing AT deficiency have been described. Analysis of mutations or sequencing are not part of current diagnostic testing, although this may be indicated in patients in whom a congenital type 2 defect is suspected or in order to differentiate between various deficiency subtypes. The $AT_{Cambridge\ II}$ Mutation (A384S), for example, is associated with slightly reduced or even normal levels of AT activity. Nevertheless, in one study, this mutation was associated with a 10-fold increase in thrombotic risk [24].

Acquired varieties of AT deficiency also exist. These may be due to defective hepatic synthesis, nephritic syndrome, disseminated intravascular coagulation, preeclampsia, acute thromboembolism, and sepsis. In liver cirrhosis, the prothrombotic component due for example to loss of AT is compensated by the simultaneous failure to produce clotting factors, so that cirrhosis rarely results in a clotting tendency.

AT activity is usually assessed by measung AT-dependent thrombin inhibition. For this reason, the antithrombotic agent hirudin and other direct thrombin inhibitors such as argatroban may lead to an incorrect measurement of high AT activity levels. AT activity is physiologically low (range 20–80%) in neonates.

Factor VIII

The factor VIII level varies widely between individuals. Factor VIII is also an acute phase protein, limiting our ability to interpret factor VIII levels in inflammatory conditions. For this reason, the factor VIII level should not be measured until at least three months have passed since an acute thrombosis. If this level is raised, a repeat measurement should be made 12 months after the thrombotic event [25]. Several studies have shown that the risk of venous thrombosis is increased about fourfold in persons with raised levels of factor VIII [26]. This relative risk increases to about tenfold in women taking oral contraceptives. The molecular reasons for this increase in risk are not completely known and include both genetic and acquired components. The concentration of factor VIII increases with age and body weight, and is also raised by hyperglycemia. About 25% of patients with acute venous thrombosis display a high factor VIII level [27]. The thrombotic risk increases as the factor VIII level increases [27]. Transient increases in the factor VIII level are seen in pregnancy and in a large number of conditions including trauma, infections, cancer and hyperthroidism [28]. Several medications such as glucocorticosteroids and desmopressin may also increase factor VIII levels [29, 30].

Resistance to activated protein C (APC resistance)

Resistance to activated protein C (APC resistance) is the commonest hereditary thormbophilia. In more than 90% of cases, this defect is due to a point mutation in the factor V gene (G to A transition at position 1691), leading to replacement of an arginine by a glutamine at amino acid position 506 in the mature protein (Factor V_{Leiden}). Activated Factor V_{Leiden} cannot be adequately cleaved and thus inactivated by protein C, leading to overactivity of the clotting cascade. About 7% of the general European population is heterozygous for the Leiden mutation in factor V, a condition that is found in about a quarter of all thrombosis sufferers. In heterozygotes the risk of thrombosis is increased three to sevenfold. Homozygosity for Factor V_{Leiden}, which affects about one person in a thousand increases the risk of thrombosis 20 to 80-fold, so that homozygotes generally require lifelong prophylactic antithrombotic medication. The factor V_{Leiden} mutation is found in about 40% of all women who develop a thrombosis during pregnancy [31].

It is of note that certain thrombophilic phenotypes are rare in carriers of the factor V_{Leiden} mutation. The risk of developing pulmonary embolism, for example, is low [32]. Current consensus is that general population screening for factor V_{Leiden} is not useful. Testing is, however, indicated in patients who have suffered a thrombosis or to investigate relatives of thrombosis patients if these are at increased risk of thrombosis for other rea-

sons [23]. Factors increasing risk are use of estrogens or oral contraceptives in women, pregnancy and extensive orthopedic surgery [23].

APC resistance can be detected directly by means of a clotting function test in plasma (phenotype) or indirectly by detection of the Leiden mutation in the factor V gene (genotype). The most common functional test for APC resistance is that described by Dahlbäck and colleagues which is based on measurement of the activated partial thromboplastin time [33]. In this test, the aPTT is measured with and without addition of APC. If APC resistance is present, the increase of clotting time in response to APC addition is less than when APC is not present. A ratio is calculated by dividing the clotting time after addition of APC by the clotting time without APC addition. A ratio of more than 2.0 is found in normal individuals, in heterozygotes this ratio is between 1.3 and 2.0, while homozygotes have a ratio under 1.3. The exact values may vary according to the equipment and the reagents used. For this reason each laboratory should calculate its own values based on a sufficient number of affected and unaffected individuals [1, 34].

APC cleaves not only factor Va but also factor VIIIa. In tests for APC resistance performed without factor V-poor plasma, very high levels of factor VIII activity may lead to competition for APC, thus reducing the inactivation of factor V and shortening the clotting time in the APC resistance test (false positive test result). This phenomenon may be almost completely avoided by use of factor V-poor plasma.

False positive APC test results may also occur in the anti-phospholipid syndrome. Protein C is activated by interaction with the thrombin/thrombomodulin complex. Lupus anticoagulant may interfere with this activation. Alternatively, antiphospholipid antibodies may block APC activity. APC resistance tests from different manufacturers show differing degrees of sensitivity to the presence of antiphospholipid antibodies.

A much rarer point mutation at position 1091 in the factor V gene (G1091C) leads to the exchange of a threonine for an arginine at amino acid position 306 of the factor V protein and is also associated with resistance to APC. This variant is known as factor $V_{Cambridge}$ and has up to now only been found in isolated individuals who were mostly of Chinese origin. The importance of the factor $V_{Cambridge}$ for risk of thrombosis is not clear and routine analysis for this mutation is not performed at the present time. The same applies to other factor V sequence variants such as factor $V_{Hong\ Kong}$ (R306Q), factor $V_{Liverpool}$ (I359T), and factor V Y1702C [17].

Homocysteine and methylene tetrahydrofolate reductase (MTHFR)

The enzyme methylene tetrahydrofolate reductase (MTHFR) plays an important role in the metabolism of free methyl groups. The vitamins B6 (pyridoxin), B12 (cobalamin) and folic acid are involved in this process. One important intermediate in this metabolic cycle is the amino acid homocysteine. About 10% of the population have raised homocysteine levels, and these are associated with an increased risk of atherosclerosis. The association with venous thrombosis is the subject of controversy. The homocysteine level is affected by diet, age and renal function.

Some rare mutations in the MTHFR gene lead to almost total loss of function of the MTHFR protein and to the syndrome of autosomal recessive homocystinuria, which leads to severe neurological deficits. These rare mutations are not the subject of the present article. Much more common is a mild form of MTHFR deficiency that is caused by a thermolabile variant of the enzyme displaying a reduction in activity of about 50%. In most such cases, a polymorphism can be detected at nucleotide positions 677 (C677T, exon 5) or 1298 (A1298C, exon 8), which results in the replacement of an alanine at postion 222 by a valine (C677T) or of a glutamine at position 429 by an alanine (A1298C). Each 1298C allele leads on average to an increase in the homocysteine level of 3%, each 677T allele to an increase of about 6%. In contrast to the classical defects in MTHFR, the thermolabile variant is not associated with neurological symptoms. Up to now, no connection could be found between the presence of these variants and an increased risk of thromboembolism.

In addition to the thrombophilic factors that have been described until now, which can be detected either by functional tests or by a combination of functional and molecular methods, a number of defects exist which can be detected only by the application of the methods of molecular biology. These are described in the following section.

G20210A mutation in the prothrombin gene

The G20210A transition in the 3′ untranslated region (UTR) of the prothrombin gene is a common mutation which leads to increased expression of the gene. After factor V_{Leiden}, this is the most common genetic risk factor for thrombophilia. About 2% of the German population are heterozygotes. Although this mutation increases the concentration of circulating prothrombin, measurement of the prothrombin concentration is not useful, since there is a wide overlap between carriers and wild-type individuals. In heterozygotes, the risk of thrombosis is increased two- to threefold. In women taking oral contraceptives however, the thrombosis risk may be increased 150-fold. Thrombotic risk may also be increased by carriage of a mutation in more than one relevant gene. For example, the risk of thrombosis in carriers of the prothrombin mutation is increased two- to threefold. However, if these persons also are heterozy-

Table 2 Classification of laboratory investigations of thrombophilia.

	Characteristics of marker of clotting disorder	Examples	Difficulties with method or interpretation
I	Common defects, unequivocal test result	Factor V_{Leiden} mutation	–
		Prothrombin 20210 mutation	–
II	Common defects, variable laboratory test results and clinical findings	Lupus anticoagulant	++
		Increased factor VIII activity	+
		Increased fibrinogen	+
III	Rare defects, heterogeneous genotype and phenotype, manifestations of thrombosis common in affected families	Antithrombin deficiency	–
		Protein C deficiency (activity and concentration)	–
		Protein S deficiency (activity and concentration)	++

Derived in modified form from Spannagl M, Mäßmer G. Hämostase in Guder WG, Nolte J. Das Laborbuch für Klinik und Praxis. Elsevier Verlag 2005:59–79.

gotic for factor V_{Leiden}, their risk of thrombosis is increased twentyfold.

It is of note that about 40% of homozygotes for the G20210A prothrombin mutation remain asymptomatic, and that a large proportion of homozygotes who do become symptomatic display additional risk factors [35]. In contrast to carriers of factor V_{Leiden}, carriers of the G20210A prothrombin mutation are prone to develop pulmonary embolus. The risk of recurrence of pulmonary embolus is also increased in heterozygotes for the prothrombin mutation. Two other polymorphisms in the 3′-UTR of the prothombin gene have been described (C20209T, C20221T). Their effect on thromboembolic risk is unknown, although they have been linked to abortion and infertility in isolated case reports [36, 37].

Marburg mutation in factor VII activator, also known as hyaluron acid binding protein 2 (HABP2) or factor VII activating protease (FSAP)

The product of the *HABP2* gene binds hyaluronic acid. It also produces proteolytic cleavage of fibrinogen, and activates both factor VII and urokinase type plasminogen activator. In animal experiments, carriers of the Marburg I variant of *HABP2* show inadequate smooth muscle cell-derived neointimal formation after vessel injury. This has been thought to explain the greatly increased risk of cardiovascular stenosis in carriers of $FSAP_{Marbug\ I}$ carriers. This polymorphism has also been found in persons with idiopathic venous thrombosis (odds ratio about 6).

Investigation for the $FSAP_{Marbug\ I}$ variant is indicated in younger patients with carotid stenosis and in cases of unclear venous thrombosis following exclusion of factor V_{Leiden}, the G20210A mutation in prothrombin and other risk factors.

Together with other risk factors such as smoking, increased Lp(a), factor V_{Leiden}, low antithrombin, raised fibrinogen and diabetes, $FSAP_{Marburg\ I}$ promotes atherogenesis. $FSAP_{Marburg\ I}$ is a significant and independent risk factor for the appearance and progression of carotid stenosis [38–40].

Conclusions

Comprehensive investigation of thrombophilia includes a large number of laboratory investigations, some of which are demanding and require experience (Table 2). An important point is that laboratory diagnostics can only be successful in close collaboration with the clinicians treating the patient. In addition to the classical risk parameters for thrombosis and thromboembolism, increased attention should be paid to the possible presence of antiphospholipid antibodies.

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