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Factor V Leiden and other coagulation factor mutations affecting thrombotic risk

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Five genetic defects have been established as risk factors for venous thrombosis. Three are protein C, protein S, and antithrombin deficiencies, defects in the anticoagulant pathways of blood coagulation. Together they can be found in ~15% of families with inherited thrombophilia. Their laboratory diagnosis is hampered by the large genetic heterogeneity of these defects. The other two genetic risk factors, resistance to activated protein C associated with the factor V Leiden mutation and increased prothrombin associated with the prothrombin 20210 A allele, are much more prevalent and together can be found in 63% of the thrombophilia families. Because both defects are caused by a single mutation, DNA analysis is the basis of their laboratory diagnosis.

Venous thrombosis is a common disease, with an estimated annual incidence of 1 in 1000 persons. The development of a thrombotic event is the final result of multiple interactions between different genetic and environmental components. This is most clearly demonstrated in inherited thrombophilia, a genetically determined tendency to venous thrombosis caused by the segregation of one (monogenetic trait) or more (complex trait) disease alleles. Because a first thrombotic event is itself a very strong risk factor for thrombosis, it is important to identify individuals at risk and to offer them adequate treatment and (or) prophylaxis.

genetic risk factors for thrombophilia

During the past 30 years, six genetic defects (listed in Table 1) have been identified that are associated with an increased risk of venous thrombosis. Dysfibrinogenemia was first described in 1965 by Beck et al. *[1]*. It is a rare disorder (prevalence of 1% among selected thrombosis patients), most commonly identified by an abnormal Reptilase® time and (or) thrombin time. Both autosomal dominant and recessive inheritances (fibrinogen Naples) have been reported *[2]*. The evidence that dysfibrinogenemia can be associated with thrombosis is scarce. The most convincing support was published in 1995 in a report from the SSC subcommittee on fibrinogen *[2]*. So far at least 15 different mutations in the fibrinogen α, β, and γ genes have been reported in patients with thrombosis that result in the phenotype of dysfibrinogenemia.

Antithrombin deficiency was first reported in 1965 by Egeberg *[3]*. It is inherited as an autosomal dominant trait, with heterozygotes having an increased risk of venous thrombosis *[4]*. Basically there are two types of antithrombin deficiency: type 1 deficiency (reduction of both functional and immunological antithrombin) and type II deficiency (presence of an abnormal molecule). At present, >79 different mutations in the antithrombin gene have been reported that are associated with a type I or type II deficiency *[5]*. Heterozygosity for antithrombin deficiency can be found in ~4% of families with inherited thrombophilia, in 1% of consecutive patients with a first deep vein thrombosis (DVT), and in 0.02% of healthy individuals *[6]*.[1]

Protein C deficiency was first reported in 1981 by Griffin et al. *[7]*. In families with thrombophilia it is inherited as an autosomal dominant disorder: In these families heterozygosity for protein C deficiency is a significant risk factor for venous thrombosis *[8]*. On the other hand, autosomal recessive inheritance has been observed in families from newborns with severe thrombosis resulting from homozygous or compound heterozygous protein C deficiency *[9]*. Also here, type I and type II deficiencies have been reported. Protein C deficiency is genetically very heterogenous: >160 different mutations in the protein C gene have been reported to be associated with a type I or II protein C deficiency *[10]*. Heterozygosity for protein C deficiency is found in ~6% of families with inherited thrombophilia, in 3% of consecutive pa-

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[1] Nonstandard abbreviations: DVT, deep vein thrombosis; APC, activated protein C; SR, sensitivity ratio; CI, confidence interval; and APTT, activated partial thromboplastin time.

Table 1. Genetic defects in inherited thrombophilia.

	Prevalence, %	Mutations
Dysfibrinogenemia	1.0	>11
Antithrombin deficiency	4.3	>79
Protein C deficiency	5.7	>160
Protein S deficiency	5.7	>69
APC resistance	45	1
Increased prothrombin	18	1
Unknown	30	?

tients with a first DVT, and in 0.3% of healthy individuals *[6]*.

Protein S deficiency was first described in 1984 by Comp et al. *[11]*. In thrombophilic families it is inherited as an autosomal dominant disorder. Heterozygotes in these families have an increased risk of venous thrombosis when compared with their unaffected family members *[12]*. Also, protein S deficiency is genetically heterogenous. Almost 70 different mutations in the protein S gene (PROS-1) have been reported now *[13]*. The large majority of protein S-deficient patients have a type I deficiency. The prevalence of heterozygotes for a type I protein S deficiency is 6% in families with inherited thrombophilia and 1–2% in consecutive patients with a first DVT *[6]*. Interestingly, heterozygosity for protein S deficiency was not identified as a risk factor for venous thrombosis in a recent large patient control study, whereas heterozygosity for protein C or antithrombin deficiency was *[14]*.

More recently, two other genetic risk factors have been reported that differ from the previous ones in two respects. They are more common and they are always associated with the same genetic defect. The first concerns activated protein C (APC) resistance due to the factor V Leiden (FV R506Q) mutation *[15]*, and the second is increased prothrombin, which is associated with the 20210 A allele of the prothrombin gene *[16]*. In the next paragraphs these genetic defects will be discussed in more detail.

Interestingly, protein C, protein S, and antithrombin deficiencies all involve defects in anticoagulant pathways, whereas the factor V Leiden mutation and the prothrombin gene mutation involve procoagulant factors. In all cases the expected result of the genetic defect is an enhanced thrombin generation.

LABORATORY DIAGNOSIS

Screening of patients for genetic defects associated with thrombophilia is done almost exclusively in specialized hemostasis and thrombosis centers. For the detection of heterozygotes of protein C, protein S, or antithrombin deficiency, we still rely on the results of specific functional and (or) immunological tests. These genetic defects are too heterogenous (see Table 1) to seriously consider the introduction of genetic testing in routine diagnostic procedures. However, genetic testing has been used successfully for prenatal diagnosis of homozygous protein C deficiency. Hopefully in the future other techniques will become available that will make sequencing of a particular gene a feasible option in the routine hematology laboratory.

The diagnostic procedures for identification of protein C and protein S deficiencies especially need further improvement. Because of biological variation and gene–environment interactions, there is a large overlap in, for instance, protein C values between proven carriers of a protein C deficiency (heterozygotes) and non-protein C-deficient family members *[8]*. In practice this leads to the definition of arbitrary cutoff points that never will prevent substantial percentages of false-positive and false-negative diagnoses. Also, it has become clear that the range of protein C values that can be measured in healthy individuals depends on age and gender *[17]*.

A further complication in the diagnosis is that many patients will be treated with oral anticoagulants. This treatment will result in a decrease both in the plasma concentration and in the degree of carboxylation of the vitamin K-dependent proteins, including protein C and protein S. This problem has been extensively discussed in the past and several solutions have been proposed but later found to be not good enough (e.g., comparison of protein C/protein S concentrations with those of factor VII or factor II).

In the case of the diagnosis of protein S deficiency, an additional complication is that in plasma part of the protein S circulates in a complex with the C4b binding protein, and that these complexes do not have APC cofactor activity *[18, 19]*. Only very recently have some hard data been presented that in families with a known type I mutation in the protein S gene, heterozygotes can be identified better on the basis of their reduced free protein S concentrations than on the basis of their reduced total protein S concentrations. Hopefully these new insights will result in the near future in new recommendations for the laboratory diagnosis of protein S deficiency.

A technical problem with many of the protein C and protein S clotting assays was discovered during the last 3 years. Most of these assays apparently will give abnormal results in plasmas of patients that are APC resistant but not protein C or protein S deficient *[20, 21]*. Therefore new functional tests are needed that are more specific for these two anticoagulant proteins.

From the foregoing it is clear that laboratory diagnosis of protein C and protein S deficiencies is still far from optimal, especially where the relatively low prevalence of these defects among thrombosis patients further contributes to the rather unfavorable positive and negative predictive values of an (ab)normal result. This situation is completely different for the laboratory diagnosis of the other two genetic defects, APC resistance associated with the factor V Leiden mutation and increased prothrombin associated with the prothrombin 20210 A allele.

APC resistance associated with factor V Leiden

The concept of resistance to APC was first introduced in 1993 by Dahlbäck et al. *[22]*. It was defined as a poor anticoagulant response of plasma to the addition of APC. The phenotype of APC resistance [APC-sensitivity ratio (SR) <2.17] was frequently found among thrombosis patients *[23, 24]*. Koster et al. reported in 1993 that this phenotype was a risk factor for developing a first DVT {odds ratio: 6.6 [95 confidence interval (CI) 3.6–12.0]} *[24]*. In 1994 we reported that >80% of the cases with APC resistance were carriers of the same mutation in the gene of factor V: a G → A transition in position 1691, in exon 10, that predicts the replacement of Arg 506 by Gln in the factor V molecule (factor V Leiden) *[15]*. All carriers of the factor V mutation (and only these) had APC-SRs ≤1.8 *[15, 25]*. Interestingly, Arg 506 is located at one of the three cleavage sites for APC in factor Va (Arg 306, Arg 506, and Arg 679). Subsequent biochemical studies in which the inactivation of factor Va Leiden by APC was compared with that of normal factor Va demonstrated that as a consequence of the replacement of Arg 506 by Gln, the inactivation of the factor Va activity (Xa cofactor activity) was considerably retarded *[26, 27]*. This finding readily explains why carriers of the factor V Leiden mutation have the phenotype of APC resistance and offered sufficient starting points for the development of new laboratory tests for the identification of factor V Leiden carriers (see below). So far no other mutations of the factor V or factor VIII gene have been found that cause functional APC resistance.

The initial observation that the phenotype of APC resistance was not only common among patients with venous thrombosis, but also rather frequent among healthy control subjects, was later confirmed with genetic tests for the factor V Leiden mutation. Table 2 shows the prevalences of factor V Leiden carriers in the Dutch population, and in consecutive and selected patients with venous thrombosis.

Similar or slightly lower prevalences have been reported from other centers during the past 2 years in various selections of thrombosis patients. Interestingly, the prevalence of factor V Leiden carriers in the healthy population varies between different geographical regions and between different ethnic groups *[28, 29]*. It appears that the factor V Leiden mutation is only found in caucasoid subpopulations (prevalence of heterozygotes in the populations varies from 2% to 13%), and is extremely rare among Africans, Southeast Asians, Chinese, Japanese, Amerindians, and Greenland Inuit. Haplotype analysis of the factor V Leiden allele of homozygously affected individuals shows strong evidence for a common haplotype, thus supporting the hypothesis of a single mutational event *[15, 29]*. Of course the prevalence of heterozygous carriers in a particular population determines also the prevalence of homozygous carriers and therefore is an important variable to consider when exploring the need to design population screening programs for this mutation.

Table 2. Prevalence of factor V Leiden mutation in the Dutch population.

Healthy control subjects	3%
DVT patients	
Consecutive	19%
Selected	50%

The increase in risk of a first DVT was found to be 7.9 (95 CI 4.1–13) in heterozygotes and estimated to be 91 (95 CI 26–322) in homozygotes *[30]*. Also, there is preliminary evidence that in factor V Leiden carriers the incidence of recurrent thrombotic events is higher than in non-factor V Leiden carriers *[31]*. Whether carriers of the factor V Leiden mutation also have an increased risk of arterial thrombosis is still a matter of debate.

Because APC resistance associated with factor V Leiden is a common genetic defect, it offered unique opportunities to study the interaction of this defect with other genetic or acquired risk factors for thrombosis. Today we know that the prevalence of factor V Leiden among families with inherited thrombophilia and protein C, protein S, or antithrombin deficiency is much higher than expected on the basis of its population frequency *[25]*. These observations, together with more detailed analyses of families in which two different genetic defects were segregating, strongly support the model of inherited thrombophilia as a multiple gene disorder *[32–34]*. Because in such families carriers of two genetic defects clearly have an increased risk of a first thrombotic event when compared with carriers of a single defect, it is important to screen thrombophilia patients for all the known genetic risk factors.

Laboratory diagnosis of APC resistance associated with factor V Leiden

In principle we have three different types of tests available to screen patients for APC resistance associated with the factor V Leiden mutation. First, the classical APC resistance test with undiluted plasma *[22, 35]*: In these tests a plasma clotting time [prothrombin time (PT), activated partial thromboplastin time (APTT), Xa-clotting time, etc.] is determined in the absence and presence of a carefully calibrated amount of APC. The results are expressed as an APC-SR (clotting time + APC/clotting time − APC) or a normalized APC-SR (APC-SR patient plasma/APC-SR normal plasma) *[35]*. Most commonly, the APTT has been used for the determination of APC-SR. Unfortunately, these assays differ largely in the sensitivity and specificity for the factor V Leiden mutation *[36]*. In most cases this is related to the APTT reagent used. For instance, the APC-SR obtained with the Chromogenix reagents can also be reduced because of increased factor VIII concentrations, use of oral contraceptives, the presence of lupus anticoagulants, or the presence of platelet residues. This clearly affects its suitability to identify

factor V Leiden carriers. In contrast, another APC resistance test—which only differs from the Chromogenix test in the source of the APTT reagent—has an almost 100% sensitivity and specificity for the factor V Leiden mutation *[37]*, when a normalized APC-SR of 0.70 is used as the cutoff value. The performance of several of these APTT-based APC resistance tests has recently been evaluated in an international collaborative study, organized by the Scientific Standardization Committee of the ISTH *[36]*. A second complication of these classical APC resistance tests is that they cannot be used for the analysis of plasmas of patients on oral anticoagulant therapy. This therapy will result in a decrease in plasma factor II and factor X concentrations, which in its turn will increase the sensitivity of the plasma for APC *[35]*.

For all these reasons, so-called modified APC resistance tests have been developed that are highly sensitive and specific for the factor V Leiden mutation. The most simple modification is to dilute a test plasma in a carefully selected factor V-deficient plasma in such a way that the influence of eventual variations in other clotting factors in the test plasma on the clotting time is minimal *[38, 39]*. Other modifications use slightly different approaches to obtain similarly high sensitivity and specificity for the factor V Leiden mutation *[40, 36]*.

The most direct test for factor V Leiden is of course the genetic test, which includes genomic DNA or RNA from white blood cells as template for amplification of the fragment of the factor V gene/mRNA that contains the mutation *[15, 41]*. The presence or absence of the mutation can be analyzed with allele-specific probes or restriction enzyme analysis (e.g., *Mnl*I digestion). One should realize that the latter is not specific for the mutation, so the possibility exists of a positive DNA test and a negative APC resistance test (see discussion in ref. *42*). The approach of the use of allele-specific primers for the amplification seems especially to be very useful for screening of large series of DNAs.

Currently our strategy for screening patients for the factor V Leiden mutation consists of determining the APC-SR with a modified APC resistance test that is sufficiently specific and sensitive for the mutation, followed by a DNA/RNA test, only to confirm an abnormal result. Apart from that we perform the classical APC resistance test that was developed in our laboratory *[34]* only for research purposes. With this test we are able to identify patients with a different type of APC resistance, the molecular basis of which is still unsolved *[25]*. For some reference laboratories the exclusive use of a PCR-based test might be recommended, especially when the expertise for blood collection and handling for the coagulation-based assays is not available. Such a test, however, should rely on specific identification of the 1691 G and A alleles (allele-specific amplification or use of allele-specific probes).

THE PROTHROMBIN 20210 A ALLELE

Recently a new genetic risk factor for venous thrombosis was identified by systematically sequencing the prothrombin genes of 28 probands from families with unexplained familial thrombophilia *[16]*. In five of these 28 patients a sequence variation was observed that was also present in 1 of 100 healthy control subjects. It concerned a G-to-A transition in position 20210 of the prothrombin gene. This mutation is located in the very last nucleotide of the 3′ untranslated region of the cDNA just before the poly A attachment site (see Fig. 1). After screening all the patients and controls of a large population-based case-control study (Leiden Thrombophilia Study) for the presence of the prothrombin 20210 A allele, it was established that this allele was associated with an increased risk of a first DVT [odds ratio: 2.8 (95 CI 1.4–5.6)].

This mutation was found in 1–2% of healthy control subjects, 6.2% of consecutive patients with a first DVT, and in 18% of patients that had been selected for unexplained familial thrombophilia *[16]*. Again there seems to exist a strong interaction of this genetic defect with other genetic risk factors for thrombosis. In two families in which both the factor V Leiden and the prothrombin 20210 A allele segregate, thrombotic events were only observed in individuals carrying both defects (unpublished observation).

An important question is how this particular sequence variation may cause an increased risk of venous thrombosis. Because of its location in the 3′ untranslated region of the gene, we hypothesized that it may affect plasma prothrombin concentrations. Indeed, we could demonstrate that the mean plasma prothrombin concentration of 40 heterozygous carriers of the prothrombin 20210 A allele was significantly higher than that of 860 noncarriers (132% ± 8% vs 105% ± 15%) *[16]*. This suggested that the increased risk of thrombosis associated with the 20210 A allele might be related to the increased prothrombin concentrations. Indeed, further analysis of the same case-control study indicated that having a prothrombin concentration >115% is itself a risk factor for thrombosis [odds ratio 2.1 (95 CI 1.5–3.1)] *[16]*. Only 25% of the individuals with prothrombin concentrations >115% carry the prothrombin 20210 A allele, suggesting that there might be other genetic or acquired defects associated with increased prothrombin concentrations.

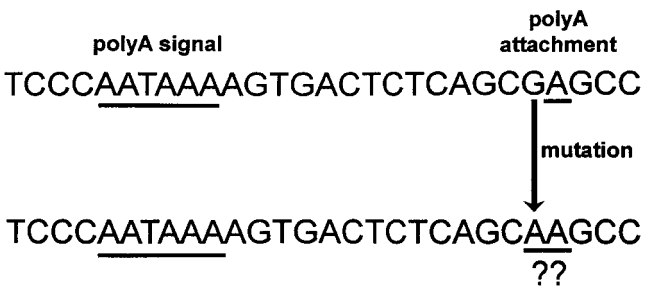

Fig. 1. Location of the G (20210) A mutation in the prothrombin gene.

The laboratory diagnosis for the presence of the prothrombin 20210 A allele relies completely on DNA analysis. In fact this mutation is the first example of a genetic coagulation defect that cannot be reliably diagnosed by standard functional or immunological hemostatic tests.

PERSPECTIVES

During the past 4 years the discovery of APC resistance associated with the factor V Leiden mutation and of the prothrombin 20210 A allele, which is associated with increased plasma prothrombin concentrations, have had a major impact on our understanding of thrombophilia as a complex disease in which interactions between genetic and environmental components contribute to the clinical phenotype.

In the diagnostic coagulation laboratory a major change has occurred in the workup of thrombophilia patients by the introduction of large-scale DNA testing. In these 4 years the yield of our screening program for thrombophilia patients has improved from ~5% to almost 25% in unselected patients and from 15% to 63% in patients from thrombophilia families. In the meantime we should not forget to think of ways to improve the laboratory diagnosis of protein C and protein S deficiencies when DNA testing is not a feasible option. Finally, the awareness that three of the five known genetic risk factors for thrombosis do interfere with the efficacy of the protein C anticoagulant pathway has stimulated the search for new screening tests that can identify most (or all) of the genetic and acquired defects in this pathway.

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