Improved functional determination of Factor V Leiden induced APC resistance

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Factor V Leiden mutations are responsible for the majority of cases of Activated Protein C (APC) resistance in which activated Factor Va is only slowly inactivated. A new functional test for Factor V Leiden induced APC resistance has been developed and shows significant advantages over current test methods.

Although in itself not life-threatening, the formation of a blood clot in one of the deep veins of the body, known as deep vein thrombosis (DVT), is often associated with potentially fatal complications. Of these the most serious is pulmonary embolism (PE) which occurs in approximately 30% of cases of DVT. Certain categories of subjects have a greater risk of developing DVT than others. Higher risk categories include the elderly, obese patients, smokers, cancer patients, pregnant women, as well as women taking oral contraception or on hormone replacement therapy. Another important category of patients with increased risk of DVT are those with acquired or hereditary risk factors, i.e. patients with thrombophilia.

Epidemiology of Factor V Leiden mutation

Observed in up to 40% of patients with idiopathic thrombotic manifestations, activated protein C (APC) resistance is the most common risk factor for venous thromboembolism (VTE). Over 80% of such cases can be explained by the Factor V Leiden mutation (FV Leiden) [1]. Overall, 3 to 8% of the general European and US populations are heterozygous for the FV Leiden mutation. Within Europe, there is a variation in the frequency, ranging from 10-15% in southern Sweden and Greece, 4-8% in Germany, to 2-3% in Italy and Spain. Homozygosity for the FV Leiden mutation is found in approximately 1 in 5000 of the general European population. Relatively high levels are found in Middle Eastern and Indian populations, while the mutation is less frequently found in East-Asian, African, and indigenous Australian populations. In addition to the FV Leiden mutation, APC resistance can also be caused by acquired conditions such as pregnancy, antiphospholipid antibodies or elevated levels of Factor VIII. The American College of Medical Genetics (ACMG) has published a consensus statement indicating the circumstances under which testing for Factor V Leiden should be carried out or considered (Table 1).

Factor V Leiden mutation and its impact on haemostasis

The efficiency of the coagulation cascade is massively increased by the activation of the non-enzymatic cofactors FVIII and FV. It is therefore of utmost importance that the activated forms of these cofactors, FVIIa and FVa, be under strict regulatory control, as well as activated coagulation proteases such as FXa or FIla. These latter are neutralised by the formation of complexes with protease inhibitors such as antithrombin III (AT III) in a reaction that is enhanced by the glycosaminoglycans of endothelial cells or by heparin-like drugs.

As for the inactivation of the cofactors FVa and FVIIa, this is primarily mediated by APC, a vitamin K dependent serine protease which has sequence homology to coagulation factors, Protein S, and probably also FV in its nonactivated form, serve as cofactors in this reaction. Although thrombin and other proteases such as plasmin can also inactivate FVa and FVIIa, it appears that in vivo, the main inactivation pathway is mediated by APC.

In subjects with the FV Leiden mutation, the rate of inactivation of Factor Va in the APC-mediated step is significantly slower; the molecule is said to have become resistant against activated protein C ("APC- resistance"). The defect is caused by a G-to-A substitution at nucleotide 1691 in the coding part of the factor V gene. This results in the replacement of the arginine residue by a glutamine residue at position 506 which is one of three APC cleavage sites in the factor Va molecule. Although this replacement slows down the inactivation of factor Va by APC, it does not impair the procoagulant function of factor Va. In practice, therefore, the consequence of the mutation is to effectively prolong the time during which FVa can contribute to thrombin formation as part of the prothrombinase complex.

Thrombin has many biological functions. It is the most potent platelet activator, and can also activate several other coagulation factors such as FV, FVIII and FXIII, as well as protein C and the so-called thrombin activatable fibrinolysis inhibitor (TAFI). Thrombin also converts fibrinogen into fibrin which, together with activated platelets, forms the clot. It is likely therefore, that any mechanism which leads to enhanced thrombin formation, such as the APC resistance described above, (but also high FVIII levels, deficiency of protein C or protein S) may finally result in an enhanced risk of thromboembolic complications. Conversely, any situation where there is a reduced rate of thrombin clearance, e.g. AT III deficiency, is likely to have the same result.

Clinical consequences of the Factor V Leiden mutation

The clinical expression of FV Leiden thrombophilia can be quite varied. Some individuals with FV Leiden never develop thrombosis at all. Others do not have their first episode of DVT until adulthood, while some already suffer recurrent thromboembolism before the age of 30 years. The clinical expression of the mutation is influenced by the number of FV Leiden alleles (i.e., homo- or heterozygosity), as well as the presence of other coexistent genetic abnormalities and risk factors. There are several such risk factors that have been identified. These include the prothrombin mutation G20210A (associated with higher plasma concentrations of FII), phospholipid or prothrombin autoantibodies, as well as deficiencies of AT III, protein C or protein S. Elevated levels of FVIII, PAI-1 (plasminogen activator inhibitor type 1) or TAFI, pregnancy, oral contraceptives or cancer are further risk factors. The combination of FV Leiden mutation with any or several of these factors may further increase the risk for DVT.

The heterozygous defect is associated with a 5 to 10 fold increase in the risk of thromboembolism, whereas the homozygous defect results in a 50 to 100 fold increase. A significant fraction of patients with venous leg ulcerations have APC resistance and FV Leiden, whereas pulmonary embolism is less common than DVT in patients with FV Leiden. FV Leiden seems to be associated with stillbirth as well as with some poor pregnancy outcomes. It has also been identified in significant numbers in women who have other obstetrical complications such as preeclampsia, placental detachment, intra-uterine growth retardation, repeated pregnancy losses or first-trimester loss. The risk of thrombosis is increased by about 35 times in young women taking oral contraceptives and with the FV Leiden mutation.

Table 1. Criteria for testing for Factor V Leiden (ACMG consensus statement).
However, FV Leiden mutations can protect against blood loss, and may thus be an evolutionary advantage in certain circumstances, e.g. during childbirth. Patients with the FV Leiden mutation have less blood loss during open heart surgery. Subjects with FV Leiden have a decreased risk of spontaneous intracranial haemorrhage, a finding that may have relevance in the risk stratification of patients who require major surgery.

**Factor V Leiden assays**

The method first used to detect the mutation was a modified aPTT. This assay is based on the principle that when APC is added to normal plasma, factors Va and VIIIa are inactivated, resulting in a slower coagulation process and thus prolonged aPTT. In practice this assay involves incubating the sample both with and without a standardised amount of exogenous APC and then carrying out a aPTT determination. The result is expressed as the ratio of aPTT with APC to that without, (aPTT + APC)/(aPTT - APC). The APC-resistant phenotype is characterised by only a small prolongation of the aPTT in response to APC, giving a correspondingly low ratio in this case.

This method is suitable for the initial characterisation of the APC resistance syndrome, but has several limitations. As with several other aPTT-dependent tests (or the aPTT itself) the results can be significantly affected by the type of instrument that was used for the standardisation of the test. Comparability of results and of appropriate cut-off values therefore becomes difficult. The assay also has limitations in patients who have a baseline-prolonged aPTT caused by vitamin K antagonists, heparin anticoagulation, other coagulation defects or a lupus inhibitor. The assay can also be affected by haemostatic changes that occur during pregnancy or during acute thrombosis.

The impact of many of these factors that influence the validity of the test can be reduced by mixing the sample with Factor V-deficient or Factor V-depleted plasma and adding a heparin antagonist. Such modified "second generation" APC resistance assays have a much higher sensitivity and specificity for FV Leiden [2]. They can be used successfully in patients with lupus inhibitors, as well as in patients with acute thrombosis, pregnancy, or inflammation.

Other functional tests for APC resistance have also been described. Some of these start lower in the coagulation cascade, thus circumventing the impact of factor VIII. Currently, however, the most frequently used test for FV Leiden is probably still the aPTT-based test with a prior incubation with FV deficient plasma.

**Clinical results with Pefakit APCR-FV Leiden**

The performance of the test is summarised in Table 2, and shows results from more than 600 samples obtained from several external centres. The samples were all characterised by repeated PCR. It can be seen from the data in Table 2 that the test clearly differentiates FV wild type samples from the heterozygous FV Leiden samples and even more so from the homozygous form. This clear distinction is seen on all four coagulometer types used. As in all aPTT-based test systems, the value of the final measured ratio can depend to some extent on the individual instrument used. There is, however, no effect of instrument type on the final clinical conclusion. The homozygote samples gave a measured ratio of approximately 1 with a relatively narrow range, whereas the heterozygotes show a somewhat broader range. The wild type form, in which FV is expressed as the ratio of aPTT with APC to that without, (aPTT + APC)/(aPTT - APC).

<table>
<thead>
<tr>
<th>Genotype of FV</th>
<th>Ratio (range) and sample size on different coagulometers</th>
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<tbody>
<tr>
<td>Wildtype</td>
<td>CA 540: 4.2 - 6.0 (n=25); ACL: 3.2 - 5.4 (n=53); BCS: 4.0 - 5.5 (n=68); KC 4: 4.2 - 6.9 (n=25)</td>
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<tr>
<td>Heterozygote F VL</td>
<td>1.4 - 1.8 (n=105); 1.4 - 2.1 (n=57); 1.4 - 2.2 (n=108)</td>
</tr>
<tr>
<td>Homozygote F VL</td>
<td>1.0 - 1.1 (n=15); 0.9 - 1.2 (n=14); 0.9 - 1.1 (n=17); 1.0 - 1.1 (n=15)</td>
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Table 2. Clinical performance of the Pefakit APCR-FV Leiden test, using four different coagulometer instruments, namely models CA540 (Sysmex), ACL (Instrumentation Laboratory), BCS (Dade Behring), KC4 (Amelung/Trinity). The ratio measured is that of aPTT with and without APC. It can be seen that the new test clearly differentiates heterozygotes from homozygotes. Standard aPTT tests generally cannot make this distinction.
was found to be due to some samples having been mis-typed in the molecular biology test. The discrepancies disappeared when the PCR method was repeated.

In summary, all current available data show that the method is 100% specific and sensitive [4]. Currently the precise biochemical explanation behind the clearly superior characteristics of the new assay system is not known. It is possible that the activation step using the snake venom-derived FV activator is the reason for this improved performance. Recent findings have indeed shown that the activation of FV by thrombin may lead to a deteriorated sensitivity in the APCR test.

**Current status and future prospects**

Clinical results obtained on hundreds of specimens have shown that the analytical resolution of the new clotting test for FV Leiden is comparable to that of PCR-based techniques. These latter are more time-consuming, more expensive and frequently subject to analytical problems. The new test is simple, rapid and can be adapted to manual or automated coagulometers. The test is therefore well suited for the rapid assessment of FV Leiden mutation, which is the most common risk factor for venous thromboembolism. Awareness of the existence of a potential thrombophilic situation caused by FV Leiden mutation is especially important since this may trigger consideration of life-style changes in subjects who are already associated with an increased risk of thromboembolism, e.g. in pregnant women or those considering oral contraception. On the other hand, given that recent data show that FV Leiden mutation can give partial protection against blood losses, the APCR-Factor V Leiden assay may even be an interesting new approach to the preoperative risk stratification before major surgery.

**References**


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