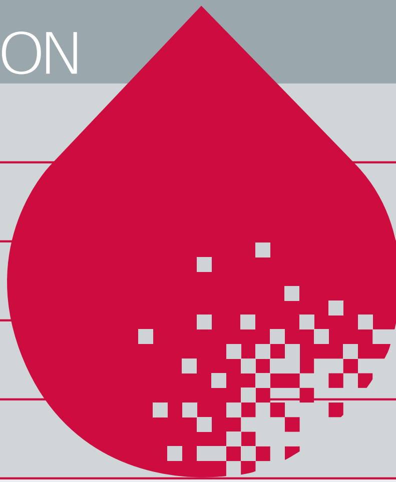

APC Resistance

Frank Axelsson, Product Information Manager, Chromogenix;
Steffen Rosén, Vice President Scientific and Medical Affairs, Chromogenix.

Instrumentation Laboratory

Frank Axelsson, Product Information Manager, Chromogenix;
Steffen Rosén, Vice President Scientific and Medical Affairs, Chromogenix.

APC Resistance

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Introduction

APC Resistance, a Common Risk Factor for Thrombosis

Activated protein C (APC) is a key anticoagulant enzyme needed for the proper down-regulation of blood coagulation.
A poor anticoagulant response to APC, denoted APC resistance, is a recently described blood defect found to be a major risk factor for venous thromboembolism in Western societies. At least 90% of cases with the APC resistance phenotype can be explained by a point mutation in the gene for coagulation factor V.
The mutation predicts the synthesis of an abnormal factor V molecule (termed FV:Q^{506} or FV Leiden) that is partially resistant to inactivation by APC, causing a life-long disposition to a hypercoagulable state.
APC resistance due to the presence of the FV:Q^{506} allele is inherited as an autosomal dominant trait and has a prevalence of 2-13% in the general population. Frequencies of APC resistance among patients with venous thrombosis, depending on the selection criteria, range from 20-60%.
The high prevalence of APC resistance and the availability of simple blood tests to detect this disorder, raises the question whether more general screening for APC resistance should be performed in conjunction with surgery, pregnancy, use of oral contraceptives and other established risk factors for thrombosis.

Venous thromboembolism

The formation of an obstructive mass of clotted blood in the venous part of the circulatory system is known as venous thrombosis. The mass itself is called a thrombus and is composed of platelets, blood cells and fibrin. A thrombus which breaks loose and is carried away with the bloodstream is called an embolus. When caught in the blood vessels of the lung it may develop into pulmonary embolism, the most feared complication of venous thrombosis.Venous thromboembolism is a major health problem in Western societies, constituting the third most common cardiovascular disease after acute ischemic heart disease and stroke.[1]
The incidence has increased steadily in recent centuries, perhaps due to longer life-spans and the adoption of more sedentary habits.
In the USA, venous thromboembolism accounts for more than 250,000 hospitalizations a year, corresponding to an incidence of about one per 1,000 individuals. The annual death rate due to pulmonary embolism is estimated to be 50,000.[1]

Thrombogenic risk factors

As described by Rudolf Virchow more than a century ago, there are three primary pathogenic risk factors for venous thrombosis: a reduced blood flow, vessel wall damage, and a change in blood components (Figure 1).
Any one of these risk factors potentiates the other and creates a hypercoagulable state in which the balance

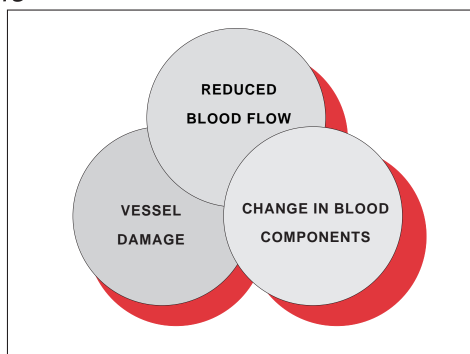


Figure 1. Virchow's triad for venous thrombosis.

between procoagulant and anticoagulant forces has shifted in favor of coagulation.[2,3] Hypercoagulability and venous thrombosis tend to develop in conjunction with circumstantial or acquired risk factors such as surgery, pregnancy, use of oral contraceptives, immobilization, cancer and old age. It is also known that genetic risk factors often play an important role in the pathogenesis, since as many as 20-40% of patients referred to a specialist laboratory may have a family history of thrombosis.[4-6] However, genetic defects associated with an inherited tendency to develop thrombosis (thrombophilia) were, until recently, identified in only a few percent of all thrombosis patients.[7]

Novel defect in the protein C anticoagulant pathway

The diagnostic situation for inherited thrombophilia improved dramatically in 1993 with the discovery of a novel defect in the protein C anticoagulant pathway. Based on the hypothesis that a poor anticoagulant response to activated protein C (APC) might predispose to thrombosis, a Swedish research group led by Björn Dahlbäck measured the anticoagulant activity of exogenously added APC in an APTT-based assay.[8] In a normal response, the addition of APC to plasma induces a prolonged clotting time.
This occurs because APC cleaves and inactivates two critical coagulation proteins, factors Va and VIIIa.
However, when the assay was run on plasma from a middle-aged man suffering from recurrent episodes of venous thrombosis, the result showed a much shorter prolongation of the clotting time than expected (Figure 2).

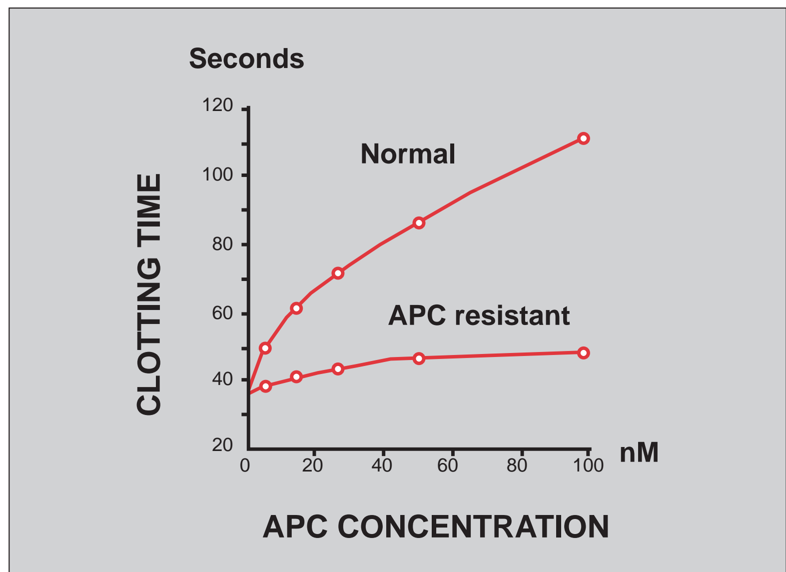


Figure 2. The poor anticoagulant response to activated protein C (APC resistance) in an APC-resistant thrombosis patient compared to a normal response (from Dahlbäck et al).[8]

Several of the man's relatives demonstrated a similar poor anticoagulant response to APC and family studies suggested that this disorder, denoted APC resistance, was inherited as an autosomal dominant trait.[8,11] Subsequent investigations carried out in Western countries showed that APC resistance was present in 20-60% of all cases of venous thromboembolism and that it was highly prevalent in the general population (1-7%).[9-12] These results proved APC resistance to be the most prevalent cause of thrombophilia, being larger than the sum of all other previously established genetic risk factors, including antithrombin, protein C and protein S deficiency.

Mutation in the factor V gene explains APC resistance

The search for the molecular mechanism of APC resistance led to the isolation of a protein from normal plasma, which was able to correct APC resistance in a dose-dependent manner.
This protein was identified as factor V, suggesting that APC resistance was caused by a genetic defect in the factor V gene.[37] Other studies reached the same conclusion and a point mutation that predicts the replacement of arginine (R) at position 506 in the factor V molecule with glutamine (Q) was soon identified.[13-15]
The mutated protein, denoted $FV{:}Q^{506}$ (or FV Leiden), is activated in a normal way and retains normal procoagulant activity, although it is partially resistant to APC cleavage and inactivation resulting in a disposition to a hypercoagulable state.
At least 90% of APC resistant cases are explained by this mutation.[16]

Diagnostic breakthrough in thrombophilia

The discovery of APC resistance and the identification of the $FV{:}Q^{506}$ mutation as its main cause means, that a genetic explanation can now be identified almost as often as non-genetic risk factors in thrombosis patients
This diagnostic breakthrough, in combination with the availability of simple laboratory tests, offers a powerful tool for preventing venous thromboembolism.
This monograph reviews the APC resistance phenomenon and describes the major tests for its phenotype and the $FV{:}Q^{506}$ genotype.
The use of these tests in the clinical environment will help establish guidelines for therapy and prophylaxis, which hopefully will lead to reduced morbidity and mortality in thrombophilic patients.

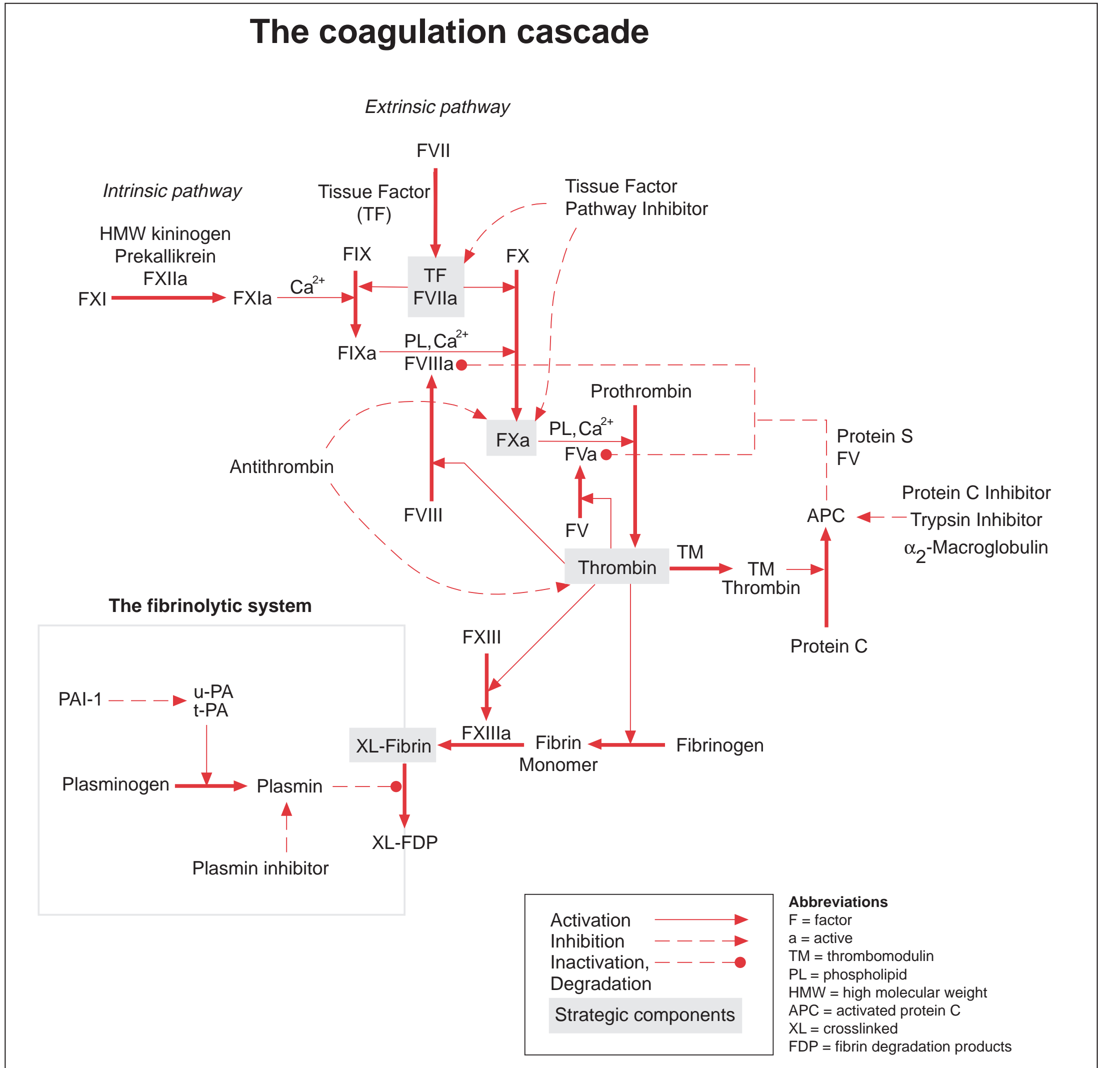


Figure 3. The coagulation cascade.
Vascular damage initiates the coagulation cascade resulting in the explosive generation of thrombin at the site of injury. Thrombin catalyzes the conversion of fibrinogen to an insoluble fibrin (clot) matrix, in the presence of factor XIIIa and calcium ions. Critical reactions are closely checked and localized by circulating anticoagulants, such as APC, TFPI and antithrombin. Fibrinolysis is initiated when fibrin is formed and eventually dissolves the clot. Inappropriate activation of blood coagulation and/or depressed fibrinolytic activity may lead to the formation of a thrombus. In contrast, a defect or deficiency in the coagulation process and/or accelerated fibrinolysis is associated with a bleeding tendency. The cascade scheme is organized into the intrinsic (factors XII, XI, IX, VIII, prekallikrein, HMW kininogen), extrinsic (tissue factor, factor VII) and common pathway (factors V, X, XIII, prothrombin, fibrinogen). The extrinsic pathway is initiated when blood is exposed to tissue factor released from damaged endothelium. The intrinsic pathway is initiated by the activation of factor XII involving "contact factors" on negatively-charged surfaces, such as glass or kaolin in vitro. Feedback activations of factors V, VII and VIII by factor Xa and the activation of factor XI by thrombin are not shown.[22-23,28]

Initiation and regulation of blood coagulation

In order to prevent dangerous blood loss following vascular injury, the hemostatic system is called into action. Within seconds of injury the damaged vessel contracts and circulating, disc-shaped cell components known as platelets are activated. These adhere to the site of injury, where they aggregate to form a loose plug that reduces or temporarily stops the bleeding (primary hemostasis). Blood coagulation is triggered simultaneously with these events (Figure 3).

The coagulation cascade

Blood coagulation involves a complex set of step-like proteolytic reactions, which can be described as a progressively amplified cascade of proenzyme-to-enzyme conversions. This biochemical process takes place preferentially on negatively-charged phospholipid surfaces provided by activated platelets and results in the rapid formation of a fibrin matrix, which stabilizes the initial platelet plug and seals the bleeding vessel.[17-18]
There are two activation pathways for blood coagulation, the intrinsic and the extrinsic pathway. Both pathways involve a number of plasma proteins as listed in Table 1. Most of the coagulation factors are zymogens (i.e. proenzymes) of trypsin-like serine proteases, which cleave arginyl peptide bonds with high specificity. Several proteins, including factors II, VII, IX and X, protein C and protein S, are subjected to vitamin K-dependent carboxylation of glutamic acid residues during their synthesis in the liver. This unique amino acid modification allows the proteins to bind calcium ion and phospholipids and thereby to participate efficiently in multimolecular complexes in the coagulation cascade.[17-18]
The extrinsic pathway is the most important for the initiation of coagulation.[17]
This pathway becomes activated when disrupted tissue and activated monocytes exposes tissue factor to the bloodstream.[19]
Tissue factor forms a complex with factor VII, which becomes activated and then activates factors IX and X. The intrinsic pathway is initiated by the exposure of "contact" factors (factor XII, HMW kininogen and prekallikrein) in plasma to a negatively charged surface, such as connective tissue in vivo or glass in a test tube.[20]

Factor /	Name	Size [kDa]	Conc. [µg/ml]	Factor /	Name	Size [kDa]	Conc. [µg/ml]
I	Fibrinogen	340	3,000	XIII	Fibrin-stabilizing factor	320	10
II	Prothrombin	69	100	-	Tissue factor pathway inhibitor	46	1
III	Tissue factor	46	-	-	Antithrombin	58	150
IV	Calcium	-	-	-	Protein C	62	4
V	Proaccelerin	330	10	-	Protein S	78	20
VI	-	-	-	-	Thrombomodulin	60	-
VII	Proconvertin	48	0.5	-	Protein C inhibitor	57	5
VIII	Antithemophilic factor	330	0.1	-	Plasminogen	92	200,000
IX	Christmas factor	55	5	-	t-PA	70	5
X	Stuart-Prower factor	59	8	-	u-PA	54	8
XI	Thromboplastin antecedent	160	5	-	PAI-1	52	50
XII	Hageman factor	80	30	-	Plasmin Inhibitor	70	70,000

Table 1: Coagulation factors and regulatory proteins.

The two pathways converge on factor X to a common pathway, which ends with the conversion of prothrombin (factor II) into the key coagulation enzyme, thrombin.[21] The serine protease thrombin converts circulating fibrinogen into clot-forming fibrin molecules and activates the transglutaminase, factor XIII, which stabilizes the fibrin matrix through covalent cross-linking.
Thrombin also stimulates hemostasis and coagulation through positive feedback, by activating more platelets and also the circulating non-enzymatic proteins, factors VIII and V.[22-23] All these feedback activations by thrombin lead to an explosive amplification of the coagulation cascade and rapid clot formation.

Thrombin control

It is evident that the autocatalytic nature of thrombin could clot the blood content of a person within minutes if uncontrolled. In humans, the necessary control involves two aspects, i.e. inhibition of thrombin already formed and prevention of further thrombin generation.
Direct thrombin inhibition is provided primarily by circulating antithrombin,[25] whereas the crucial prevention of thrombin generation is provided indirectly by thrombin itself. This self-regulating function of thrombin is expressed in its binding to thrombomodulin, a specific, high-affinity receptor protein located on undamaged endothelium.[26,27]

The protein C anticoagulant pathway

On binding to thrombomodulin, thrombin loses all its procoagulant properties. Instead, it becomes a potent activator of protein C, the key component of the protein C anticoagulant pathway (Figure 4). APC is a serine protease that rapidly prevents thrombin generation by cleaving and inactivating the phospholipid-bound, activated forms of coagulation factors Va and VIIIa. APC in turn is only slowly neutralized by three inhibitors, protein C inhibitor, trypsin inhibitor and α_2-macroglobulin.
The relatively long half-life of APC in vivo (15-20 minutes) is a prerequisite for its function as a circulating anticoagulant. The anticoagulant activity of APC is potentiated and supported by protein S, a vitamin K-dependent, non-enzymatic plasma protein synthesized in the liver.[27-28]
Protein S probably exerts its function by promoting the binding of APC to phospholipid surfaces and by removing the factor Xa and factor IXa-mediated APC protection of factors Va and VIIIa respectively.[28] Protein S has also been suggested as having an APC-independent anticoagulant activity, by inhibiting prothrombin activation through direct interaction with factor Va and factor Xa.[28] The normal concentration of protein S in plasma is 20-25 µg/ml. Approximately 60% of this is bound to C4bBP, a regulatory protein of the classic complement system. Only the free form of protein S has the APC cofactor function. Recently, it has been found that non-activated factor V functions in synergy with protein S as an APC cofactor in the degradation of factor VIIIa and possibly also factor Va.[29] This has been confirmed in other studies,[30-31] although the view of intact factor V as an anticoagulant APC cofactor has been challenged. Instead, it has been suggested that the central B-domain released on activation of factor V expresses the APC cofactor activity (Figure 5).[32] The in vivo relevance of these findings awaits further investigation.Taken as a whole the protein C pathway constitutes an ingenious mechanism by which procoagulant thrombin attains anticoagulant properties in the absence of vascular injury.[28] The physiological importance of this mechanism is demonstrated clinically by the serious thrombotic disorders associated with its malfunction.

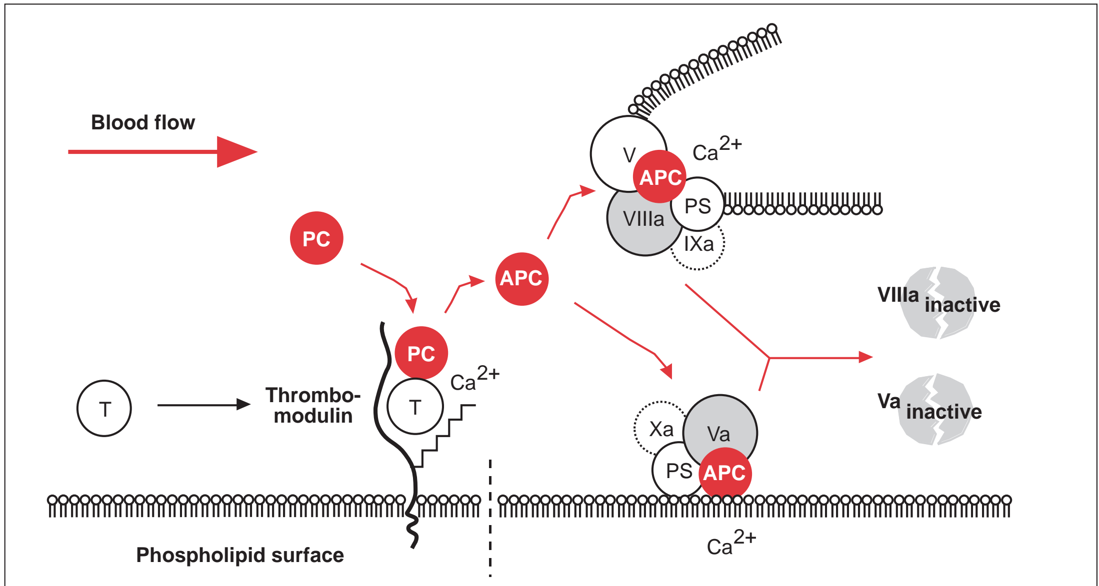


Figure 4. The protein C anticoagulant pathway.
Thrombin escaping from a site of vascular injury binds to its receptor thrombomodulin (TM) on the intact cell surface. As a result, thrombin loses its procoagulant properties and instead becomes a potent activator of protein C. Activated protein C (APC) functions as a circulating anticoagulant, which specifically degrades and inactivates the phospholipid-bound factors Va and VIIIa. This effectively down-regulates the coagulation cascade and limits clot formation to sites of vascular injury. The activity of APC is potentiated by protein S and possibly intact factor V. APC is slowly neutralized by circulating inhibitors. Thrombin bound to TM will eventually be inhibited by antithrombin or removed through endocytosis of the thrombin/TM complex. Symbols: T= thrombin, PC= protein C, PS= protein S. Based on reference.[28]

Natural substrates of APC, factors VIIIa and Va

Factors V and VIII are two large, relatively unstable, plasma proteins of about 330 kDa, with similar structure and function.[22-24]
Factor V is an essential component for the rapid conversion of prothrombin to thrombin, whereas factor VIII is needed to accelerate the activation of factor X to factor Xa. The essential role of these non-enzymatic cofactor proteins in hemostasis is evidenced by the severe bleeding tendency associated with their deficiency.[22,33]
Both factor V and factor VIII are synthesized mainly in the liver and circulate in plasma as inactive molecules with little or no procoagulant activity. A unique feature of factor VIII is that it circulates in a stabilizing, noncovalent complex with the von Willebrand factor, an adhesive protein that is important for the proper function of platelets.[23] The plasma concentration of factor V is about 10 µg/ml, which is up to a 100-fold higher than that of factor VIII (0.1-0.2 µg/ml).[28] About 20% of the total amount of factor V in blood is synthesized by megacaryocytes and stored in platelets. This stored form of factor V is released in conjunction with platelet activation and has an important role in normal hemostasis.
The genes for factor V and VIII are located on chromosomes one and X respectively. They code for mature, single-chain proteins of roughly 2200 amino acids.

Prior to secretion into the bloodstream, the factor VIII molecule is processed to a calcium ion-linked heterodimer, whereas factor V circulates as a single-chain protein. [22-23] Computer-aided comparison of the primary amino acid sequence of factors V and VIII reveals a high degree of homology, with an overall identity of about 30%.[24] Both proteins contain several types of similar internal repeats, termed A1-A2-B-A3-C1-C2.

Activation of factors V and VIII

Factors V and VIII are activated through limited proteolysis by thrombin or factor Xa.[22-23] During its activation, factor VIII is released from the protective influence of the von Willebrand factor and converted to a calcium ion-dependent trimer (A1, A2 and A3-C1-C2). The active factor V molecule (factor Va) is a dimer that consists of a heavy chain (A1-A2), non-covalently linked via calcium ions to a light chain (A3-C1-C2) (Figure 5). The activated factors VIIIa and Va bind to negatively-charged phospholipid in the presence of calcium and serve as cofactors/receptors for factors IXa and Xa respectively. The importance of these multimolecular complex assemblies, better known as the tenase and prothrombinase complexes, is evidenced by the over 100,000-fold increase in the combined rate of activation of factor X and prothrombin when compared to the activation catalyzed by their respective enzyme alone.[24]

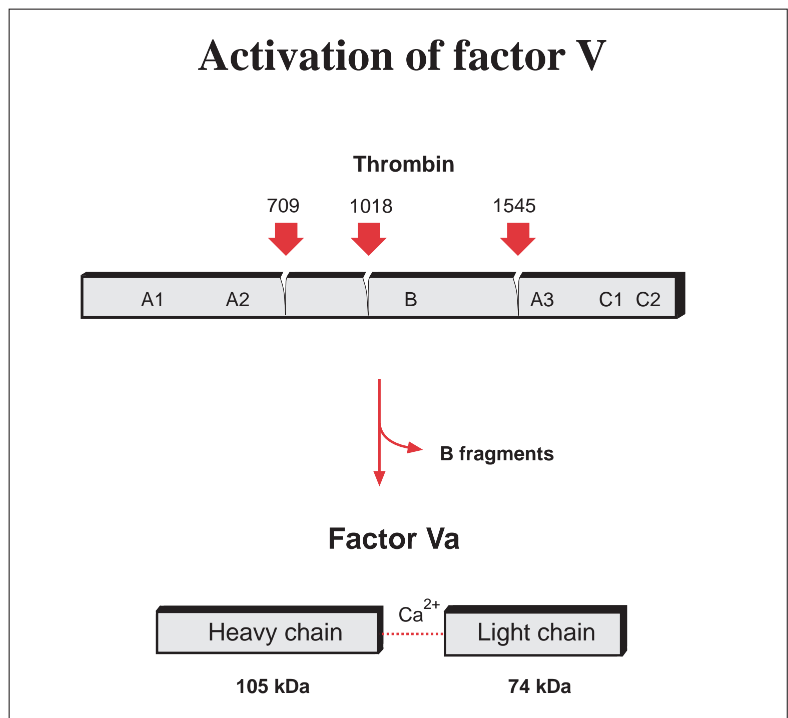


Figure 5. Activation of factor V.
Thrombin or factor Xa cleave peptide bonds, as indicated by the arrows. The A1-A2 heavy chain and the A3-C1-C2 light chain form a calcium-ion dependent complex.[22]

Inactivation of factors Va and VIIIa

APC effectively degrades phospholipid-bound factors Va and VIIIa. In contrast, the native forms of the proteins are poor substrates for APC. The inactivation of factor Va takes place through the APC-mediated cleavage in the heavy chain of the molecule of three peptide bonds at Arg^{506}, Arg^{306} and Arg^{679} (Figure 6).[34]
Cleavage at Arg^{506} is needed for the efficient exposure of the cleavage sites at Arg^{306} and Arg^{679}. The lipid-dependent cleavage at Arg^{306} appears to be the major inactivating cleavage site and results in a loss of about 80% cofactor activity, whereas cleavage at Arg^{679} is lipid-independent and is responsible for the loss of most of the remaining cofactor activity.[34] Potential structural differences between platelet factor Va and plasma factor Va may influence the extent to which the cofactor is cleaved initially at Arg^{306}.[35] APC inactivates factor VIIIa by cleavages at Arg^{336}, Arg^{562} and Arg^{734}. The main loss of factor VIIIa cofactor activity is associated with the cleavage at Arg^{562}.[36]

Molecular explanation of APC resistance

The initial observation that normal factor V mixed with APC resistant plasma was able to correct the APC response in a dose-dependent manner, suggested to several independent research groups that APC resistance was due to a defect in the factor V molecule.[13,37,38] However, the precise molecular explanation was discovered first by a Dutch group led by R. Bertina.[13] The APC resistance phenotype in this seminal study was linked to a single-point mutation in the factor V gene, which substitutes G (codon CGA) with A (codon CAA) at nucleotide 1691 in exon 10.[13] This mutation replaces Arg (R) with Gln (Q) at position 506 in the factor V molecule, thus modifying one of the three APC cleavage sites (Figure 6).
The mutant FV:Q^{506} molecule expresses normal procoagulant activity when activated by thrombin or factor Xa, although its rate of inactivation is about 10-fold slower than that of normal factor Va.[39-42]

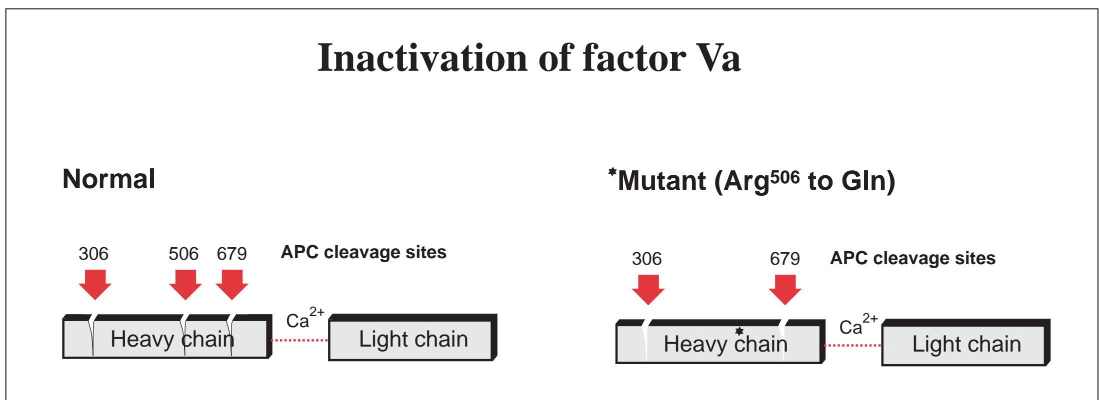


Figure 6. Inactivation of factor Va by APC.
APC inactivates membrane-bound factor Va through proteolytic cleavages at specific sites in the heavy chain. Cleavage in the light chain (not shown) appears to have no effect on the factor Va cofactor activity. APC resistance is mainly caused by a mutation, which substitutes Arg^{506} with Gln, making this site inappropriate for APC cleavage. The mutant factor Va molecule can still be inactivated at the remaining cleavage sites, although the inactivation rate is slower than that of normal factor Va. See main text for references.

This "resistance" to degradation by APC allows for a larger duration of thrombin generation, which is reflected by increased levels of prothrombin fragment 1+2, thrombin-antithrombin (TAT) complex and D-dimer.[43-46] Recent data also suggest that a reduced ability to slow down thrombin generation may stabilize a blood clot by weakening the profibrinolytic effect of APC.[48] An antifibrinolytic mechanism could thus be an additional factor contributing to the prothrombotic tendency observed in APC resistant patients.
The fact that mutant FVa:Q^{506} can still be inactivated by APC cleavage at Arg^{306} and Arg^{679} might account for the relatively mild hypercoagulable state observed in APC-resistant individuals and help explain why additional genetic and/or acquired risk factors are required for thrombosis to develop.[41]

Heterogeneous phenotype

The phenotype of APC resistance as determined by the classic APTT-based test is clearly heterogeneous (see Figure 8 and "normals") and several reports have shown that about 10% (range 4-20%) of APC resistant cases among Caucasians, do not involve the FV:Q^{506} mutation.[13,16,41,47]
The cause of this type of APC resistance is not known but may be the result of other genetic defects or of acquired APC resistance.
Analogous to the FV;Q^{506} mutation, APC resistance could be explained by mutations at the APC cleavage sites of factor VIII, for example a mutation at Arg^{336} or Arg^{562}. Although, to present date no such mutations have been found.[49]

Clinical aspects

Hypercoagulable state and thrombophilia

A hypercoagulable state is a condition that favors coagulation, as recognized by increased thrombin generation. Hypercoagulability can be due to a number of factors, which can be either inherited (primary) or acquired (secondary) (Table 2).
Thrombophilia is the clinical term for a hypercoagulable state that causes an increased tendency to thrombosis. Several genes have been implicated with inherited thrombophilia, although only factor V (APC resistance), antithrombin, protein C and protein S have been clearly linked to an increased risk of venous thromboembolism (Table 3).[7,50] Of these, APC resistance is the most common, both among patients and in the general population (Table 4).[9-12]

Diagnosing APC resistance

The development of a simple, APTT-based assay that measures the anticoagulant response in plasma to added purified APC, facilitated the characterization of the APC resistance phenotype.[8]
In the classic test kit, two APTT reactions are performed, one in the presence of a carefully-defined quantity of APC and the other in its absence.[51]

Primary hypercoagulable states
Inherited thrombophilia (Table 3)

Secondary hypercoagulable states
Advanced age
Heart disease
Immobility
Lupus anticoagulants
Malignancy
Obesity
Oral contraceptives
Pregnancy
Trauma and surgery
Varicose veins

Table 2.
Causes of hypercoagulability

Established:
APC resistance (factor V:Q[506])
Antithrombin deficiency
Protein C deficiency
Protein S deficiency

Non-established
Dysfibrinogenemia
Plasminogen deficiency
Elevated PAI-1
Heparin Cofactor II deficiency
Factor XII deficiency
Hyperhomocysteinemia
Elevated prothrombin levels

Table 3.
Causes of inherited thrombophilia

Genetic defect	General population	Unselected VT cases	Selected VT cases	No. of mutations
APC resistance	5%	20%	50%	1
Antithrombin deficiency	0.1%	1%	4%	>79
Protein C deficiency	0.2%	3%	5%	>160
Protein S deficiency	n.d	2%	5%	>13

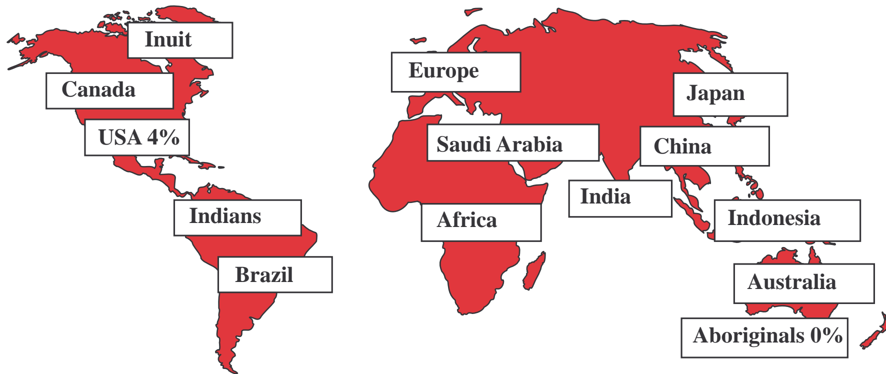
Table 4. Prevalences of inherited thrombophilia in various populations. Prevalences are estimates based on references. VT=venous thrombosis.[4,47,50]

The relationship between the two clotting times is expressed as a ratio, called the APC ratio. Healthy individuals have an APC ratio in the range 2-5, whereas APC-resistant individuals are recognized by an APC ratio below or equal to about 2. The precise cut-off for a diagnosis may vary slightly depending on the instrument type used as well as the individual condition of the instrument.[226-228] The phenotypic APC ratio reflects the severity of the hypercoagulable state and provides information on the thrombotic risk associated with inherited and possibly acquired APC resistance.
A modified APC resistance test (IL Test™ APC™ Resistance V), which exclusively detects factor V-related APC resistance is available, i.e. APC resistance due to the FV:Q^{506} mutation.[52]
The assay modification involves a predilution of plasma samples with an excess of stabilized factor V-deficient plasma (Factor V Reagent Plasma) containing a heparin antagonist.
Since the predilution with Factor V Reagent Plasma normalizes the basal APTT reaction, it safely allows for APC resistance-testing of plasma from patients on oral anticoagulant or heparin therapy.
It also produces a complete discrimination for FV:Q^{506}, which makes the modified assay highly suitable for factor V mutation screening.
Test results are expressed as an APC-V ratio calculated in the same way as the APC ratio obtained from the classic test.
The APC-V ratio provides genotypic information concerning factor V and is generally lower than the APC ratio for the same sample, regardless of the instrument used. Typical APC-V ratio ranges for different factor V genotypes are 2.2 - 3.2 for normal FV:R^{506}, 1.4 - 1.8 for heterozygous FV:Q^{506}, and 1.1 - 1.3 for homozygous FV:Q^{506}.

World distribution of APC resistance and FV:Q^{506}

An overall cumulative analysis of different patient groups with venous thromboembolism gave a prevalence of APC resistance of about 20% (range 0-64%, see Tables 5 and 6).[9-12,53-69]
The variation in the prevalence of APC resistance between clinical studies are related mainly to differences in selection criteria and the uneven distribution of the FV:Q^{506} allele in the general population in different parts of the world (Figure 7).
The highest prevalences of APC resistance and the FV:Q^{506} mutation have been found among healthy controls in several European populations of Caucasian origin, most notably in Cypriot Greek (13%),[77] Swedish (11%),[78] French (10%),[75] British (9%),[77] German (9%)[72] and Dutch (5%)[10] people.
In contrast, the mutation appears to be rare among Chinese[70,77,83] and absent among Japanese[60,76,79] and Africans (Negroid).[77,92] This could account for the relatively low incidence of venous thromboembolism reported in these ethnic populations.[71,77]
The average FV:Q^{506} carrier frequency among healthy European controls is about 5%. Approximately 0.1% of a Caucasian population can be expected to be homozygous for this mutation.

Distribution of the FV:Q506 mutation in the world population



1) FV:Q506 mutation determined with a DNA-based assay.

Country/region	FV:Q506 n/n test.	%	Ref.
Australia, Aborigines	0/73	0	77
Brazil, Indians	0/83	0	81
Brazil, Blacks	137	0.7	81
Brazil	2/100	2	57
Canada	19/356	5.3	82
China, Han	1/618	0.2	83
China, Hong Kong	0/293	0	84
Finland	4/137	2.9	93
France, Paris	5/229	2.2	74
France, Strasbourg	17/176	9.7	75
Germany, South	14/180	7.8	85
Germany, North-East	58/814	7.1	86
Germany, North-West	18/190	9.5	72
Greece	17/203	8.4	83
Greek Cypriots	25/187	13	77
Greenland, Inuit	0/133	0	87
Iceland	3/96	3.1	77
India, North	3/70	4.3	88
Indonesia, Sumatra	0/105	0	77
Italy	9/344	2.6	89
Japan	0/192	0	76
Jamaica	0/91	0	77
Kenya	0/60	0	77
Mongolians	0/36	0	77
Netherlands	14/474	3.0	54
Papua New Guinea	0/95	0	77
Peru, Indians	0/19	0	77
Saudi Arabia	5/200	2.5	90
Senegal	0/96	0	77
Sweden	11/101	11	78
Taiwan, Aborigines	0/83	0	77
UK	21/237	8.9	77
UK	5/144	3.5	73
USA, Blacks	3/214	1.4	91
USA	42/704	6.0	69
Zambia	0/95	0	77

2) APC resistance phenotype determined with the original APC resistance test.

Country	APC resistant n/n test.	%	ref.
Austria	1/50	2	55
France	1/75	1.3	12
France	2/50	4	56
Italy	20/1212	1.2	64*
Japan	3/291	1.0	59
Netherlands	14/301	4.6	10
Poland	1/110	0.9	62*
Spain	3/107	2.8	65
Sweden	9/130	6.9	11
USA	2/39	5.1	67

* abstract

3) APC resistance due to FV:Q506 in USA determined with the modified APC resistance test.

USA ethnic origin	US pop. millions	% FV:Q506 mutation
Caucasians	185	5.3
Hispanic	25.8	2.2
Afroamericans	32.7	1.4
Native Indians	2.1	1.3
Asians	7.5	0.46

Data was presented by Dr. J Miletich at the 42nd ISTH Subcommitte Meeting in Barcelona in June 1996. In total 2242 plasma samples were investigated from individuals of different ethnic origin representing a typical US population.

Figure 7. Reported prevalences of APC resistance and the factor V:Q^{506} mutation in the global population.

Country	Venous thrombosis n pts.	n. FV:Q506		References
Austria	40	7	(17%)	Halbmayer et al[55]
France	175	29	(17%)	Trossaërt et al[56]
France	48	9	(19%)	Cadroy et al[12]
France	183	24	(13%)	Samaha et al[53]
Italy	20	2	(10%)	*Tosetto et al[64]
Italy	118	33	(28%)	De Stefano et al[68]
India	28	6	(21%)	Pati et al[63]
Japan	43	5	(12%)	Kambayashi et al[59]
Japan	22	4	(18%)	Fujimura et al[60]
Netherlands	301	64	(21%)	Koster et al[10]
Poland	72	9	(12%)	*Lopaciuk et al[62]
Sweden	104	34	(33%)	Svensson et al[11]
Spain	72	3	(4%)	*Borell et al[65]
Spain	176	14	(8%)	Ortega et al[61]
USA	25	16	(64%)	Griffin et al[9]
USA	37	9	(24%)	Chusman et al[67]
Total	1,464	268	(18%)	

Table 5. Prevalence of APC resistance phenotype in patients with venous thrombosis.

* abstract paper

Country	Venous thrombosis n pts.	n. FV:Q506		References
USA	121	14	(11%)	Ridker et al[69]
Japan	22	0	(0%)	Fujuimura et al[60]
France	87	14	(16%)	Alhenc-Gelas et al[66]
Netherlands	301	53	(17%)	Bertina et al[13]
Netherlands	27	10	(37%)	Voorberg et al[14]
Netherlands	471	92	(19%)	Rosendaal et al[54]
Brazil	40	8	(20%)	Arruda et al[57]
Australia	45	12	(26%)	Ma et al[58]
Total	1,114	203	(18%)	

Table 6. Prevalence of the FV:Q^{506} mutation in patients with venous thrombosis according to gene analysis.

The origin of the FV:Q^{506} mutation

Several investigators have suggested that the high prevalence of the FV:Q^{506} mutation could be due to the evolutionary advantage it would confer, which has helped to maintain and spread the mutation.[71,77,81] It is possible that the selective disadvantage of a life-long hypercoagulable state could be balanced by, for example, the protection against excessive blood loss during delivery and menstruation. The selective risk of the FV:Q^{506} mutation would also be of less historical importance, as people in ancient times were not exposed to modern risk factors for thrombosis (e.g. oral contraceptives, surgery, sedentary life-style etc.). The high allelic frequency of FV:Q^{506} in Caucasian populations and its linkage to different polymorphisms in the factor V gene, supports the hypothesis that the mutation occurred as a single event in the ancient European population.[13,74,75,94] The time of this event would be approximately 30,000 years ago, i.e. after the diversion of Africans from non-Africans (140,000 years ago) and after the diversion of Caucasoid from Mongolic populations (70,000 years ago), but before the diversion of Caucasian subpopulations. However, the possibility of recurrent mutations in other races is not altogether unlikely, since the FV:Q^{506} mutation involves a CpG dinuclotide, which is an established hot-spot for mutation.[88]

Clinical manifestations of APC resistance

The clinical manifestations of inherited, heterozygous protein defects in familial thrombophilia involving antithrombin, protein C, protein S and factor V (APC resistance) are fairly similar. Mutations affecting the qualitative or quantitative function of these proteins often result in venous thromboembolism at a young age (before the age of 45 years) and are followed by a tendency towards recurrent thrombotic episodes.[50] The most common manifestation of APC

resistance is deep venous thrombosis (DVT) of the lower limbs, with or without pulmonary embolism, which accounts for about 90% of all thrombotic episodes.[50,53,96] Other, less frequent, manifestations include superficial thrombophlebitis[96] and unusual sites for thrombosis such as the mesenteric,[96] central retinal,[97-101] portal,[102,103] internal jugular,[104] and cerebral veins.[105,106] The relative risk of DVT for carriers of the FV:Q^{506} mutation compared to non-carriers has been estimated to increase 7-fold for heterozygotes (single defect) and 80-fold for homozygotes (double defect).[54] A similarly increased risk of pulmonary embolism has been observed by some investigators,[216] although not by others, who observed a significantly lower increased risk.[217,218] Since aging itself is a risk factor for thrombosis, the absolute risk increases with age.[69] The risk of recurrent thrombosis in carriers of the FV:Q^{506} mutation has been shown to increase 4-fold,[53,223] but again this has not been confirmed by others.[224] The penetrance of clinical manifestations among APC-resistant individuals is variable, and a majority of heterozygous carriers of FV:Q^{506} actually never experience any symptoms. In fact, not even homozygous carriers will necessarily be affected by thrombosis during their lifetime.[43] These facts illustrate that FV:Q^{506} is a mild risk factor per se and that the probability of APC-resistant individuals developing thrombosis is dependent on the coexistence of other risk factors. About 60% of APC-resistant patients have their first thrombotic event in combination with pregnancy, oral contraceptives, trauma or surgery.[16,53,96] Because of the high prevalence of APC resistance in the general population, its combination with other genetic defects is not unusual.

A wide range of disorders have been reported in connection with APC resistance, implicating its part in the development of thrombotic complications. These include the Budd-Chiari syndrome,[107-109] nephrotic syndrome,[110] leg ulcers,[111,112] heparin-induced thrombocytopenia,[113] priapism,[114] polycythemia vera,[115] essential thrombocythemia,[115] child-thrombosis,[116-119] cutaneous skin necrosis,[120,121] neonatal purpura fulminans,[165] acute lymphoblastic leukemia,[166] and systemic sclerosis.[170]

Arterial thromboembolism

Although there is a clear link between APC resistance due to the FV:Q^{506} mutation and venous thrombosis, the same link to arterial thrombosis has been enigmatic. Several studies have reported the presence of APC resistance in young stroke patients, suggesting that it contributes to the pathophysiology.[55,122-126,163] Halbmayer et al found that 20% (6 out of 30) of young Austrian stroke patients were APC-resistant according to the classic APC resistance test.[55] However, other studies of stroke patients, involving both functional and DNA-based assays, found no increased prevalence of either the APC resistance phenotype or FV:Q^{506} mutation compared to healthy controls (Table 7).[67,69,93,133-135,169]

Country	Arterial n pts.	thrombosis n. FV:Q^{506}	Controls	References
Australia	222	11 (5.0%)	(4%)	van Bockxmeer et al[129]
Finland	358	16 (4.5%)	(2.9%)	Kontula et al[93]
Germany	224	21 (9.4%)	(4.1%)	März et al[132]
Sweden	101	18 (18%)	(11%)	Holm et al[78]
UK	386	16 (5.6%)	(5.6%)	Catton et al [133]
USA	583	32 (5.5%)	(5.5%)	Ridker et al[69]

Table 7. Prevalence of the FV:Q^{506} mutation in patients with arterial thrombosis compared with controls according to gene analysis.

The possible correlation between the FV:Q[506] mutation and the risk of ischemic heart disease, particularly myocardial infarction, has also been investigated. With the exception of two papers,[78,132] the general conclusion is that the FV:Q[506] mutation is not an important risk factor for arterial thrombosis in heterozygotes,[67,69,72,75,93,127-131,164] but may have a role in homozygotes.[126,137] Some papers have appeared recently which may help to clarify the situation. These suggest that acquired (or inherited) APC resistance, independent of the FV:Q[506] mutation, may indeed be an important risk factor for arterial thrombosis.[139-141] These observations are most interesting and they call for an evaluation in different patient groups, using the classic APC resistance test, to explore whether the APC ratio may be predictive for both venous and arterial thrombotic events.

APC ratios in thrombophilic families

Zöller et al investigated 50 thrombosis-prone Swedish families with APC resistance (Figure 8).[16] In three of these families the FV:Q[506] mutation was not present, suggesting another, as yet unidentified, cause of APC resistance. In total, 308 family members were investigated; 146 normal, 144 heterozygotes and 18 homozygotes.
APC ratios were low in all the homozygous and most of the heterozygous cases. APC ratios in the APC-resistant individuals who lacked the mutation ranged from 1.3 to 2.0. Heterozygotes with a history of thrombosis had significantly lower APC ratios than those without thrombosis and none of the heterozygotes with APC ratios >2.0 had experienced thrombosis. Moreover, relatives without the mutation but with thrombotic histories had on average lower APC ratios than those without thrombosis. Significant differences in thrombosis-free survival curves and APC ratios were observed between the groups (Figure 9), thus confirming that APC resistance is an important risk factor for thrombosis. By the age of 33, 8% of the normals, 20% of the heterozygotes and 40% of homozygotes had experienced manifestations of venous thrombosis. The average age for the first thrombotic event was 25 (range 10 to 40 years) for homozygotes and 36 (range 18 to 71 years) for heterozygotes. In the thrombosis-prone families the observed incidence of thrombosis was higher than expected, suggesting that these families have been affected by additional genetic defects.

Polygenetic familial thrombophilia

The underlying cause of familial thrombophilia has long been considered to be single-gene defects. However, the notion that this idea was too simple has been reported repeatedly in recent years, particularly in connection with protein C deficiency.[142-143] It was found that the same type of mutation could affect different families differently, giving rise to the idea that several genetic risk factors in combination are usually needed for clinical manifestations to occur. Strong evidence supporting this view came with the discovery of APC resistance. Its high prevalence in the general population and the observation that individuals with combinations of inherited risk factors (e.g. FV:Q[506] and protein C deficiency) suffer more severely from thrombosis, and at a younger age, than those with single defects, has led to the idea of familial thrombophilia being primarily a polygenetic syndrome.[47,144,145,168] Most studies have confirmed this by showing a relatively high incidence of FV:Q[506] among clinically symptomatic probands in thrombophilic families with protein C,[146-147] protein S,[148,149,167] or antithrombin deficiency (Table 8).[150,151]

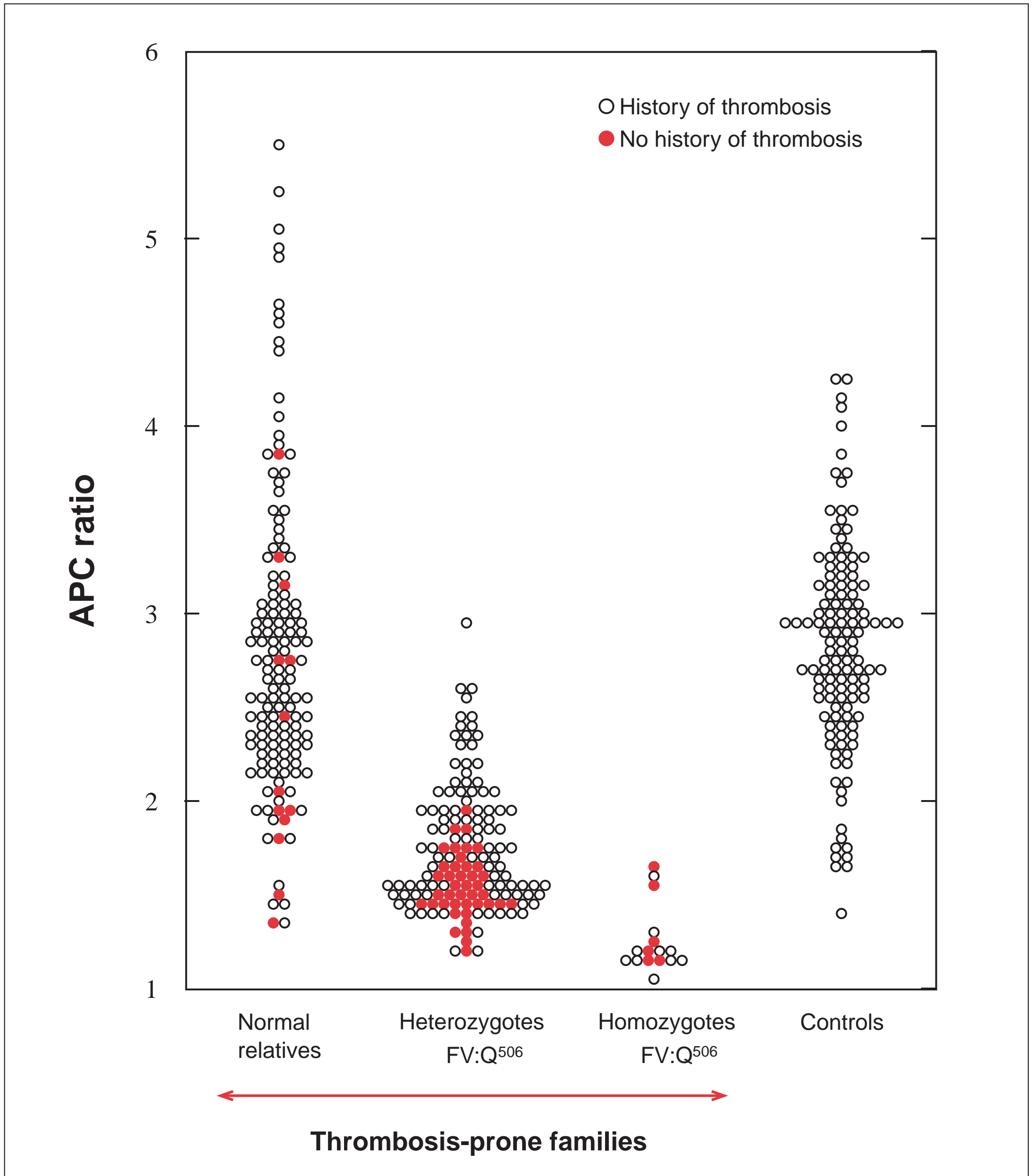


Figure 8. Relationship between APC ratios and the FV:Q506 allele in families with APC resistance. APC ratios in non-anticoagulated carriers of the FV:Q506 mutation and in family members without the mutation (normals), compared with unrelated healthy controls. The APC ratios were determined by the original APC resistance test method. Using a cut-off value of 2.0, the sensitivity and specificity for the FV:Q506 allele would be 85% and 87%, respectively. APC-ratios (mean±SD) in normals 2.8±0.8, heterozygotes 1.7±0.3, homozygotes 1.3±0.2, and controls 2.8±0.6. Reproduced by permission of Zöller et al and The American Society for Clinical Investigation.[16]

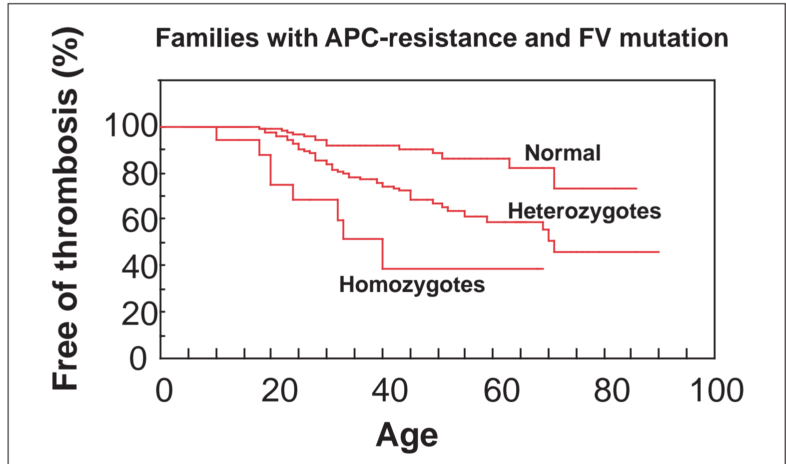


Figure 9. Thrombosis-free survival curves for different FV:Q506 genotypes. The probability of being free from thrombotic events at a certain age in family members without the FV:Q506 mutation, compared with family members with the mutation. At the age of 33, 8% of normals, 20% of heterozygotes and 40% of homozygotes had had venous thrombotic events. Reproduced by permission of Zöller et al and The American Society for Clinical Investigation.[16]

One hit	Two hits	Ref.
20% FV:Q506, 54% AT def.	92% FV:Q506 + AT def.	150
13% FV:Q506, 31% PC def.	73% FV:Q506 + PC def.	147
19% FV:Q506, 19% PS def.	72% FV:Q506 + PS def.	148

Table 8. The incidence of thrombotic episodes in thrombophilic families, related to the number of genetic defects ('hits'). AT=antithrombin, PC=protein C, PS=protein S.

The observed frequency variation of the FV:Q506 mutation among these probands is probably related to population differences.[152-153] Other interesting candidates for polygenetic familial thrombophilia involving FV:Q506, include hyperhomocysteinemia (due to either cystathione-b-synthase or methylenetetrahydrofolate reductase deficiency),[154-155] familial antiphospholipid syndrome,[156] heparin cofactor II deficiency,[157] plasminogen deficiency,[158] and possibly also elevated prothrombin levels due to a 20210 AG genotype in the prothrombin gene.[281] The influence of FV:Q506 on inherited bleeding disorders has also been studied. A possible moderation of the hemophilia A (factor VIII deficiency) phenotype has been observed in some cases,[159] although this was not seen in others.[160] A more surprising influence of FV:Q506 is seen in cases of parahemophilia (factor V deficiency). Heterozygotes for this rare bleeding disorder are often asymptomatic, since they have one functional factor V allele that maintains adequate factor V levels in blood (about 50%). However, the coinheritance of a factor V deficiency mutation on one allele and FV:Q506 on the other leads to a severe APC resistance phenotype similar to the homozygous FV:Q506 state.[161-162]

APC resistance and circumstantial risk factors for thrombosis

Pregnancy

During normal pregnancy the plasma concentrations of several of the proteins involved in the hemostatic mechanism change towards a hypercoagulable state. Although these changes are of physiologic importance in minimizing the risk of blood loss at delivery, they also increase the risk of thrombotic complications. In developed countries the overall incidence of thrombosis has been reported to be around 0.09% during pregnancy, with the risk being two to three-fold increased during puerperium.[171] APC resistance appears to be an important risk factor for thrombosis in connection with pregnancy.[68,172-176] In two Swedish studies, 45-60% of women with a history of pregnancy-related thrombosis were found to be APC resistant.[172-173] Carriers of the FV:Q^{506} mutation appeared to be especially prone to developing thrombosis in early pregnancy and after delivery, compared to non-carriers of the mutation.[173] APC resistance also seems to be associated with an increased risk of second trimester miscarriage related to placental infarction.[177-178] In general, women have slightly lower APC ratios compared to men.[179] This difference becomes more pronounced during pregnancy and indeed a substantial proportion of pregnant women may even develop an acquired APC resistance.[180-183] Although it has been demonstrated that increased factor VIII levels lower the APC ratio,[184] and that increased factor VIII levels are common during pregnancy,[180-181] other hormonally-influenced factors probably contribute since the actual correlation between the APC ratio and the concentration of factor VIII is low (Figure 10). Until more is known about the mechanism and clinical relevance of acquired APC resistance, the effect of pregnancy should be taken into consideration when interpreting the APC ratio obtained using the classic APC resistance test. The detection of factor V-related APC resistance using the modified APC resistance test is straightforward, since predilution in factor V-deficient plasma eliminates any pregnancy-induced changes in the patient's plasma.[182]

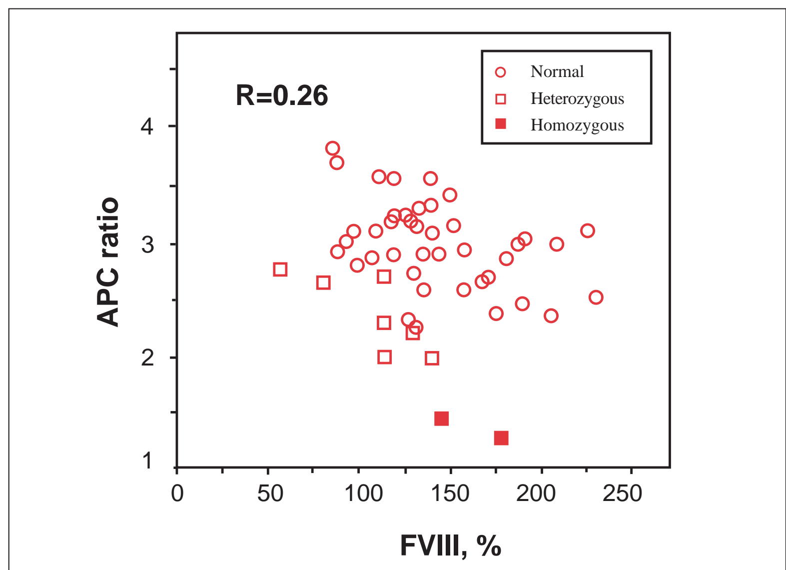


Figure 10. Correlation of factor VIII activity and the APC ratio from analysis with APC Resistance. Instrument ACL.

Oral contraceptives

The use of oral contraceptives (OCs) is a much-debated risk factor for thrombosis, associated with a two to nine-fold increase in the risk of thrombosis, depending on the active substance used, compared to non-users.[185,186] Similar to pregnancy, increased levels of fibrinogen and procoagulant factors, as well as reduced APC ratios and reduced levels of antithrombin and free protein S, have been reported in women using OCs.[179,187-190] For the majority these procoagulant changes are negligible, although for a small number of women with a genetic or acquired predisposition for thrombosis, the added prothrombotic influence of OCs may be sufficient to trigger a thrombotic event.[191]

The risk of venous thrombosis in OC users with APC resistance has been investigated by Vandenbroucke et al.[185] The risk of thrombosis among OC users in this study was shown to increase 4-fold when compared to women not using OCs (baseline risk about 0.01% annually). Women heterozygous for the FV:Q^{506} mutation who did not use OCs showed an 8-fold increase in thrombotic risk, whereas a 35-fold increase in risk was shown in OC users heterozygous for the mutation. Thus, the joint effect of the two risk factors appears to be multiplicative. Through a similar effect, the increase in risk for homozygotes using OCs is several 100-fold (Table 9).[54,185]

Clinical studies also demonstrate that OCs leads to an unacceptably high risk of venous thrombosis in females with homozygous FV:Q^{506}.[192]

Hellgren et al investigated 28 women with a history of thrombosis in connection with OCs and they found that nine (32%) of the women had APC ratios <2.0, indicating APC resistance.[172] None of the women investigated were using OCs at the time the blood samples were taken and in only one of nine patients with APC resistance was an additional risk factor for thrombosis identified.

Genotype	Non user -	OCs (overall)	OCs (levonorgestrel)	OCs (desogestrel)
Normal	1	4	4	9
Heterozygotes	8	32	15	48
Homozygotes	80	320	150	480

Table 9. Relative risks associated with oral contraceptives (OCs) and the factor V:Q^{506} mutation.[185-186]

Considering the high prevalence of APC resistance in the general population, a substantial number of women are placed at a higher risk by using OCs. In North America alone there are about 10 million OC users.[193]

Assuming the allele frequency for FV:Q^{506} is 0.025, then roughly 500,000 would be heterozygous and 6,000 homozygous for the mutation.

Since the annual thrombotic risk is about 0.3% for heterozygotes and 3% for homozygotes using OCs, it can be calculated that around 2,000 thrombosis cases per year in North America are caused by the combination of APC resistance and OCs.

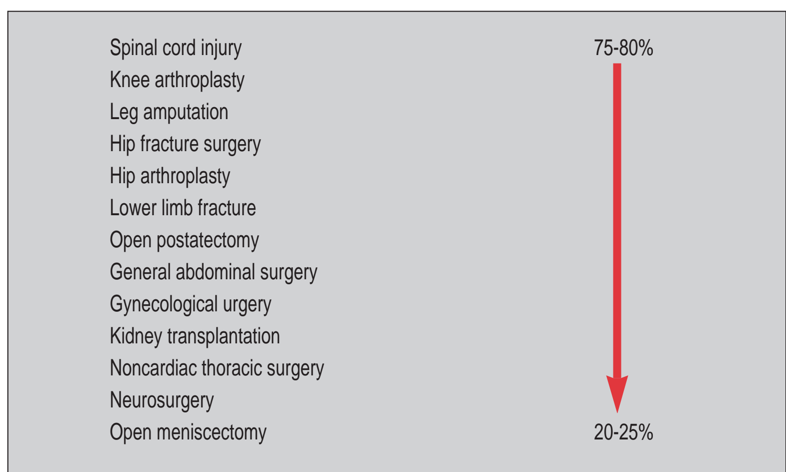
This figure should be compared to the number of thrombosis cases in OC users without the mutation, which is about 4,000 cases per year.

Thus, in the presence of both risk factors (OCs + mutation), venous thrombosis appears to develop in a substantial number of women who would never have had thrombosis in the presence of either risk factor alone.

It can be estimated that 1-2% of all cases of venous thrombosis caused by OCs will have a fatal outcome due to pulmonary embolism. Overall, it would appear that a significant number of women with APC resistance should avoid OCs. Physicians who prescribe OCs generally interview the woman about her family history to learn whether there are any cases of thrombosis. However, since APC resistance is only a mild risk factor per se, with many families being asymptomatic, it would not be suspected in the vast majority of women from interview-data alone. This raises the question as to whether all women should be screened for the FV:Q^{506} mutation prior to prescribing OCs.[190,194-197]
The potential reduction in mortality and morbidity cases due to thromboembolism caused by the pill must, however, be weighed against the risk of thrombosis in connection with unwanted pregnancy.[198]

Surgery

Patients undergoing major trauma or surgery, in particular orthopedic or abdominal surgery, generally run a high risk of experiencing hromboembolic events (Table 10). The precise risk for an individual is not only determined by the type, extent, and duration of the surgical trauma, but also by the accumulation of predisposing risk factors, e.g. advanced age, morbidity, malignancy, obesity, varicose veins, prior history of thrombosis, and thrombophilia.[199] There are several indications that APC resistance is a predisposing risk factor for postoperative thrombosis.
Lindblad et al anecdotally reported three cases of APC resistance and thrombosis in connection with peripheral vascular reconstruction.[136]
One of these cases, had severe APC resistance and later developed a fatal arterial occlusion. Ouriel et al investigated 76 patients (10 with APC resistance) with lower extremity occlusive disease who underwent infrainguinal bypass.[138] Six (60%) of the 10 patients with APC resistance had failed arterial reconstructions, versus 16 (24%) of 66 patients without APC resistance. In a Danish study it was shown that 30% of patients who developed deep venous thrombosis after knee arthroplasty were APC resistant.[200]



Spinal cord injury	75-80%
Knee arthroplasty	
Leg amputation	
Hip fracture surgery	
Hip arthroplasty	
Lower limb fracture	
Open postatectomy	
General abdominal surgery	
Gynecological urgery	
Kidney transplantation	
Noncardiac thoracic surgery	
Neurosurgery	
Open meniscectomy	20-25%

Table 10. Risk groups in trauma and surgery in order of decreasing frequency of DVT using no thromboprophylaxis.[199]

Although the studies and number of patients are small they raise the question of whether it may be justified to screen for APC resistance preoperatively and whether APC-resistant individuals require more aggressive or prolonged anticoagulant thromboprophylaxis.
The latter approach, using one-month, post-operative prophylaxis with a low molecular weight heparin, was recently shown to be beneficial when applied generally to patients undergoing hip replacement.[201-202]

Antiphospholipid antibodies

Lupus anticoagulant and anticardiolipin antibodies are acquired antiphosholipid antibodies (APAs) which were initially described in patients with systemic lupus erythematosus (SLE).
Recently, the interest in these types of antibodies, which are more correctly described as antibodies directed against phospholipid-protein complexes, has focused mainly on their strong correlation with thrombotic disease, thrombocytopenia and recurrent fetal loss. The association of one of these complications and the presence of APAs has been termed the antiphospholipid syndrome.[203] The mechanism underlying the syndrome is at present unknown, although various interferences in the protein C pathway have been suggested as being a possible pathogenic mechanism for thrombosis.[204] One such interference may be the selective blocking of APC on phospholipid surfaces by specific types of APAs, leading to an acquired APC resistance.[41,205-207] Several reports have also demonstrated acquired APC resistance to be a relatively common feature in patients with the antiphospholipid syndrome,[206,208-211] even when the uncertainties of lowered APC ratios caused by APA-induced prolongation of the basal APTT were considered.[206] At present, there is no clear clinical evidence to support a higher thrombotic risk among patients with the antiphospholipid syndrome and acquired APC resistance.[210,214,215]
Furthermore, the FV:Q^{506} mutation does not seem to be over-represented in patients with the syndrome compared with healthy controls.[210-212]

When to test for APC resistance phenotype and FV:Q^{506} genotype

Up to now, the classic APC resistance test has been used mainly as a simple screening assay for the FV:Q^{506} mutation. However, several limitations have been reported for this application. Firstly, this test has a sensitivity and specificity for the mutation which is usually in the range 75-90%.[219,220]
Secondly, the test is only reliable if the basal APTT-reaction is within the normal range,[221] which therefore disqualifies many APC-resistant patients on anticoagulant therapy from testing.[222]
As the classic APC resistance test stands today, it is not recommended for FV:Q^{506} mutation screening if the modified test can be performed instead (see below). The question that arises is when should the classic test be used? Interestingly, the APC ratio is not a one-variable reflection of the APC response in vivo, but rather an indication of an anticoagulant system response that in general seems to decrease under a hypercoagulable state.[139] A poor anticoagulant response to APC, independent of the FV:Q^{506} mutation, may therefore be a thrombotic risk factor or risk marker in a wide range of conditions, including venous thromboembolism,[16,139] antiphospholipid protein syndrome,[209] second trimester miscarriage,[177] systemic sclerosis,[170] ischemic stroke,[140,141] occlusion after vascular surgery,[138] pregnancy[174] and the use of oral contraceptives.[188-190] Future studies will clarify whether the phenotypic APC ratio obtained from the classic test may serve as a predictor of venous and arterial thrombotic events.

Screening for FV:Q506

The modification of the APC resistance test, in which sample plasma is prediluted in an excess of stabilized factor V-deficient plasma, improves the discrimination for the FV:Q506 mutation dramatically.[229-236]

The modification also allows for the testing of patients on anticoagulant therapy and strongly reduces the influence of preanalytical variables such as storage and plasma handling. Evaluation of this test using different categories of clinical samples showed that the specificity and sensitivity for the presence of the FV:Q506 mutation was 100% (Figure 11).[278]

The robustness and simple format of the modified test, together with its high discrimination between normal and mutated factor V genotypes, makes it an ideal tool for FV:Q506 mutation screening.

An important point to recognize when using the modified test for screening, is that by prediluting the sample plasma in factor V-deficient plasma phenotypic expressions related to factors other than factor V are lost.

In order to obtain the optimal information for thrombophilia diagnosis, one approach could be to analyze each plasma sample using both the classic and the modified APC resistance test.[233] This strategy identifies APC-resistant patients, both with and without the FV:Q506 mutation, and keeps the need for confirmatory gene analysis to a minimum.

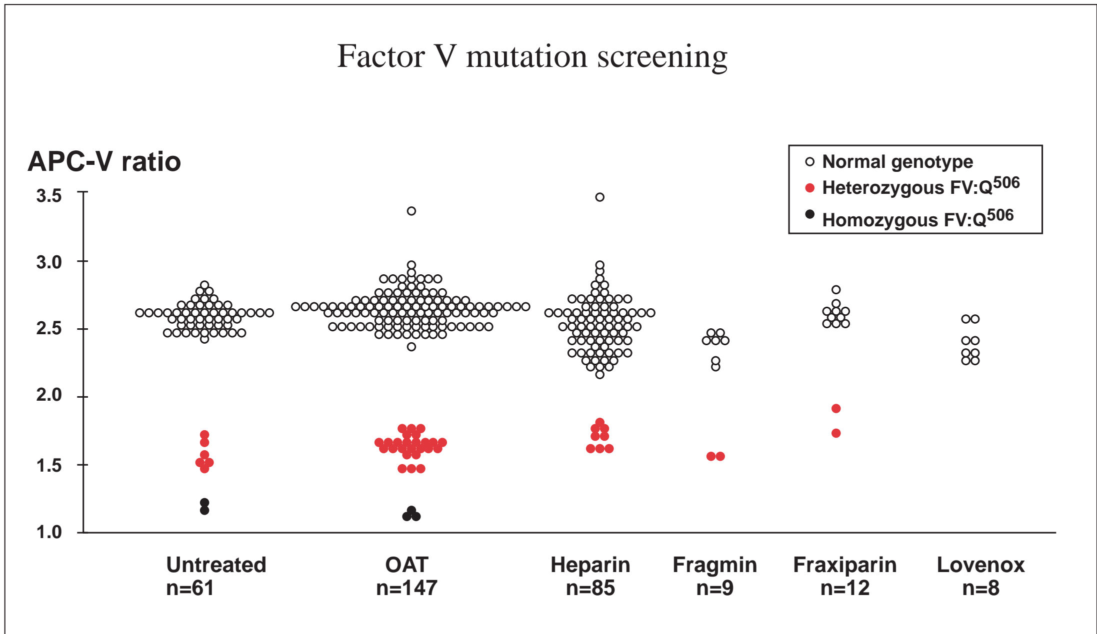


Figure 11. Relationship between APC-V ratios and the factor V:Q506 allele in various sample categories as determined using the APC Resistance V method. Complete discrimination was obtained between the normal and mutated factor V genotypes. Instrument: ACL. OAT= oral anticoagulant therapy (vitamin K-antagonist).[278]

Management of APC resistant patients

A potentially large number of thrombosis-prone individuals with APC resistance will most likely be identified in the near future.
This of course raises the question of patient management.
At present there are no established guidelines for managing thrombotic patients with APC resistance, although it is generally agreed that they should be treated in the same way as the patients with antithrombin, protein S and protein C deficiency.[249] An acute thrombotic episode should be treated conventionally with heparin for 5 to 10 days, followed by an oral anticoagulant (warfarin) within 24 hours to produce an International Normalized Ratio (INR) of 2.0 to 3.0.
Patients should be given general advice on how to minimize the thrombotic risk, including dietary advice, cessation of smoking and avoiding long periods of immobility.
Thrombophilic women should avoid oral contraceptives and all patients should be notified that they may require special treatment prior to surgical, medical or obstetric procedures that carry an increased thrombotic risk.
Women with a history of thrombosis and who have a known genetic defect may require anticoagulation throughout pregnancy, preferably by using dose-adjusted subcutaneous heparin.[68]
As a rule, all thrombophilic women should be offered thromboprophylaxis in conjunction with delivery and puerperium. Women with no previous history of thrombosis, but who do have a genetic defect, require individual consideration.
Homozygous and heterozygous patients with a second anticoagulant defect should be given preventive therapy in all risk situations and long-term therapy should be considered if thrombosis is recurrent.
When a woman has experienced venous thrombosis after oral contraceptive use it is recommended that she is tested for the possible presence of the FV:Q^{506} mutation. If she is heterozygous for the mutation she should be carefully informed about her thrombotic risk and counseled about the type of contraceptive she should use in the future.
The mere fact that she has had a thrombosis indicates that the risk in her case is significant. If she is homozygous for the mutation, she should be strongly recommended to discontinue the use of oral contraceptives.
As in any investigation of a young patient with venous thrombosis and APC resistance, it is also recommended to search for other causes of inherited thrombophilia.
Because only a proportion of the subjects with a heterozygous defect develop thrombosis, it is unjustifiable to put symptom-free family members on antithrombotic prophylaxis solely on the basis of having a genetic defect. It is, however, essential that these asymptomatic family members are carefully counseled with respect to their defect and offered short-term prophylaxis in special situations where there is an extra risk of thrombosis.

Laboratory analysis of APC resistance

The laboratory analysis of poor anticoagulant response to APC is based on an activated partial thromboplastin time (APTT) assay, which is modified in the manner described by Dahlbäck et al.[8]
The standard APTT reaction begins by adding a surface-activating agent (e.g. kaolin, silica, ellagic acid) and a phospholipid preparation to citrated, platelet-poor plasma, thereby achieving maximum activation of factor XI. The plasma is then recalcified in order to activate the coagulation cascade and the time for clot formation is measured.
The name APTT originates from the fact that the phospholipid reagents were originally derived from a lipid-enriched extract of complete thromboplastin (now called tissue factor), hence the term partial thromboplastin. The APTT reaction is dependent on factors of both the intrinsic and the common pathway of the coagulation system (Figure 12).

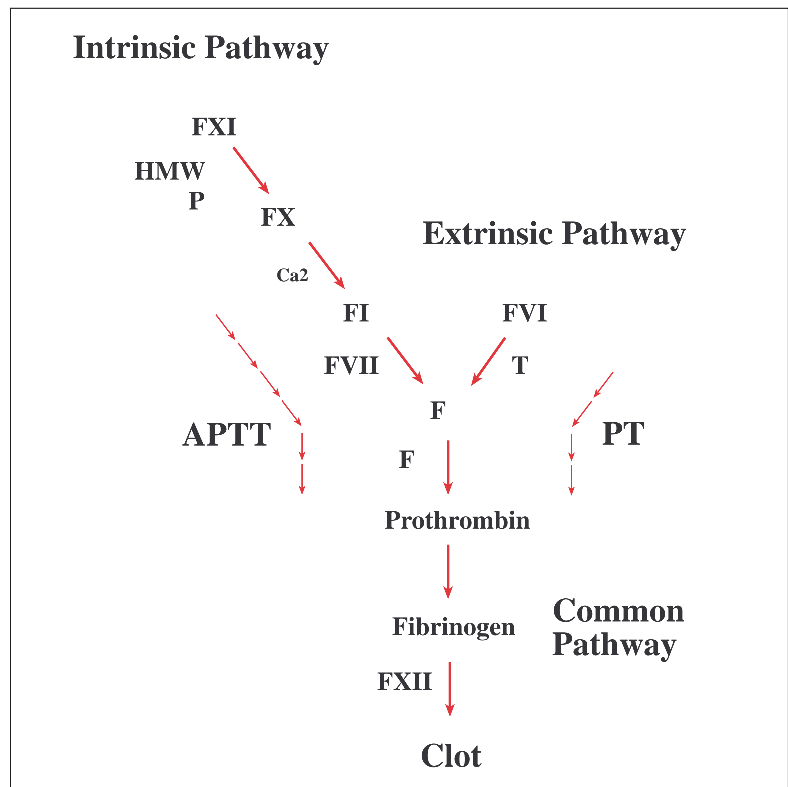


Figure 12. Coagulation pathways.
The intrinsic and extrinsic pathways can be tested independently using the activated partial thromboplastin time (APTT) and prothrombin time (PT) assay methods, respectively.

The classic APC resistance test

In the classic APC resistance test, two APTT reactions are performed, one in the presence of a carefully defined amount of APC and the other in its absence. The result can be calculated either as a prolongation of the clotting time in presence of APC or as the ratio between the clotting times in the presence and absence of APC. Use of the APC ratio for expression of the results is preferred, mainly due to its lower susceptibility to preanalytical sample handling variations.[225]
As for any APTT-based assay, it is important to follow a standardized procedure for blood sampling and storage. The APTT reaction without the addition of APC should be within the normal range (25-40 sec) in order to obtain valid APC ratios.[221] This means that plasma from patients undergoing therapies causing deficiencies in clotting factors, for example treatment with warfarin or heparin, will not allow for reliable APC resistance analysis. Anticoagulant therapies must therefore, if possible, be discontinued for at least one week to allow re-establishment of the baseline prothrombin level. An alternative method in the case of heparin therapy is to neutralize heparin in the sample using a heparin antagonist.[244]

Instrument effects

The APC ratios obtained from the analysis, of plasmas from healthy individuals on different coagulation instruments is typically within the range 2-5. The precise cut-off value for a diagnosis may vary slightly due to the instrumentation and the condition of the specific instrument.[226-228]
In general, coagulation instruments with a turbidimetric or photometric clot detection principle offer better discrimination than instruments using an electromechanical detection principle.[225,227]

The normal APC ratio range

Since the cut-off ratio for an abnormal APC response may vary slightly between laboratories and instruments it is recommended that laboratories establish their own normal range.
The normal range can be determined by measuring the APC ratios from at least 50 healthy non-APC resistant individuals in the age range 20-65 years and calculating the median APC ratio. The APC ratio cut-off is then obtained by multiplying the median value by either 0.75 (median above 3) or by 0.8 (median below 3). Alternatively, the cut-off can be calculated as the mean minus two standard deviations.If it is not possible to perform factor V genotyping in the determination of the 50 healthy non-APC resistant individuals, a practical approach is to exclude the lowest 10% and highest 10% of the APC ratios before calculating the APC cut-off ratio.

Normalized APC ratio

In addition to the instrument effect on the APC ratio, different sources of APTT reagents as well as different batches of the same APTT reagent may show varying sensitivity to APC.[225,277,247] In order to compare results from different laboratories accurately it may be advantageous to present APC ratios after having normalized them against a pooled normal plasma, PNP (APC ratio/PNP-APC ratio).[225,277]
The properties of the PNP are important to consider as mixing studies of different plasmas show that a 10% contribution or more of APC-resistant plasma in the PNP greatly affects the normalized APC ratios obtained.[221]

Effect of plasma preparation and handling

To ensure reliable results it is necessary to obtain proper control of preanalytical variables.

Thus, blood samples must be centrifuged thoroughly and the plasma removed carefully in order to minimize the number of platelets in the sample.[240-243] The main reason for this being that freeze-thaw cycles reduce the APC ratios in the presence of platelets due to rupture of platelet membranes leading to the exposure of procoagulant phospholipids and platelet-bound factor V. As a rule, samples should be handled in the same way as the controls used to establish the normal range, i.e. "like should be compared to like".[234]

A routine single centrifugation of the blood sample at 2,000 x g for 20 minutes will normally be adequate to obtain platelet-poor plasma (<1% normal platelet count).[221]

Centrifugation should take place at room temperature in order to avoid cold activation since this may eventually lead to the activation of factors VIII and V. Careful separation of plasma from platelets is achieved by leaving 0.5-1.0 cm of plasma above the cell layer. If plasma is to be frozen, it should be frozen rapidly in volumes of ≤ 1ml, at -20 °C or below.

Antiphospholipid antibodies

The presence of acquired antiphospholipid antibodies, such as lupus anticoagulant, is a relatively common finding in thrombophilic plasma. Because the antibodies react with anionic phospholipids they may give rise to a prolonged clotting time and thus may influence the APC ratio (decreased ratio).[237]

It has been observed that the effect of lupus anticoagulants can be surmounted by the addition of excess phospholipid, however further studies are needed to confirm this observation.[239]

Influence of factors V and VIII

Mixing experiments of different plasmas have demonstrated that changes in factor V levels between 12-100% do not have any significant effect on the APC ratio. [245]

Very high factor VIII levels, for example in connection with pregnancy and inflammatory states, have been reported to lower the APC ratio,[183,184,246] although the actual correlation between factor VIII activity and the APC ratio appears to be weak (Figure 10).

Influence of factors II, IX, X, protein S and protein C

Prothrombin and factor X concentrations below 50% tend to produce higher APC ratios. In contrast, the APC ratio is not influenced to any great extent by variations in factor IX and protein S concentrations down to 30% of normal.[225] Variations in plasma levels of protein C have no influence on the APC ratio since a standardized amount of exogenous APC is added in the assay. On the other hand, it should be noted that the factor V:Q[506] mutation may influence certain clotting-based functional protein S and protein C assays leading to the incorrect diagnosis of APC resistance as either protein S or protein C type II deficiency.[250-251]

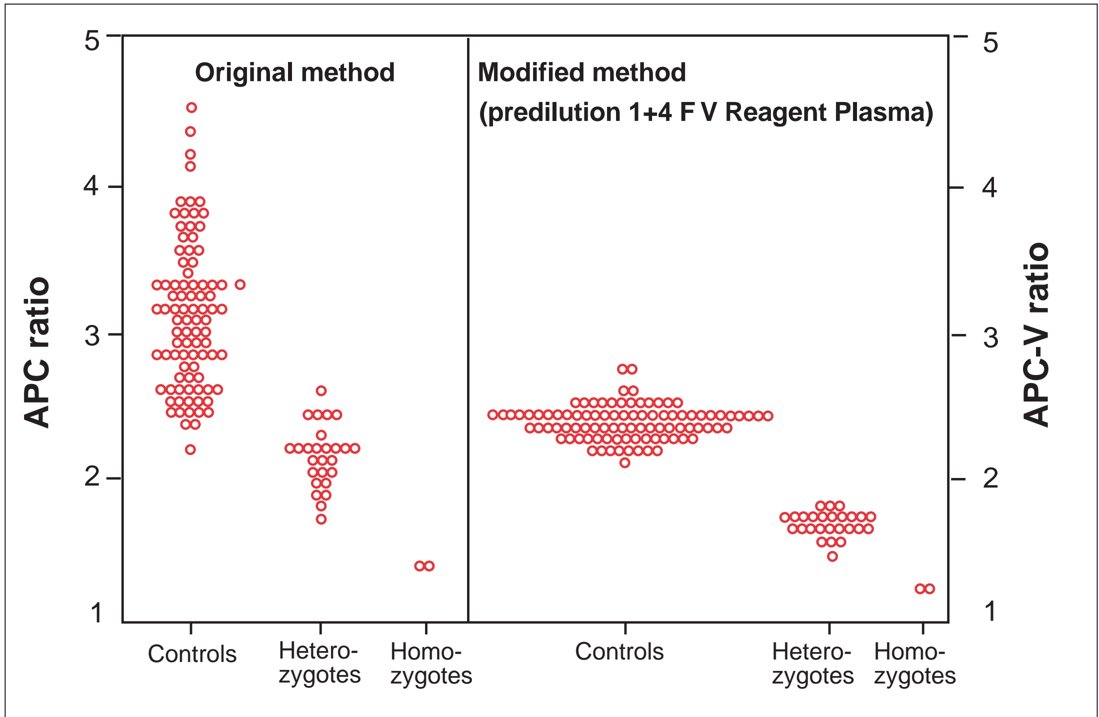


Figure 13. Comparison of APC ratios obtained from the classic APC resistance test, with APC-V ratios obtained from the modified test method. Both these methods complement each other.
The original method detects thrombosis-prone individuals with APC resistance due to the FV:Q[506] mutation, unknown mutations or acquired factors and indicates the severity of their hypercoagulable state.
The modified method provides a high discrimination for the factor V:Q[506] mutation and allows for analysis of plasma from patients on OAT or heparin therapy. The same individuals were tested in both groups.
Instrument ACL.

The modified APC resistance test

A modification of the classic APC resistance test has been described in which the sample plasma is prediluted with an excess of factor V-deficient plasma.[229-232] The predilution stage normalizes the concentration of plasma proteins involved in the formation and regulation of thrombin, except for factor V, resulting in an improved discrimination for the factor V:Q[506] mutation (Figure 13).[234-236]
Because prediluted plasma samples obtained from patients undergoing oral anticoagulant therapy have an APTT reaction within the normal range independent of treatment intensity, it is now possible to test for APC resistance due to factor V mutation in a large group of patients previously disqualified from APC resistance testing.
The inclusion of a heparin antagonist such as Polybrene® in the factor V deficiency plasma also makes it possible to analyze samples with heparin levels ≤ 1 IU/ml (unfractionated and LMW preparations).[278]
The use of a stabilized factor V-deficient prediluent (F V Reagent Plasma) in the modified test, strongly reduces the influence of pre-analytical variations such as plasma handling and storage.

Furthermore, no significant difference between fresh and frozen plasma is obtained and platelet counts up to 15,000/μL are easily tolerated.[279] Predilution (1+4) reduces most sources of interference, although it cannot be excluded that the analysis of plasma from patients with antiphospholipid antibodies may result in an abnormal APTT.
In such cases, increasing the dilution factor may correct the test result (e.g. 1+9 or 1+19). In neonates and infants (< 6 months of age) a predilution factor of 1+9 is needed because of their special hemostatic condition.[248]

Alternative functional assays for APC resistance

The present detailed understanding of the mechanism behind APC resistance has produced a number of alternative assay concepts for APC resistance (Table 11).[252-259] A novel chromogenic APC resistance assay is presented in Figure 14.[280] The assay is based on the addition of factor Xa to diluted test plasma in the presence of calcium ions, phospholipids and APC. Presence of the factor V:Q[506] mutation decreases the rate of inactivation of factor Va by APC and allows for appreciable thrombin generation. This is measured by the hydrolysis of a chromogenic thrombin substrate. Preliminary results show complete concordance between the chromogenic assay and the modified APC resistance test.[280]

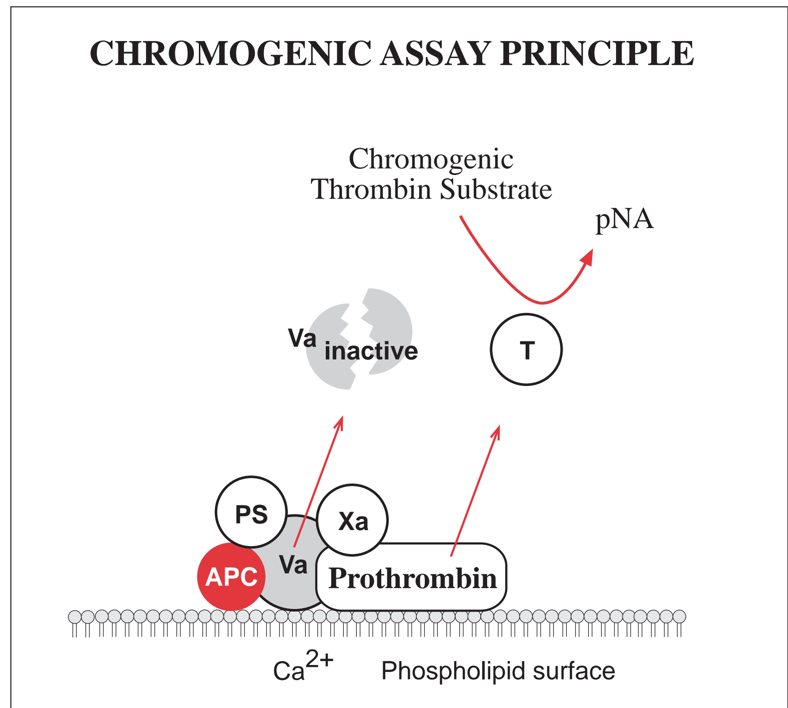


Figure 14. Chromogenic APC resistance assay. Endogenous factor V is activated by thrombin. In competing reactions, factor Va then serves as a cofactor to factor Xa or as a substrate to APC. Presence of the factor V:Q[506] mutation decreases the rate of inactivation of factor Va by APC and allows for appreciable thrombin generation. The amount of pNA formed by cleavage of the chromogenic substrate is measured at 405 nm.

Different assay concepts for APC resistance
APTT ± APC (± FV def. plasma)
APTT ± Protac (± FV def. plasma)
RVV ± APC
RVV ± Protac
FXa ± APC
Tissue factor/FV def. plasma ± APC
Chromogenic using a FXa substrate
Chromogenic using a thrombin substrate

Table 11. Overview of assay methods for APC resistance. (±APC: test with and without added APC; Protac: protein C activator; RVV: factor X activator; FV def.: factor V deficient.

PCR-based assays for FV:Q^{506}

Identification of a mutation is generally accomplished by amplification of genomic material containing the mutation followed by a mutation detection procedure. Amplification is achieved by the polymerase chain reaction (PCR) technique using either DNA or mRNA as a template.[274,275] The key components in a standard PCR include a pair of oligonucleotide primers (around 20 bases long), the DNA building blocks; deoxyribonucleoside triphosphates, and a heat-resistant DNA polymerase (e.g. Taq). These components are added to a closed test tube containing the template DNA. A temperature-driven cyclic process is then initiated, consisting of three stages: 1) DNA strand separation, 2) Annealing of primers, and 3) DNA synthesis (Figure 15). A central point of the PCR is that all new DNA strands serve as templates in successive cycles. The new DNA, consisting of the target sequence flanked by primers, increases exponentially in subsequent cycles. After n cycles the DNA target sequence is amplified 2n-fold. The amplification is a million-fold after 20 cycles and can be carried out in less than an hour.

In the work by Bertina el al,[13] in which the FV:Q^{506} mutation was first identified, the detection stage was performed by enzymatic digestion of the amplification product using the Mnl I restriction enzyme, followed by agarose gel electrophoresis. Presence of the mutation removes one of normally two cleavage sites for Mn I.

The resulting number and size of the fragments indicates whether the mutation is present or not.

Apart from methods involving direct sequencing of cDNA,[14] a vast number of PCR-based methods for the determination of the factor V:Q^{506} mutation have been published. These involve improvements of the original method,[262] , chemiluminescent detection,[261]whole blood PCR,[260] two-stage PCR using restriction enzymes Mnl I and Nla III,[264] use of allele-specific probes,[263,269,271] with the introduction of a Taq 1,[267,270] or a Hind III recognition site,[272] in combination with Elisa,[265] or with capillary electrophoresis.[266]

A PCR assay using allele-specific primers after microwave irradiation of leukocytes is a novel method which eliminates the need to extract the genomic DNA.[268] The determination of FV:Q^{506} can also be achieved by using the direct RNA amplification technique (NASBA), together with the detection procedure ELGA (enzyme-linked gel assay).[273]

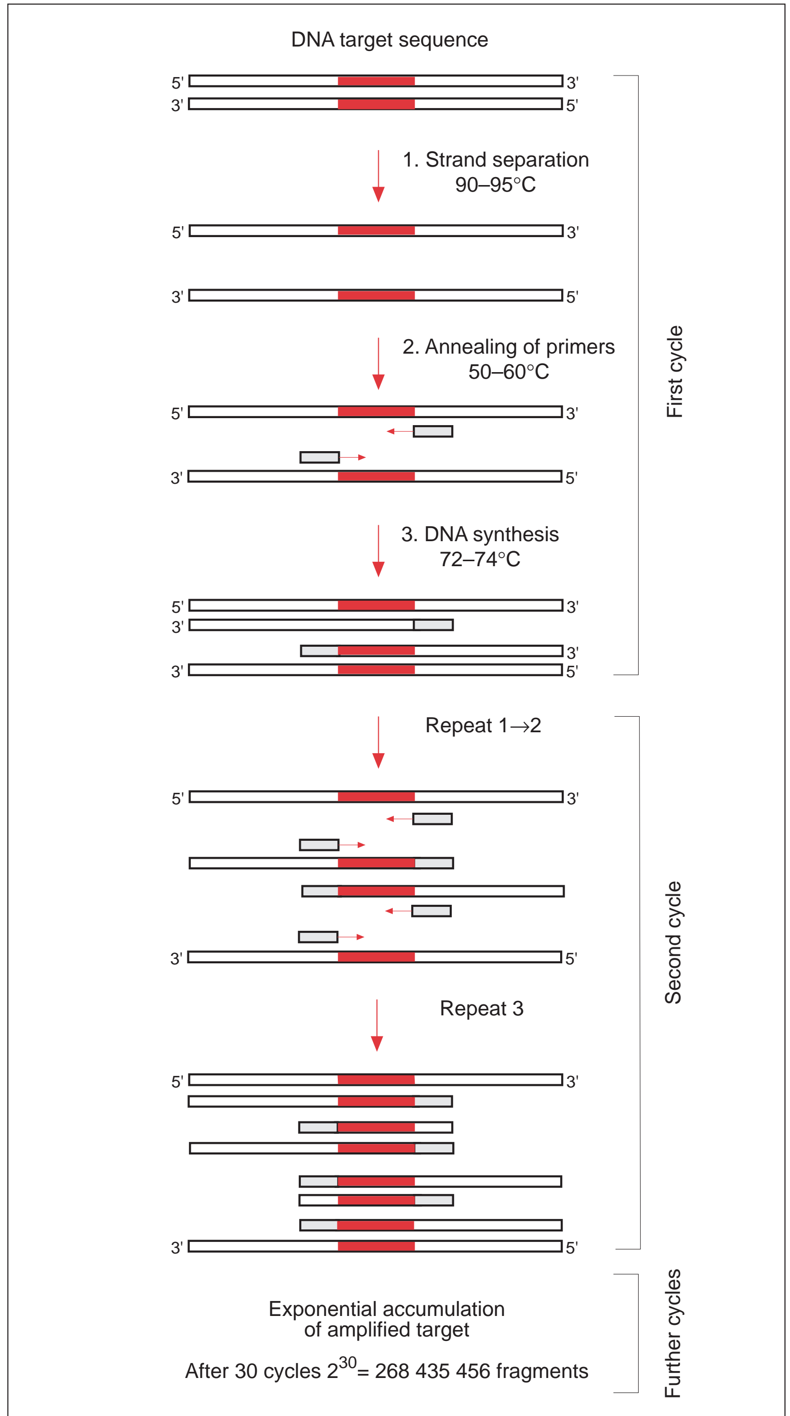


Figure 15. The general principle of the polymerase chain reaction (PCR).

IL Test™ APC™ Resistance V

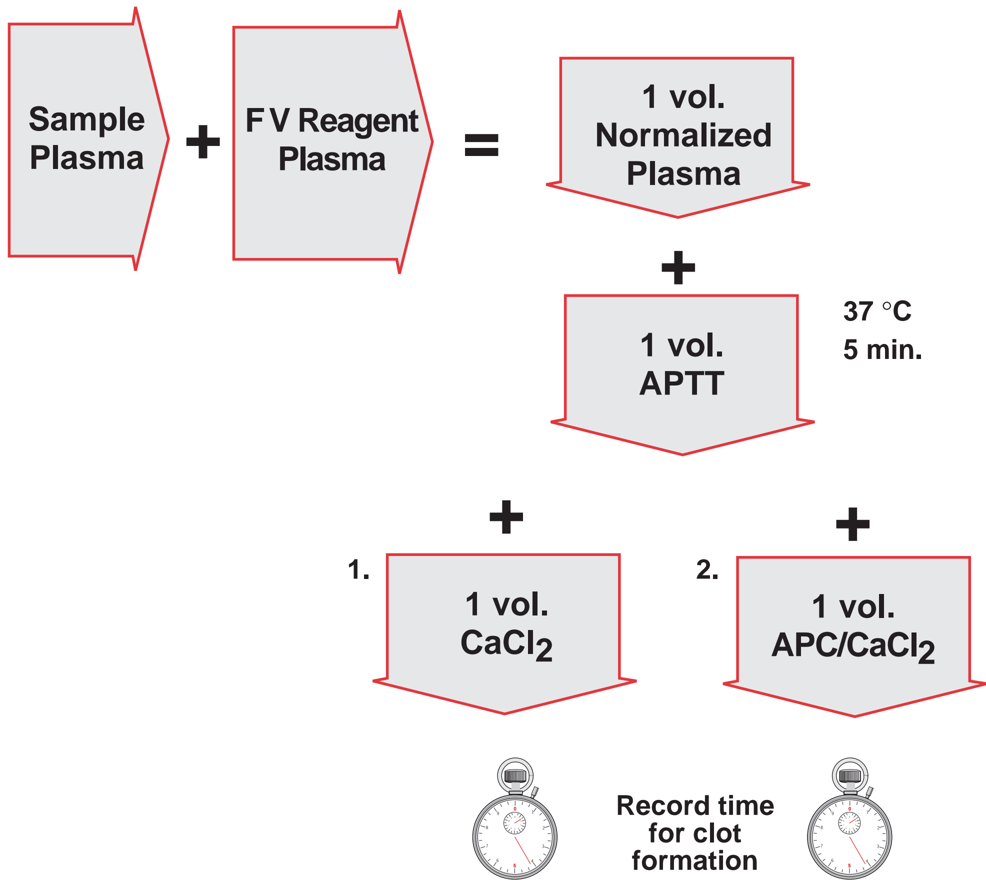
Principle

APC resistance is a blood coagulation abnormality characterized by an impaired anticoagulant response to activated protein C (APC).
Inherited resistance to APC is the most common pathogenic risk factor for venous thrombosis as it is found in approximately 20% of unselected patients with DVT and in up to 50% of cases with familial venous thrombophilia.
The APC resistance phenotype is, in more than 90% of cases, due to a mutation in the Factor V gene, resulting in a replacement of Arg ^{506} (R) with Gln (Q) in the Factor V protein. The selectivity for the Factor V:Q^{506} is increased by normalizing the concentrations of other plasma proteins involved in formation and regulation of thrombin. Performing the APTT-based APC resistance assay in the presence of an excess of a stabilized Factor V deficient plasma not only gives a 100% sensitivity for the FV: Q ^{506} mutation, but also allows for APC resistance analysis of plasma from patients on oral anticoagulant or heparin therapy and also strongly reduces the influence of pre-analytical variations such as plasma handling and storage.

Composition

The IL APC Resistance V kit (Cat. No 200087-00) consists of:	
APTT reagent	2 x 4 mL
Factor V Reagent Plasma	2 x 4 mL
APC/$CaCl_2$	2 x 2 mL
$CaCl_2$	2 x 2 mL
APC Control Plasma Level 1	2 x 1 mL
APC Control Plasma Level 2	2 x 0.5 mL
The IL Factor V Reagent Plasma - APC Resistance V kit (Cat. No 200088-00) consists of:	
Factor V Reagent Plasma	5 x 4 mL
Tests on the ACL system	
	60 samples* (120 determinations) per kit.
Tests on the ACL Futura system	
	80 samples (160 determinations) per kit.

*Note: Using one additional Factor V Reagent Plasma vial (Cat. No 200088-00)



$$\text{APC-V ratio} = \frac{\text{Clot time APC/CaCl}_2}{\text{Clot time CaCl}_2}$$

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A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 10, 3698-3703 (1996).

Appendix

Key reports in the history of APC resistance

1993

February
Proc Natl Acad Sci USA 90,1004-1008 (1993).
First report by Dahlbäck et al on the discovery of APC resistance and its association with familial thrombophilia.

July
First commercial test for APC resistance (Coatest® APC Resistance) available.

December
Lancet 342,1503-1506 (1993).
Leiden Thrombophilia study showing APC resistance as a major risk factor for venous thrombosis.

1994

February
N Engl J Med 330, 517-521 (1994).
Second major study reported by Svensson et al showing APC resistance to be the major cause of venous thrombosis.
Proc Natl Acad Sci USA 81, 1396-1400 (1994).
Article published by Dahlbäck and Hildebrand suggesting APC resistance to be caused by a genetic defect in the factor V gene.

May
Nature 369, 64-67 (1994).
Bertina et al demonstrate the association between a single point mutation in the factor V gene and APC resistance. The mutation results in the substitution of Arg^{506} with Gln, which causes impaired APC inactivation of factor Va.

July
J Biol Chem 269, 18735-18738 (1994).
Article by Shen et al which demonstrates that intact factor V and protein S are synergistic cofactors to APC in the degradation of factor VIIIa.

October
Lancet 344, 1162-1163 (1994).
Jorquera et al suggests a modification of the original APC resistance test involving the dilution of sample plasma in factor V-deficient plasma.

December
J Clin Inves 94, 2521-2524 (1994).
Zöller et al identifies the same $FV{:}Q^{506}$ mutation in 47 out of 50 thrombophilic families with APC resistance.

1995

J Biol Chem 270, 24, 4053-4057 (1995).
Kalafatis et al explain the mechanism by which the $FV{:}Q^{506}$ mutation leads to the APC phenotype. It is shown that the replacement of Arg^{506} by Gln delays the inactivation of factor Va.
N Engl J Med 332, 912-917 (1995).
Prospective study by Ridker et al of healthy men showing APC resistance to an important risk factor for venous thrombosis but not for myocardial infarction.
Lancet 346, 1133-1134 (1995).
Rees et al report on the distribution of $FV{:}Q^{506}$ in 24 populations.
The allele frequence was high among Europeans (4.4%), but low or absent among other ethnic groups.

Glossary

Allele. One of an array of possible mutational forms of a gene at a specific locus.
Amino acids. Basic building blocks of all proteins.
Antibody. A molecule produced by animals in response to antigen.
Anticoagulant therapy. Prevention of intravascular clotting by influencing the coagulation system with drugs.
Anticoagulants. Endogenous or exogenous clot inhibitor substances.
Antigen. A molecule which induces the formation of an antibody.
Antithrombin. The major serine protease inhibitor of thrombin.
APC resistance. Blood defect associated with thrombophilia characterized by a poor anticoagulant response to activated protein C (APC).
Assay sensitivity. True-positive results as a proportion of the total of true-positive and false-negative results TP/(TP+FN).
Assay specificity. True-negative results as a proportion of the total of true-negative and false-positive results TN/(TN+FP).
Autosome. A chromosome other than a sex chromosome.
Chromogenic substrates. Synthetic peptides that react with proteolytic enzymes producing a yellow color.
Chromosome. The darkly staining bodies within the cells made up of a large number of genes and a centromere region.
Complement system. A group of more than twenty serum proteins, some of which can be serially activated and participate in a cascade resulting in cell lysis.
Embolism. Obstruction or occlusion of a vessel by a transported clot.
Endothelium. Cells lining blood vessels and lymphatics which control the passage of materials into and out of the bloodstream.
Enzymes. A protein with catalytic power that can convert a molecule called the substrate into a new form called the product.
Familial. Affecting more members of the same family than can be accounted for by chance.
Fibrin. An elastic filamentous protein derived from fibrinogen by the action of thrombin, which releases fibrinopeptides A and B from fibrinogen.
Fibrinogen. Factor I; a globulin of the blood plasma that is converted into the coagulated protein, fibrin, by the action of thrombin in the presence of calcium ions.
Fibrinolysis. The hydrolysis of fibrin by plasmin.
Gene. The unit of inheritance, located at a specific region on the chromosome.
Genotype. The genetic constitution of an individual; may be used with respect to gene combination at one specified locus or with respect to any specified combination of loci.
Glycoprotein. One of a group of protein-carbohydrate compounds.
Hemostasis. Process which arrests the escape of blood from injured vessels. Vascular constriction, platelet aggregation and fibrin formation take part.
Hemostatic balance. Physiological balance between coagulation and fibrinolysis.
Heparin. Intrinsic substance produced in mast cells, chiefly acting as thrombin inhibitor by accelerating antithrombin activity.
Hepatocytes. Cells in the liver that are arranged in folded sheets. They produce many of the blood proteins.
Heterozygous. Having dissimilar alleles at one or more loci.
Homozygous. Condition of having identical alleles at one or more loci under consideration.
Intron. Gene segment between exons not encoding protein.

Locus. The position on a chromosome at which a particular gene is found.
Phenotype. In genetics, a group or category to which an individual may be assigned on the basis of one or more characteristics, observable clinically or by laboratory means, that reflect genetic variation or gene-environment interaction.
Phospholipid. A fat soluble substance containing phosphorus that is extracted from animal or vegetable cells by nonpolar solvents.
The basic constituents of biomembranes.
Platelets. A small disk-shaped blood cell, containing granules in the central part and peripherally, clear protoplasma, but no nucleus. Number: 200,000 to 300,000/µl.
Polymorphism. Occurence in more than one form in the same species.
Proteases, proteinases. Enzymes hydrolyzing native protein, or polypeptides, making internal cleavages.
Proteins. A class of macromolecules that are built from a repertoire of twenty amino acids.
Proteolysis. Enzymatic cleavage of protein.
Receptor. A cell surface molecule which binds specifically to particular proteins or peptides in the fluid phase.
Sepsis. A clinical syndrome of serious bacterial infection.
Serine protease. Proteolytic enzyme with a serine residue at its enzymatically active site.
Serum. The watery portion of blood remaining after fibrinogen has been removed from the plasma
Thrombi. Composed predominantly of fibrin and red cells, with variable amounts of platelets and leukocytes.
Thrombin. Active protease deriving from prothrombin (factor II). Induces conversion of fibrinogen into clot-forming fibrin monomers resulting in the coagulation of blood.
Thrombocyte. Blood platelet
Thrombocytopenia. A condition in which there is an abnormally small number of platelets in the circulating blood (usually less than 150,000/µl).
Thromboembolism. Refers to either thrombosis or embolism or a combination of both.
Thrombolytics. Biological and synthetic substances capable of activating the fibrinolytic system in plasma.
Thrombophilia. An inherited or acquired disorder in which there is a tendency to develop thrombosis.
Thrombosis. The formation of a thrombus (blood clot).
Thrombotic. Relating to, caused by, or characterized by thrombosis.
Zymogens. The enzymatically inactive precursors of proteolytic enzymes.

Notes

Patent

The APC resistance test method has been patented worldwide (US 5443960, EP 0608235) and licensed to Chromogenix AB (Dahlbäck patent), Möndal, Sweden.

Research on the method can be freely carried out, but any test for APC resistance done with 'in house" methods which are commercialized will infringe the patent.

IL Test™ APC™ Resistance V and IL Test™ Factor V Reagent are made in Sweden by Chromogenix AB, an IL Company

Instrumentation Laboratory

Part. No 98083-77