Diplomarbeit

Evaluation of two functional APC resistance assays in comparison with the genetic test method

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Simon Michaelis

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unter der Anleitung von

Univ. Prof. Dr. Harald Mangge

OA Dr. Florian Prüller

Graz,Datum……..

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ZUSAMMENFASSUNG

Einführung: APC Resistenz ist ein labormedizinischer Phänotyp, der durch eine herabgesetzte antikoagulatorische Reaktion auf Zugabe von aktiviertem Protein C (APC) charakterisiert ist. In den allermeisten Fällen ist sie bedingt durch eine Mutation auf dem Faktor V Gen (FV^{LEIDEN}), welche einen Austausch von Arginin zu Glutamin an Position 506 des Faktor V Moleküls zur Folge hat. Dadurch wird eine Schnittstelle von APC auf Faktor V eliminiert und die Inaktivierung von aktiviertem Faktor V wird gestört. Innerhalb der kaukasischen Bevölkerung ist die APC Resistenz die häufigste Ursache für eine genetisch bedingte Thrombophilie. Bei heterozygoten Trägern der FV^{LEIDEN} Mutation ist das thrombotische Risiko etwa 2- bis 8-fach erhöht, bei homozygoten 20- bis 50-fach. Daher ist die labormedizinische Bestimmung der APC Resistenz und der FV^{LEIDEN} Mutation von großer klinischer Bedeutung. Als Goldstandard gilt die direkte Erfassung der Mutation mittels PCR Methoden. Diese sind teuer und zeitintensiv, weshalb die Etablierung funktioneller APC Resistenz Tests angestrebt wird. Das Ziel dieser Studie ist die Evaluierung zweier funktioneller APC Resistenz Tests hinsichtlich ihrer diagnostischen Wertigkeit im Vergleich zur genetischen Bestimmungsmethode.

Material und Methoden: Die Daten von 3086 Patienten, die ein Thrombophilie Screening durchlaufen haben, wurden statistisch ausgewertet. Alle Patienten waren genetisch getestet worden, dazu entweder mit einem APTT-basierten funktionellen Test (Zweitgenerationentest), mit einem PT-basierten Test (Drittgenerationentest) oder mit beiden. Die Cut-off Werte, deskriptive Statistiken und diagnostische Parameter wurden berechnet.

Resultate: Der Drittgenerationentest zeigte hohe Sensitivität und Spezifität in der Unterscheidung zwischen Wildtyp-Trägern und Heterozygoten (0.997 und 1.0) und zwischen Heterozygoten und Homozygoten (1.0 und 0.994). Die Cut-off Werte waren klar definierbar. Der Zweitgenerationentest zeigte geringere diagnostische Sicherheit (Sensitivität 0.973, Spezifität 0.989).

Diskussion: Der Drittgenerationentest ist sehr gut als Screeningtest für die APC Resistenz geeignet und kann die Notwendigkeit für die kostspieligere genetische Testmethode reduzieren. Falsch klassifizierte Proben waren großteils zusätzlichen Mutationen auf dem Faktor V Gen zuzuordnen.

ABSTRACT

Introduction: APC resistance is a laboratory phenotype characterized by poor anticoagulant response to activated protein C (APC). The vast majority of cases is caused by the factor V Leiden (FV^{LEIDEN}) mutation leading to a substitution of arginine to glutamine at position 506 of the factor V molecule. Thus, an APC cleavage site is eliminated and inactivation of activated factor V is altered. Among Caucasians, APC resistance is the most common cause of familial thrombophilia, a genetically based disposition to venous thromboembolism. The thrombotic risk is increased about 2- to 8-fold for heterozygous FV^{LEIDEN} carriers, and 20- to 80-fold for homozygotes. Hence, testing for APC resistance and FV^{LEIDEN}, respectively, is of great clinical importance. The gold standards in testing are PCR methods directly determining the mutation. Due to their high expenses and long turnaround times, more cost-effective and easy applicable functional assays are sought to be implemented in the clinical routine. The aim of this study is the evaluation of the diagnostic usability of two commercially available functional APC resistance assays in comparison to the genetic test method.

Materials and Methods: Data from 3086 patients, who underwent thrombophilia screening, was statistically analyzed. All patients had been tested genetically, and either with a functional APTT-based test (2^{nd} generation test), a functional PT-based test (3^{rd} generation test), or both. The cut-off limits, descriptive statistical values and diagnostic values (sensitivity, specificity, positive predictive value, negative predictive value) were calculated.

Results: The 3^{rd} generation assay showed great sensitivity and specificity in discriminating between wild-type factor V individuals and heterozygotes in FV^{LEIDEN} (0.997 and 1.0, respectively) and between heterozygotes and homozygotes (1.0 and 0.994, respectively). The cut-off levels were clear with considerable gaps. The 2^{nd} generation assay showed lower discriminatory efficiency with sensitivity and specificity between wild-types and heterozygotes of 0.973 and 0.989, respectively.

Discussion: The 3^{rd} generation test is appropriate as a screening test for APC resistance helping to reduce the necessity of genetic testing. Misclassified samples were predominantly attributable to additional mutations on the factor V gene.

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1 INTRODUCTION

1.1 BLOOD COAGULATION

1.1.1 PHYSIOLOGY

The blood coagulation system is balanced between procoagulant and anticoagulant forces (see **Figure 1**). Normally, this balance tends to anticoagulation, thus it prevents from thrombosis. On sites of vascular injury the coagulation cascade is triggered and locally the system shifts to a procoagulant state leading to blood clotting. This mechanism prevents from bleeding.

The initial response to vascular injury is the formation of a platelet plug, which occludes the lesion (primary hemostasis). Concomitantly, the blood coagulation system is activated by the contact of blood with tissue factor. Tissue Factor is a transmembrane protein, which complexes with both zymogen and activated factor VII (FVII), which acts as a serine protease. The TF/FVII-complex promotes, together with Ca^{2+}, the conversion of factors IX and X to their active forms (FIXa and FXa) by specific cleavage. The action of factor VII is limited by the tissue factor pathway inhibitor (TFPI)(1)(2). Activated factor X is a part of the prothrombinase complex, which also includes activated factor V (FVa) as a cofactor. Bound on negatively charged phospholipids and in the presence of calcium ions, it promotes the activation of prothrombin to thrombin (3). Thrombin amplifies the coagulation cascade by activating factors V, VIII and XI. The activated factors VIII (FVIIIa) and IX (FIXa) constitute the tenase complex, which activates factor X to FXa (3). The assembly of the tenase and the prothrombinase complexes require negatively charged phospholipids, particularly phosphatiylserine and phosphatidylcholine. During the activation of platelets, phospholipids are converted from the inner to the outer layer of the membrane, so that the complexes are able to assemble on (4). Coagulation factors VII, IX, X and thrombin are interacting with phospholipids by γ-carboxyglutamic acid residues in the N-terminal part of the proteins. These resi-

dues are formed vitamin-K-dependently in a post-translational modifying step. Furthermore, it is essential for calcium binding (4). Antagonizing the effect of vitamin K, leads to a decline of coagulation activity. Anticoagulant drugs, like warfarin or phenprocoumon, are acting on this basis (3).

Another form of initiation of blood coagulation, beside the described tissue factor-dependent pathway, is the intrinsic pathway. Factor XI is activated by factor XII, high-molecular-weight kininogen and prekallikrein. The physiological role is not fully elucidated, because it is not involved in trauma mediated coagulation (3).

Central step of the coagulation cascade is the activation of prothrombin to thrombin, catalyzed by the prothrombinase complex. Thrombin acts as a procoagulant factor by activating fibrinogen to fibrin. Furthermore, it activates factor XIII to XIIIa, which is responsible for solid cross-linking of fibrin. Eventually, it leads to a compact fibrin clot, which covers the vascular injury. Also, thrombin exerts procoagulant functions by activating several factors, as mentioned above (5).

But thrombin also has anticoagulant properties. It activates the serine-protease inhibitor antithrombin. Antithrombin mostly inhibits free enzymes, whereas enzymes located in the tenase- or prothrombinase-complex are less likely to be targeted. The purpose of this specificity is to restrict the coagulation process on the site of injury (3).

The protein C anticoagulant pathway is activated by thrombin binding to thrombomodulin. Activated protein C acts as a modulator of the activity of the cofactors VIIIa and Va (6).

Dissolving of a fibrin clot is managed by plasmin, the most important fibrinolytic protease. Its zymogen, plasminogen, is circulating in the blood and can be activated by tissue plasminogen activator (tPA) or urokinase. The activity of tPA is enhanced significantly by the presence of fibrin. Furthermore, fibrin binds both plasminogen and tPA, resulting in generation of plasmin on the site of a fibrin clot. Fibrinolysis is regulated by inhibitors of plasmin, namely α_2-plasmin inhibitor and α_2-macroglobulin, and inhibitors of tPA, namely plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2) and C_1-esterase inhibitor (7).

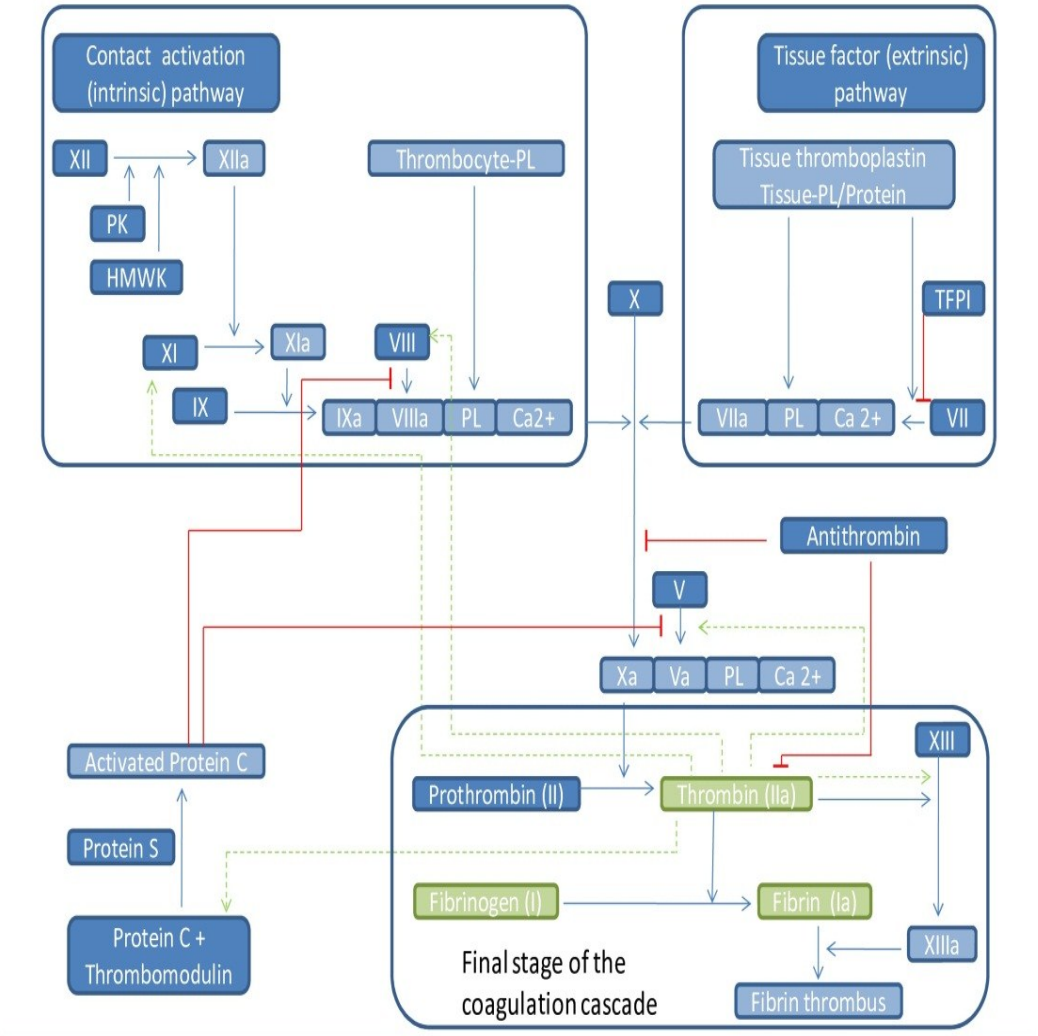

Figure 1 Coagulation cascade (8). HMWK High Molecular Weight Kininogen, PK Prekallikrein, PL Phospholipids, TFPI Tissue Factor Pathway Inhibitor,

1.1.2 FACTOR V

Coagulation factor V (FV) plays a crucial role in both, procoagulant and anticoagulant pathways. Disturbances in its function can lead to either bleeding or thrombosis. The most common genetic cause of thrombophilia, APC resistance, is mostly based on a mutation within the factor V gene (factor V^{LEIDEN}) (9). Thus, it is important to have a closer look on its structure and function.

Structure and synthesis

The FV gene is located on the long arm of chromosome 1. Its size is about 80kB and it consists of 25 exons and 24 introns (10) (11).

The factor V molecule is a single chain, 330kDa, glycoprotein. About 75-80% of factor V in blood circulates as free protein, the rest is stored in platelets (12). The factor V portion in the platelets is located in the α-granules, partially proteolyzed, partially complexed to multimerin1 (MMRN 1) (13).

Factor V is synthesized in the liver. It is not fully elucidated if there is biosynthesis of factor V in the megacaryocytes, the precursor cells of platelets. But there is high evidence, that a great amount of factor V in the platelets origins from blood plasma. It is supposed to be endocytosed and further processed within the platelets (13).

The amino acid sequence of the factor V molecule has similarities to those of coagulation factor VIII (FVIII) and ceruloplasmin, the major copper binding protein. It consists of 3 A domains, a connecting B domain, and two C domains. The arrangement is A_1-A_2-B-A_3-C_1-C_2. The activation of factor V to factor Va is accomplished by thrombin. It cleaves the molecule at certain cleavage sites and releases the B domain. Accordingly, the amino-terminal domains A_1-A_2 are referred to as heavy chain (105kDa) and the carboxy-terminal domains A_3-C_1-C_2 as light chain (74kDa). In the heavy chain region there is a 40% sequence identity between FV and FVIII, about the same between the light chain regions. There is no similarity in the connecting regions of the two molecules. Moreover, the A domain of both proteins has a ~30% structure identity to that of ceruloplasmin (14).

The molecule undergoes extensive post ribosomal processing. Especially the differences in glycosilation influence the cofactor activity of FV. Two different variants of FV, referred to as FV_1 and FV_2, are formed via different processing of the C_2 domain. The activated forms of these variants (FV_1a and FV_2a) differ in their ability to bind to negatively charged membranes. FV_2a has a much higher affinity to negatively charged phospholipids. Since the assembly of the prothrombinase complex relies on the binding on phospholipid membranes, FV_2a expresses a higher cofactor activity compared to FV_1a (15).

Procoagulant function

Coagulation factor V circulates in blood as a procofactor to FX. In this state it obtains only little of the possible cofactor activity. To exert its full activity as part of the prothrombinase complex it needs to be activated first. Thrombin is the key activator in this process. It cleaves FV at three sites within the B domain: Arginine^{709} (Arg^{709}), Arg^{1018} and Arg^{1545}.The cleavage at Arg^{1545} is crucial for the activation of factor V. It leads to the release of the connecting B domain and the assembly of FVa (see **Figure 2**) (16). The activated factor V is a heterodimer, composed of the heavy chain (A_1-A_2; 105 kDa) and the light chain (A_3-C_1-C_2; 74 kDa), and linked by a Ca^{2+} ion (17).

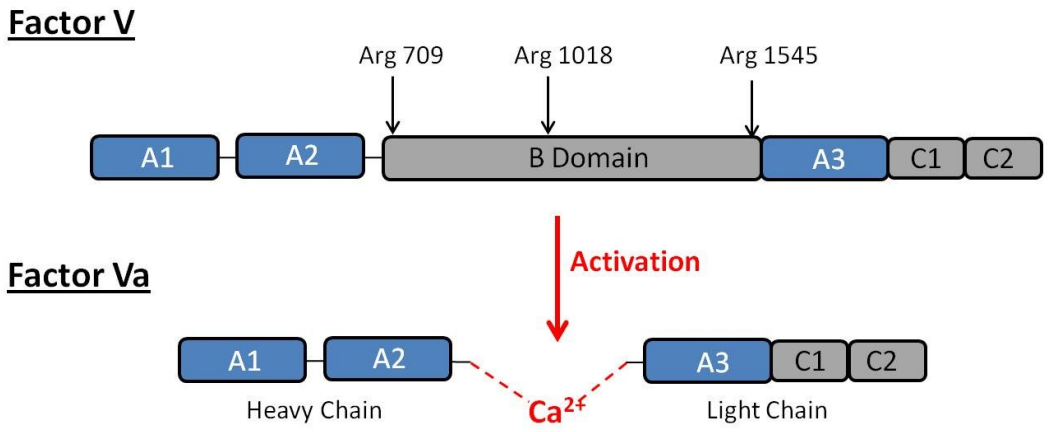

Figure 2 Organisation of the FV molecule (17). The configuration of the inactive form of factor V contains a connecting B domain, which is dissociated after activation of FV to FVa by limited proteolysis. The arrows pointing at the B domain are denoting the cleavage sites (Arg^{709}, Arg^{1018}, Arg^{1545}) for FV activation by thrombin (Adapted from 17).

Accordingly, FVa constitutes the prothrombinase complex, together with FXa, Ca^{2+} and phospholipids. The prothrombinase complex administers the activation of prothrombin to thrombin by sequential cleavage of the molecule at two sites (18). The phospholipids are provided by platelets at the site of injury. Activated platelets switch the inner layer of their membrane to the outside, so that phospholipids, mainly phosphatidylserine and phosphatidylcholine, are exposed to the plasma. On this negatively charged surface FVa and FXa constitute the prothrombinase complex (19). The interaction between FVa and phospholipids is due to both electrostatic and hydrophobic forces. The A_3 domain as well as the C_2 domain contains elements involved in binding to phospholipid membranes, respectively (20).

The mechanisms of FVa cofactor activity are still not fully elucidated, but there is evidence that FVa alters the FXa-phospholipid interaction. Activated factor X interacts with phospholipids in a calcium-dependent manner. In particular, the Gla domain of FXa is linked to negatively charged phosphate residues of phospholipids via Ca^{2+} ions. The Gla domain is rich in γ- carboxyglutamic acid, which is added to the molecule in a vitamin K- dependent posttranslational glycosilation step. Activated factor V accelerates the interaction between FXa and phospholipids about two orders of magnitude (21). Furthermore, there is evidence that FVa enhances the catalytic activity of FXa about 3,000-fold. As cause for this interaction the conformational alteration of the active site of FXa by FVa has been discussed (22). On the whole, FVa increases the rate of thrombin formation by FXa approximately five orders of magnitude, highlighting the importance of FVa as cofactor in the prothrombinase complex (21).

The inactivation of FVa is performed by activated protein C (APC). APC is an anticoagulatory protein, which acts as a serine protease. There are three cleavage sites identified, each within the heavy chain. They are located at Arg^{306}, Arg^{506} and Arg^{679}.The cleavage at Arg^{506} is the fastest reaction among them, but the cleavage at Arg^{306} seems to be the essential step for full inactivation (23). There are two possible ways of inactivation. The first one starts with cleavage of FVa at Arg^{506}. The peptide bond at Arg^{506} is the preferred site for initial cleavage of FVa. This step is then followed by cleavage at Arg^{306}, which leads to full inactivation of FVa. The second option is inactivation directly via Arg^{306}. Structurally, the complete loss of FVa activity is reached by the dissociation of the A_2 domain, which can only be accomplished by the cleavage at Arg^{306} (24).

Anticoagulant function

In addition to its procoagulant property, factor V also exerts an anticoagulant function. It can act, synergistically with protein S, as a cofactor to the inactivation of FVIIIa by APC (25). Only the cleavage of intact FV at the cleavage site at Arg^{506} by APC leads to the anticoagulatory active form of factor V. Once FV is activated to FVa, it loses its anticoagulant potential. This suggests an important role of the connecting B domain in the anticoagulant pathway, because it is dissociated in FVa. Namely, the C-terminal section of the B domain, which is neighboring the A_3

domain of FV, is essential for anticoagulant cofactor activity. As soon as FV is cleaved at Arg^{1545} the connection between B and A_3 is disrupted, thus making a turn to the anticoagulant properties impossible (26) (27).

These findings suggest a dual pathway in the activation of FV, either to a procoagulant or an anticoagulant protein (see **Figure 3)**. The local concentrations of enzymes, like thrombin, FXa or APC, are the main determinants in this system. High concentrations of thrombin or FXa account for a high probability of activating factor V to its procoagulant form. Contrariwise, locally high concentrations of APC are directing FV rather to its anticoagulant pathway. Accordingly, FV constitutes a sensor for procoagulant and anticoagulant influences (28).

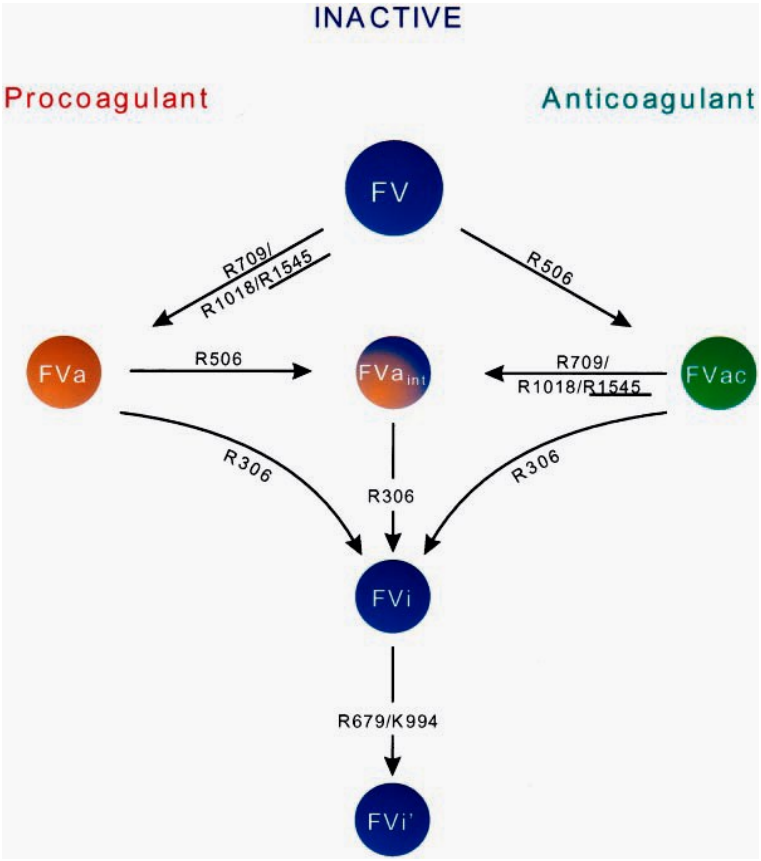

Figure 3 Dual pathway of factor V activation (28). Based on the local concentrations of both procoagulant and anticoagulant enzymes, inactive factor V (FV; blue) is either activated to FVa (red) or to its anticoagulant form FVac (green). Deactivation of FVa usually starts with the cleavage at Arg^{506}, which leads to a semiprocoagulant molecule (FVa_{int}; red and blue). Finally the cleavage at Arg^{306} leads to full inactivation (FVi). Further cleavage at Arg^{679} or Arg^{994} (FVi') has no influence on cofactor activity (27).

1.1.3 APC ANTICOAGULANT SYSTEM

The protein C anticoagulant pathway affects the coagulation system by regulating the activity of the cofactors FVa and FVIIIa. Protein C, a vitamin K-dependent protein, is the major component of the system. In its activated form, activated protein C (APC), it acts as a serine protease, which inactivates FVa and FVIIIa by cleaving at certain peptide bonds. The activity of APC is increased by the two cofactors protein S and anticoagulant factor V (6).

Protein C is activated by a molecular complex, containing thrombin and thrombomodulin (TM). Thrombomodulin is a transmembrane glycoprotein located on the vascular endothelium. When thrombin is binding to TM, it loses all procoagulant properties. In addition, it is converted to an activator of protein C (29).

The activation of protein C needs a negatively charged phospholipid surface, on which the γ-carboxy-glutamic acid (Gla) domains of protein C can bind. The attachment of the Gla domain in the posttranslational processing of the protein is Vitamin K-dependent. The Gla domain also binds the endothelial receptor of protein C (EPCR), which is important for the proper activation of protein C. The EPCR is responsible for the adjustment of protein C and the thrombin-TM complex on the surface of the endothelium. The activation of protein C is accelerated up to 20-fold by the presence of the EPCR (30) (31). Eventually, protein C is activated to APC via dissociation of an activation peptide and conversion of the serine protease domain to its active form (32).

In FVa, there are three APC-cleavage sites described, at Arg^{306}, Arg^{506} and Arg^{679}, as mentioned above. The cleavage at Arg^{506} is kinetically favored over the cleavage at Arg^{306}. Moreover, the Arg^{506} cleavage has a lower dependence on protein S and phospholipids (28). However, the cleavage at Arg^{306} is essential for the complete inactivation of FVa via dissociation of the A_2 domain (33).

Since FVIIIa has the same domain structure as FVa, it is inactivated via limited proteolysis and subsequent dissociation of the A_2 domain. In addition to protein S, FV acts as a cofactor to APC. There for, intact FV has to be cleaved at position Arg^{506} before, so that it can act as an anticoagulant cofactor (28).

The inhibition of APC is managed by protease inhibitors, e.g. protein C inhibitor (PCI), α_1-antitrypsin and α_2-macroglobulin (34).

The result of the APC anticoagulant pathway is the down-regulation of the tenase and the prothrombinase complex, respectively. This leads to less generation of thrombin and thus to an anticoagulant impact.

1.2 FAMILIAL THROMBOPHILIA

1.2.1 VENOUS THROMBOSIS

Venous thromboembolism (VTE), which primarily manifests as deep vein thrombosis (DVT) of the lower limbs or pulmonary embolism (PE), occurs with an incidence of 1 in 100,000 per year in young people up to 1% per year in people of old age (35). Moreover, venous thrombosis may result in major complications, like the post-thrombotic syndrome or even death, in the case of fatal PE. Owing to the importance of VTE for public health, interest for basic mechanisms of venous thrombosis has brought some insight into the development of the disorder.

In 1856, Virchow postulated three major causes of thrombosis, referred to as the *Triad of Virchow*: (1) damage of the vessel wall (2) stasis of the blood flow (3) alterations of the blood composition (36). This hypothesis is still valid, in terms of a basic model of thrombosis. Particularly the latter two points have been proven to play a crucial role in the development of thrombosis (37).

According to newer models, the pathogenesis of VTE is multifactorial. In the proposed model, a certain threshold of prothrombotic influences (risk factors) has to be surpassed, so that a thrombotic event may occur. Normally more than one risk factor is needed to elicit a thrombotic event. Moreover, the interaction between different risk factors can alter the risk of thrombosis significantly. Two classes of risk factors are distinguished: acquired and genetic risk factors (38).

Acquired factors predisposing to VTE are well known. They include older age, immobilization, trauma, surgery, pregnancy, puerperium, cancer, lupus anticoagulants and oral contraceptives (37)(39).

Familial thrombophilia is defined by genetically caused disposition to venous thromboembolism (VTE). The clinician's attention should focus on familial thrombophilia, if a patient presents with one or more of the characteristic features. Early onset of thrombosis, namely under the age of 45, recurrence of thrombotic events and a positive family history are signs of a possible genetic background. In the case of a female patient, recurrent obstetrical complications, like multiple abortions or stillbirth, are suspicious to an underlying disorder of blood clotting (39) (40) (41).

Numerous genetically determined alterations of the blood coagulation system have been discovered over the last 60 years. A handful of them have gained clinical importance, in terms of high prevalence among the general population and high incidence of VTE among the carriers of the genetically caused alterations, respectively. The most common disturbances associated with familial thrombophilia are APC resistance, prothrombin G20210A variant, antithrombin deficiency, deficiency of protein C, and protein S deficiency (41).

1.2.2 Resistance to activated protein C (APC resistance)

APC resistance is defined by a poor anticoagulant response to APC. It was first described by Dahlbäck et al in 1993 on the basis of a thrombophilic family (42). Later its relevance as the most common genetic risk factor for venous thrombosis has been elucidated (43) (44).

Genetic background

About 90 to 95% of all cases of APC resistance are caused by the FV Leiden mutation (FV^{LEIDEN})(45). This makes it to the most common genetic risk factor for venous thromboembolism. About 8% of Caucasians are carrier of the mutation. The highest prevalence was found among Greeks with an allelic frequency of 7%. Among populations outside Europe the mutation is rare. It does not occur among populations of Africa, Southeast-Asia and indigenous populations of America and

Australia. The confinement of the mutation on Caucasians could be due to a founder effect (46). In heterozygotes, the risk of thrombosis is increased ~7-fold, in homozygous carriers up to ~80-fold with a markedly enhanced risk of developing thrombosis at young age (47).

The FV Leiden mutation is a single point mutation, changing Arginine (Arg) into Glutamine (Gln) at position 506 of the FV gene. Thus, the important APC cleavage site at Arg^{506} is eliminated (9). Since the cleavage at Arg^{506} counts for both, inactivation of FVa and generation of APC cofactor activity of FV, the blood coagulation system is highly influenced by the mutation. Normally, the inactivation of FVa starts with the cleavage at Arg^{506} promoting the subsequent cleavage at Arg^{306} and Arg^{679} for full inactivation. Instead, the inactivation of activated FV^{LEIDEN} is accomplished by APC via the cleavage sites at Arg^{306} and Arg^{679} alone. Actually, it is much slower than the inactivation via Arg^{506} but with the help of protein S, which selectively accelerates the reaction at Arg^{306}, the difference is reduced (48) (49). The loss of the cleavage site at Arg^{506} also prevents the single chain FV from being converted into an anticoagulant cofactor. The loss of APC cofactor activity leads to an inadequate inactivation of FVIIIa (50). Eventually, both mechanisms contribute to the alteration of blood coagulation observed in FV^{LEIDEN} plasma (51).

Several other rare mutations affecting the function of FV and leading to APC resistance have been found. Two point mutations at the Arg^{306} cleavage site ($FV^{CAMBRIDGE}$ and $FV^{HONG\ KONG}$) have been described (52). $FV^{CAMBRIDGE}$ is characterized by a substitution of Arg^{306} by Threonine. The genotype is associated with a mild type of APC resistance and thrombosis (53). Among Hong Kong Chinese a substitution of Arg^{306} to Glycin has been found, referred to as $FV^{HONG\ KONG}$ (54). $FV^{LIVERPOOL}$ is caused by an introduction of a consensus sequence for N-glycosilation at Asn^{357}. This missense mutation confers to APC resistance by a reduced rate of inactivation and loss of APC cofactor activity (55).

The HR2 haplotype is defined by several polymorphisms within the FV gene. It may account for mild APC resistance. If a patient bears both FV^{LEIDEN} and the HR2 haplotype, a more severe form of APC resistance may arise (56).

Clinical association and epidemiology

There have been two large studies, which contributed to determine the association between APC resistance and venous thromboembolism: The Leiden Thrombophilia Study (LETS) and the Longitudinal Investigation of Tromboembolism Etiology (LITE) study (57) (58).

The LETS was designed as a case-control study. The purpose of the study was to investigate risk factors for the development of venous thromboembolism. Among other risk factors, APC resistance was one of special interest. The patients participating in the study were recruited from three clinics in the Netherlands. 474 patients younger than 70 years with a first episode of deep vein thrombosis were included. The mean age of patients and controls was 47 years. 301 patient-control pairs were able to be examined for APC resistance. The assessment of APC resistance was carried out by using the APC ratio, the ratio between two activated partial thromboplastin times, one with and one without the addition of APC. As the lower normal limit, an APC ratio of 2.17 was chosen. The relative risk of venous thrombosis in the presence of APC resistance was 6.6 (95% CI 3.6-12.0) for heterozygotes. The relative risk for homozygotes of 80 was calculated by Hardy Weinberg equilibrium, since no homozygous cases were found among study participants. Among 70 individuals in the study who were tested positive for APC resistance 56 carried the FV^{LEIDEN} mutation (57).

In line with the LITE study an assessment of venous thromboembolism associated to FV^{LEIDEN} was conducted. It was designed as a nested case-control study, examining the association between FV^{LEIDEN} and APC resistance, respectively, and future thromboembolic events. The Odds ratio (OR) for heterozygotes was 3.67 (95% CI 2.20-6.12), for homozygotes 25 (95% CI 10-66). The OR of VTE in patients who were classified as APC resistant was 2.58 (95% CI 1.62-4.10). Moreover, the Odds ratios of recurrent and idiopathic VTE among carriers of the FV^{LEIDEN} mutation were higher compared to the OR of VTE with a clear association to acquired risk factors. The difference to the LETS may arise due to the older age of study population and the inclusion of African Americans in the study (58).

The median age of the first thromboembolic episode is about 44 years for heterozygotes and 31 years for homozygotes (47). The annual risk of developing a

thromboembolic event in heterozygous FV^{LEIDEN} patients ranges between 0.28% and 0.45% (59) (60) (61) (62). The annual incidence of VTE among homozygotes is about 0.5%-1.0% (44).The thrombosis free survival among APC resistance patients is approximately 0.9 at the age of 45 (44) (60). The incidence of VTE among FV^{LEIDEN} carriers is age dependent with an increase from 0.25% per year (age 15-30 years) to 1.1% per year (age over 60 years) (61). The pathogenetic model for venous thromboembolism implies that additional acquired or genetic risk factors are necessary for the presentation of a thromboembolic event. Genetic risk factors include antithrombin deficiency, prothrombin G20210A polymorphism and deficiency of protein C or S, among the acquired risk factors are oral contraceptive use, pregnancy, surgery and immobilization (38).

In the case of both a patient carrying the FV^{LEIDEN} mutation and use of oral contraceptives, the overall risk of a thromboembolic event is greatly enhanced. Whereas the risk for VTE with oral contraception alone is elevated about 4-fold compared to the normal population and the risk with FV^{LEIDEN} alone is about 8-fold, the risk in patients exhibiting both risk factors is about 30-fold (63).

According to Bounameaux (64), who compared different studies to this issue, the most common thrombotic event patients are presenting with is isolated deep vein thrombosis (DVT) with an odds ratio of 6.0 (95% CI 4.6-7.8). The odds ratio for DVT plus pulmonary embolism (PE) is 5.2 (95% CI 3.6-7.5). Much less common is PE alone (odds ratio 2.5 (95% CI 1.8-3.5)). This could be due to a more stable composition of the fibrin clot in FV^{LEIDEN} plasma, making it less susceptible to embolization. Furthermore, if embolization occurs, it could be more likely to be partially, leaving somewhat of the clot in the deep veins. This would explain the high prevalence of DVT and PE together. Further forms of thromboembolic disease, like superficial venous thrombosis and thrombosis of cerebral, visceral and axillary veins, are rare (65).

The role of APC resistance in the pathogenesis of arterial thrombosis is still not fully elucidated. Results of studies differ in their significance. However, there is evidence that APC resistance is associated with myocardial infarction (66).

Women with APC resistance are significantly overrepresented among patients with obstetrical complications. Preeclampsia, abruptio placentae, fetal growth retardation and stillbirth are severe complications during pregnancy, which may be due to an inaccurate maternal-fetal circulation. As a cause of this situation disturbances in placental vessels and alterations of hemostasis are discussed. The latter is suggested by the association between inherited states of thrombophilia (e.g. FV^{LEIDEN}, prothrombin G20210A, AT III deficiency) and major obstetrical complications (67). Furthermore, since pregnancy is a risk factor for venous thrombosis, the incidence of venous thromboembolism during pregnancy and puerperium is increased among women with APC resistance. Thromboembolic events, particularly pulmonary embolism, play a crucial role in morbidity and mortality during pregnancy and puerperium (68).

During pregnancy several plasma proteins involved in coagulation are circulating in a changed concentration. Overall, the blood coagulation system shifts towards a procoagulant state. Hence pregnant women are, in general, more susceptible to thrombosis. It may be an evolutionally developed mechanism against bleeding during delivery (69). In APC resistant subjects the prothrombotic effect is markedly enhanced. This may explain the high prevalence of the FV^{LEIDEN} mutation in the general population, because higher protection against excessive blood loss has probably acted as an evolutionary advantage (70).

Diagnosis and Treatment

The gold standard for APC testing, at least for FV^{LEIDEN} positive cases, is the direct determination of the mutation by PCR methods. Nevertheless, various functional tests for screening and diagnostic purposes are available. A deeper insight in testing for APC resistance is given further down.

Key element at the workup of familial thrombophilia is the assessment of thrombosis risk. Therefore, patients who are diagnosed as FV^{LEIDEN} positives should be tested for other common disturbances associated with familial thrombophilia, such as prothrombin G20210A, protein C and S deficiencies and antithrombin deficien-

cy. Furthermore, assessment for antiphospholipid antibodies is recommended (71).

The acute therapeutic management of VTE comprises the administration of low molecular weight heparin (LMWH) or intravenous unfractionated heparin. Concomitantly, therapy with an oral anticoagulant is started. The INR target is about 2 to 3. The length of treatment depends on the individual risk for recurrence. By default, treatment for patients with a known reversible risk factor lasts 3 months. Individuals heterozygous for FV^{LEIDEN} are not recommended for long term anticoagulation, since the risk for bleeding complications exceeds the benefit. However, it is recommended for homozygotes and patients with multiple thrombophilic alterations. The use of anticoagulant prophylaxis in high risk situations, such as surgery, pregnancy or immobilization, should be evaluated for the individual case (72).

1.2.3 PROTHROMBIN G20210A VARIATION

The transition of Guanin to Arginin at position 20210 in the 3´-untranslated region of the prothrombin gene gives rise to an increased prothrombin level. Since this leads to a moderate procoagulant state, G20210A is a risk factor for venous thromboembolism with an OR of about 2.8 (73). It is seen in approximately 2.7% of the general caucasian population. According to that data it is the second-most common genetic alteration associated with thrombophilia (41).

The variant predominantly occurs in Caucasians. About 6% of patients with venous thrombosis are carriers of the variant (73). For heterozygotes the annual risk of developing a thromboembolic event is 0.55% (74) (61) (60) (62).

1.2.4 ANTITHROMBIN DEFICIENCY

In the sixties of the last century, antithrombin deficiency was the first form of familial thrombophilia to be discovered (75). Antithrombin acts as an anticoagulant protein by binding on heparan sulfate, which is located on the endothelium, and sub-

sequent neutralization of thrombin and clotting factors XIa, IXa and Xa. Hence, deficiency of antithrombin prolongs the half life of these procoagulant factors (41).

Type I antithrombin deficiency is characterized by low levels of antigen and low activities, whereas in Type II deficiency the antigen plasma level is in the normal range (41).

Antithrombin deficiency is prevalent among 0.02% of the general population and among 2% of VTE cases (41). For heterozygotes the thrombotic risk is 0.87-1.6 per year (41). Since no subject being homozygous in type I antithrombin deficiency has been found, this may be due to incompatibility with life. However, rare cases of homozygous states in type II antithrombin deficiency are known, owing to a defect affecting the heparan sulfate binding site (41) (76).

1.2.5 Protein C Deficiency; Protein S Deficiency

Deficiencies in both protein C and protein S are resulting in an elevated activation of prothrombin to thrombin, and hence putting procoagulant forces in favor against procoagulant mechanisms. Just as mentioned at antithrombin deficiency, type I and type II deficiencies are distinguished (41).

The incidence of protein C deficiency in the general population is 0.2-0.4%. The incidences of protein C and protein S among VTE patients are 3.7% and 2.3%, respectively (41).

A homozygous deficiency of proteins or protein s is presenting with purpura fulminans in infants. Due to the massive thrombophilic state in these patients, multiple venous thromboses occur, eventually leading to skin necrosis (41).

1.3 TESTING FOR APC RESISTANCE

Since the first description in 1993 (42), several systems for functional laboratory determination of APC resistance have been created. The common purpose of these methods is the detection of a poor response to activated protein C. Owing to the major role of the FV^{LEIDEN} mutation in APC resistance, the test results of functional methods are to be showing discrimination between wild-type- and mutation-carriers. Modern test systems are performing this assessment with sensitivity and specificity rates of about 100%. Nevertheless, the gold standard in testing for FV^{LEIDEN} is DNA analysis of the point mutation by PCR methods. Especially for determination, whether the patient is a heterozygous or homozygous carrier of the mutation, DNA-analysis is indispensable.

1.3.1 INDICATION FOR TESTING

In general, testing for APC resistance should be evaluated on the possibility of a therapeutic intervention afterwards. General testing for thrombophilia of patients presenting with venous thromboembolism (VTE) is not reducing the risk for recurrent thromboembolic events (77). Thus, testing should be restricted on selected patients. There are some clinical circumstances, which indicate testing (see. **Table 1**).

A first unprovoked VTE at any age (especially age < 50 years).
A history of recurrent VTE.
Venous thrombosis at unusual sites (e.g., cerebral, mesenteric, hepatic, and portal veins).
VTE during pregnancy or the puerperium.
VTE associated with the use of estrogen contraception or hormone replacement therapy (HRT).
A first VTE with a first-degree family member with VTE before the age of 50 years.

Table 1 APC resistance: Indication for testing; Source: (71)

Testing is also appropriate in some other clinical conditions, such as recurrent pregnancy loss, severe eclampsia or a thromboembolic event in the context of tamoxifen use (71).

General testing is not recommended for pregnant women, prior to the use of contraceptives, HRT or tamoxifen. Also, testing for patients with arterial thrombosis is not recommended (71).

1.3.2 ORIGINAL TEST SYSTEM

In 1993, Dahlbäck et al. described a patient with multiple thrombotic events. The first event was a deep vein thrombosis of one leg when he was 19 years old. Two decades later a series of more episodes of deep vein thrombosis began. Moreover, several family members presented a similar history. These findings indicated a genetic cause for the hypercoagulable state of the proband and his family members. As a pathogenetic explanation the authors suggested a newly found mechanism for thromboembolic disease: a poor anticoagulant response to APC (42).

To illustrate this theory they invented a new APTT (activated partial thromboplastin time)-based assay. Blood samples were collected and the plasma was tested twice. First, the clotting time with addition of APC was measured, and then the same test was performed without addition of APC. Since APC is an anticoagulant protein, it prolongs the clotting time. The difference between the two clotting times was calculated. The proband in the study showed a reduced response to APC, indicated by a considerably shorter prolongation of clotting time compared to control plasma. Also, 14 of 19 family members participating in testing showed a prolongation time below the fifth percentile of controls. This suggests the genetic character of the disorder, which is characterized by poor response to APC (42).

In this APTT assay two plasma samples were first incubated with a reagent, which is an activator of coagulation. This APTT reagent comprises a surface activator and a phospholipid component. The surface activator initiates clotting via the intrinsic pathway and phospholipid is essential for the assembly of the tenase and prothrombinase complex, respectively. Then one sample was incubated with ex-

ogenous APC. Alternatively exogenous PC together with a PC activator can be used to generate APC. In the same step $CaCl_2$, was added. Calcium is an essential cofactor in coagulation. The time between addition of Calcium and the generation of a fibrin clot, referred to as the clotting time, was measured (78) (42).For better comparability a new parameter in APC resistance diagnostics, the APC ratio, was introduced. It is calculated by dividing the clotting time measured with addition of APC by the clotting time measured without adding it (44). A ratio over 2.0 indicated a normal response to APC, a ratio below 2.0 indicated a defect in the APC pathway (79). A normalized form of the APC ratio, the n-APC ratio, was established for better differentiation between carriers of the mutation and healthy individuals. It is calculated by dividing the APC ratio of the patients' plasma by the APC ratio of a normal reference plasma pool. The n-APC ratio showed no considerable improvements over the APC ratio alone (80).

For reliable results the thorough preparation of samples is of utmost importance. The plasma has to be prevented from contamination with platelets. Studies showed that contaminated frozen plasma gives rise to false positive APC ratios after thawing. The reason for this phenomenon is the rupture of platelets due to the freeze-thaw cycle. The ruptured platelets release phospholipids and platelet bound FV, which leads to shorter clotting times (81) (82). In order to prevent this situation the samples should either be tested immediately without freezing or the centrifugation should be performed thoroughly before freezing (82).

Antiphospholipid Antibodies, e.g. Lupus anticoagulant (LA), are another source of influence on the APTT-based assay. Mostly, they are acquired in the context of autoimmune disease and inflammation. Because of their reaction with phospholipids, they are interfering the assembly of the tenase and prothrombinase complex and the inactivation of FVa by APC. Accordingly, Lupus anticoagulant causes a prolongation of the APTT. APC ratios are reported to be decreased by the presence of LA, giving rise to false positive results (83).

The results of the original test system showed associations with various plasma proteins. A lowering of the APC ratio, for example, has been reported to be associated with high factor VIII levels, commonly associated with inflammatory conditions or pregnancy (84) (85). Furthermore, low levels of prothrombin and factor X

seem to cause falsely high APC ratios. Thus, testing on patients who are receiving anticoagulatory drugs, such as Heparin or Vitamin K antagonists, is not reliable, since they are decreasing the named factors in the plasma. A deficiency of protein S (levels less than 20%) could lead to a false positive outcome (86).

1.3.3 Modified APC resistance Test (Second Generation Test)

In the second generation of APC tests one step in the preparation of the sample plasma is added. A predilution of the sample plasma with factor V-deficient plasma is performed. Thus, all proteins involved in blood clotting, except factor V, are supplemented to nearly the same levels. This enables the reliable testing of patients, who are receiving anticoagulatory drugs (87). The dilution ratio between the sample plasma and the factor V-deficient plasma is reported as 1:5 to 1:10, depending on the test system (87) (88).

The modified test also uses polybrene, which neutralizes low-molecular-weight heparin or unfractioned heparin in the sample (89). In the original test, low levels of protein S could produce false positive results (86). In the modified system, the factor V-deficient plasma is supposed to supply exogenous protein S to compensate the low protein S level. Anyway, protein S can become inactivated during the production of factor V-deficient plasma, which leads to the perpetuation of a protein S deficiency, and therefore an influence on the test result (90).

In the modified assay, lupus anticoagulant (LA) still remains a strong factor of influence. Because the test is phospholipid-based, LA can produce interference in the assay. Patients, who are tested positive for LA, should be tested genetically (89). One report showed a correlation between the APTT-based assay and the genetic test when a dilution of 1:40 instead of 1:5 was used (91). Another study proposed the extra addition of phospholipid, in order to neutralize LA (92).

The sensitivity and specificity of the modified test were shown to be between 93% and 100% (87) (93). However, the discrimination gap between wild-type FV and FV^{LEIDEN} is narrow (94). Discrimination between heterozygous and homozygous

FV^{LEIDEN}-carriers is insufficient, indicated by a sensitivity of 0.93, a specificity of 0.96 and a positive predictive value of 0.76 (95).

Various commercial APTT-based APC resistance test kits have been developed. A widely used one is the COATEST® APC V resistance test (Chromogenix, IL, USA) (78). An example for assays, which are using protein C activators instead of exogenously added APC, is the DIAGEN APC-R test (Diagnostic Reagents, Thame United Kingdom) (96).

1.3.4 THIRD GENERATION TEST

For the optimization of sensitivity and specificity, new APC resistance assays have been developed. Particularly, the influence of LA and anticoagulant therapy on the test results has been a central issue. A common feature of new assays is the use of snake venoms, which act as specific activators of different constituents of the coagulation system.

The PEFAKIT® APC-R Factor V Leiden assay (Pentapharm, Basel, Switzerland) is a commercially available functional, prothrombin-based, clotting assay (see **Figure 4**). The test plasma is mixed with FV-depleted plasma. Furthermore, polybrene, a heparin inhibitor is added. Then snake venom from *Daboia russelli* (Russell viper venom-factor V; RVV-V) is added. It is a specific activator of FV. In the same step APC is added. After an incubation period the plasma sample is mixed with Noscarin, a FV-dependent prothrombin activator isolated from the venom of *Notechis scutatus scutatus,* and Ethylenediaminetetraacetic acid (EDTA). Subsequently, the clotting time is measured. A second test is run without the addition of APC, which enables the calculation of an APC ratio (94).

This functional assay is based on the activity of two components isolated from snake venoms. Russell viper venom-factor V (RVV-V) activates FV to FVa, which is subsequently inactivated by added APC. This elimination step is not working, if FV is mutated on the APC-cleavage site at Arg506. As a consequence, more FVa is present after the reaction. In the second reaction phase, Noscarin activates pro-

thrombin in an FVa-dependent manner. This leads to blood clotting and the clotting time is measured. The more residual FVa is present the shorter is the clotting time. After the run of two measurements, one time with addition of APC, the other time without it, the APC ratio is calculated. A low APC ratio is indicative for APC resistance (94).

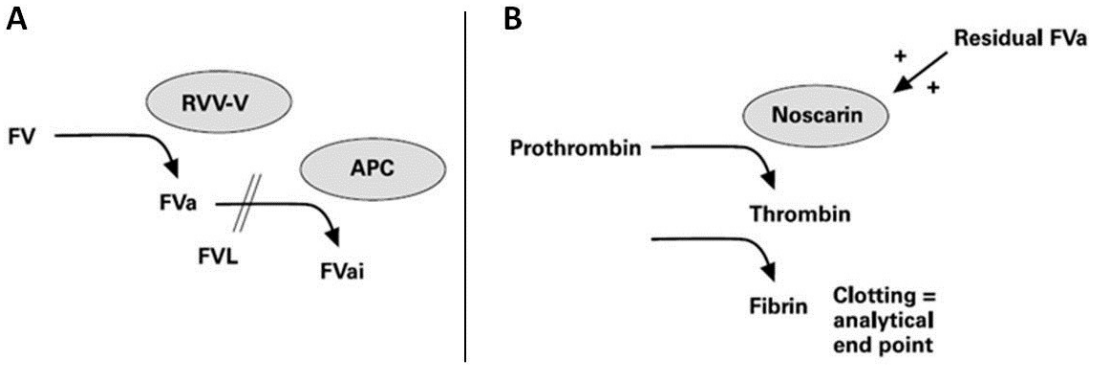

Figure 4 PEFAKIT® APC-R Factor V Leiden assay **(94)**. A. RVV-V activates FV to FVa in a first reaction step. APC eliminates activated FV. FV^{LEIDEN} (FVL) is not cleaved by APC. B. In the second step Noscarin acts as a sensor for residual FVa. The more FVa has remained the higher the activity of Noscarin, and accordingly the velocity of clotting **(94)**.

The results of several studies showed perfect discrimination between wild-type (wt)–carriers and patients being heterozygous for FV^{LEIDEN}. Genotyping of patients was obtained by PCR-methods. Sensitivity and specificity were 1.0, respectively. Moreover, the discrimination gap was broad, enabling a clear distinction between wt- and heterozygous carriers (93)(94)(95)(97). The cut-off value was determined between 1.9 (97) and 2.5 (95). The evaluation of distinction between heterozygous and homozygous carriers of FV^{LEIDEN} is difficult, owing to the low incidence of homozygous carriers. The data from different studies show a narrow discrimination with a cut-off value of 1.1 or 1.2, respectively(94)(95). A special genetic condition, referred to as pseudohomozygosity, leads to false positive results. It is characterized by a heterozygous genotype, a low APC ratio and a low FV-activity. It's explained by a heterozygous FV^{LEIDEN}-state and a mutation in the other allele, causing a decreased FV-activity. The net effect is a steep decrease of the APC ratio (95)(98).

A major cause of the improved sensitivity and specificity compared to APTT-based assays is the elimination of the influence of Lupus anticoagulants (LA) on the assay. Since LA is forming complexes with phospholipids only in the presence of calcium ions, the lack of Ca^{2+} in the plasma sample is expected to hamper the effect of LA. In the assay outlined above, calcium is bound by the chelating agent EDTA. APTT-based assays need calcium ions in order to promote the coagulation cascade, so that LA interference is possible. Available data show no interference on the assay by LA (93)(94)(95).

There seems to be no influence of oral anticoagulation (OA) or heparin on the assay. This fact has a great practical implication, since numerous patients are tested after starting anticoagulant therapy (93). Oral anticoagulants work by an antagonizing effect on Vitamin K, inhibiting the production of clotting factors II, VII, IX and X as well as protein C and protein S. Heparin acts as a promoting agent to antithrombin III (ATIII), which inactivates thrombin and factors IX and X (97). OA and heparin are prolonging the baseline APTT, an important interference-factor on APTT-based assays, giving rise to false positive test results. That doesn't apply to the newer assays, obviously. The effect of heparin can be diminished by the addition of polybrene, a heparin antagonist, to the sample plasma, which is recommended both for the APTT- and prothrombin-based assays (97).

Deficiencies of FV, protein S and protein C had no effect on the APC ratio (93)(97). A surplus of Factor VIII (>150%) also had no influence on the assay (93)(97). Since concentrations of clotting factors are increased during pregnancy, this is a potential interference factor. However, there was no effect on the assay (93)(97).

Another commercial assay is the CRYOCHECK® Clot APCR (Precison BioLogic, Dartmouth, Canada). It also uses Russell Viper Venom to activate factor V. Instead of exogenous APC, it uses snake venom from *Agkistrodon contortrix contortrix* as specific activator of protein C. To obtain the APC ratio, one determination is run with the venom, another one without it. There were no differences in sensitivity and specificity to the assay described above (97).

The HEMOCLOT® Quanti-V-L (Hyphen BioMed, Paris, France) is a recently developed APC resistance assay, which quantitatively measures the presence of FV^{LEIDEN} in a plasma probe. The sample plasma is mixed with two reagents. The first reagent contains clotting factors (fibrinogen, prothrombin, protein S, APC) in a pre-defined concentration. The second reagent comprises purified factor X and phospholipids. Finally, Ca^{2+} is added and the clotting time is measured. On the basis of different mixtures of plasma from heterozygous and wild-type plasma pools a calibration curve for the clotting time against the concentration of FV^{LEIDEN} is determined. Accordingly, the clotting time is inversely correlated to the FV^{LEIDEN} concentration. Hence, the concentration of FV^{LEIDEN} in the patients´ plasma sample can be determined by comparison of the measured clotting time in this assay with the calibration curve. Thus, the assay comprises only one measurement in contrast to other common APC resistance assays, which require two measurements (99).

1.3.5 FV^{LEIDEN} Genetic Testing

Genetic testing for the point mutation at position 1691 of the factor V gene is the gold standard in APC resistance diagnostic. Different methods have been developed. A common method uses MnlI, a restriction endonuclease, since the FV^{LEIDEN} mutation alters the restriction site of this enzyme. After the amplification of the DNA sequence surrounding the restriction site by PCR, the product is digested with MnlI. Then electrophoresis is performed to determine the length of gene products after digestion. If the restriction site is intact, specific bands in the electrophoresis are to be seen. That is not the case, if the restriction site is mutated, indicating for the FV^{LEIDEN} mutation (89).

PCR-based methods for the detection of the FV^{LEIDEN} mutation are unbeaten in their diagnostic validity, but they are costly and labor intensive. Therefore, they are neither used as screening tests nor as first line test in laboratory thrombophilia workup.

2 MATERIALS AND METHODS

In this retrospective study data from patients undergoing thrombophilia screening at the Clinical Institute of Medical and Chemical Laboratory Diagnostics was overviewed. From the time between July 2005 and November 2011 data was available from a total of 27165 patients. Out of these, 3086 patients were included in the study. Inclusion criteria were the presence of an APC ratio and a genetic factor V^{LEIDEN} evaluation. There were no exclusion criteria.

Test methods

The determination of the APC ratio had been determined either by a second-or third-generation test. The COATEST® APC V resistance test is a modified APC resistance assay used in the laboratory before the PEFAKIT® APC-R Factor V Leiden became the method of choice. The principles of both assays are described above, respectively.

As gold standard serves the genetic PCR test for factor V^{LEIDEN} as described above.

Statistical analysis

Values for sex, age, APC ratio and the FV^{LEIDEN} mutation were included in the statistical evaluation. Furthermore, statistical analysis of the prothrombin G20210A variant, protein C deficiency and protein S deficiency was performed. In the framework of descriptive data analysis incidences and statistical measures were calculated. Cut-off levels for the discrimination between wild type, heterozygous and homozygous individuals were established by ROC analysis for both APC resistance assays, respectively. To determine test validity, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for both second- and third generation APC resistance test compared to the ge-

netic assessment. Statistical analysis was performed using SPSS Statistics 20 (IBM, Armonk, USA).

Medical data

For further investigation on patients showing discrepancies between the genetic and the functional test method, medical data from the MEDOCS Hospital Information System at the LKH Univ.-Klinikum Graz was obtained.

Ethic committee vote

The study was approved by the ethic committee of the LKH Univ.-Klinikum Graz on November 11 2011.

3 RESULTS

3086 patients were tested for factor V^{LEIDEN}. 732 were tested heterozygous for the FV^{LEIDEN} mutation, 31 were tested homozygous. Of 2218 tested females, 567 (25.6%) were tested heterozygous for the mutation, 21 (0.9%) were tested homozygous. Among 868 male patients, 165 (19.0%) were heterozygotes and 10 (1.1%) were determined homozygous.

3.1.1 3RD GENERATION TEST

Patient characteristics

APC ratios were determined by the PEFAKIT® APC-R Factor V Leiden assay, a third generation test kit previously described in this thesis. It was performed in 2900 of the included patients. 2088 (72.0%) of the tested patients were females, 812 (28%) were males. The age at the time of testing ranged between 5 and 92 years, with a mean age of 41.94 (±19.5) years in the overall group and mean ages of 37.75 (±18.5) and 52.70 (±17.8) years for women and men, respectively. The genetic test yielded 713 (24.6%) patients heterozygous and 29 (0.01%) patients homozygous for FV^{LEIDEN}. Among female individuals, 553 (26.5% of females) were tested heterozygous and 20 (0.96% of females) homozygous. Of the male probands, 160 (19.7% of males) were tested heterozygous, 9 (1.1% of males) were determined homozygous.

The prothrombin G20210A variant was determined genetically in 2602 patients, of whom 151 (5.8%) were tested heterozygous for the variant. 1 patient was tested homozygous for the variant. 30 patients were determined both being heterozygous for the variant and being heterozygous in FV^{LEIDEN}. 2 individuals were tested heterozygous in the prothrombin variant and homozygous in FV^{LEIDEN}.

To assess protein C and protein S deficiency, respectively, the activity of both was tested in numerous patients. A deficiency in either protein C or protein S was defined by an activity of less than 60%. Accordingly, 16 (1.1%) of 1396 tested individuals were deficient in protein C and 167 (9.3%) of 1789 tested were deficient in protein S.

4 patients were determined deficient for protein C and protein S. 2 individuals with protein C deficiency were also genotyped as being heterozygous in FV^{LEIDEN}, whereas 21 (20 females, 1 male) of the patients deficient in protein S were classified as heterozygous in FV^{LEIDEN}.

Cut-off limits

The cut-off limits for the discrimination between wild-type FV carriers and heterozygous and homozygous FV^{LEIDEN} carriers were determined by ROC analysis (see **Table 2**). The cut-off between heterozygotes and wild-types used in the laboratory so far is higher than the one calculated in the present study (>2.9 versus >2.2; see **Table 3**).

APC ratio	**< 1.2**	**1.2 – 2.2**	**> 2.2**
FV Leiden	homozygous	heterozygous	wild-type

Table 2 Cut-off limits for the 3rd generation APC resistance test determined by ROC analysis in the present study

APC ratio	**< 1.2**	**1.2 – 2.9**	**> 3.0**
FV Leiden	homozygous	heterozygous	wild-type

Table 3 Cut-off limits for the 3rd generation APC resistance test routinely used in the laboratory

Test discrimination

Of the 2158 individuals being negative for the FV^{LEIDEN} mutation (wild-type configuration), all were correctly classified by the functional test. Of the 742 heterozygous and homozygous individuals, 707 were correctly classified as being heterozygous and 29 patients as being homozygous. 2 patients were misclassified as being

negative for FV^{LEIDEN} and 4 patients were misclassified as being homozygous (see **Table 4** and **Table 5**) The diagnostic values for the discrimination between negative individuals and individuals bearing the mutation in either one or both alleles are shown in **Table 6**. The mean values of the APC ratio categorized on the basis of the cut-off levels are depicted in **Table 7**. The discrimination between wild-type and heterozygous FV^{LEIDEN} carriers was broad, since no values between 2.2 and 2.4 were measured. The discrimination gap between heterozygotes and homozygotes was somewhat narrower. The distribution of results is shown in **Figure 5**.

	3rd gen. test **neg.(wt)**	3rd gen. test **pos.(het+hom)**
FV^{LEIDEN} **negative (wt)**	2158 (TN)	0 (FP)
FV^{LEIDEN} **pos.(het+hom)**	2 (FN)	740 (TP)

Table 4 3rd generation test discrimination between negatives (wild-type; wt) for FV^{LEIDEN} and positives (heterozygotes and homozygotes) for FV^{LEIDEN}; TN true negatives, FN false negatives, FP false positives, TP true positives

	3rd gen test **pos.het.**	3rd gen.test **pos.hom.**
FV^{LEIDEN} **pos.het.**	707 (TN)	4 (FP)
FV^{LEIDEN} **pos.hom.**	0 (FN)	29 (TP)

Table 5 3rd generation test discrimination between positive heterozygotes (pos.het.) and positive homozygotes (pos.hom.) in FV^{LEIDEN}; TN=true negatives, FN=false negatives, FP=false positives, TP= true positives

	Neg versus Pos.(het.+hom.)	**Pos.Het. versus Pos.hom.**
Sensitivity	0.997	1.0
Specificity	1.0	0.994
PPV	1.0	0.879
NPV	0.999	1.0

Table 6 3rd generation test diagnostic values. PPV positive predictive value; NPV negative predictive value

	Mean	SD	Range (without outliers*)
homozygous	1.024	0.043	1.0-1.1
heterozygous	1.562	0.167	1.4-1.9
Wild-type	3.909	0.533	2.4-6.3

Table 7 Mean values, standard deviation and range of results of the 3rd generation test; * 2 heterozygotes classified as wild-types (APC ratios 2.5 and 3.3, respectively), 4 heterozygotes classified as homozygotes APC ratios 1.0 and 1.1, respectively)

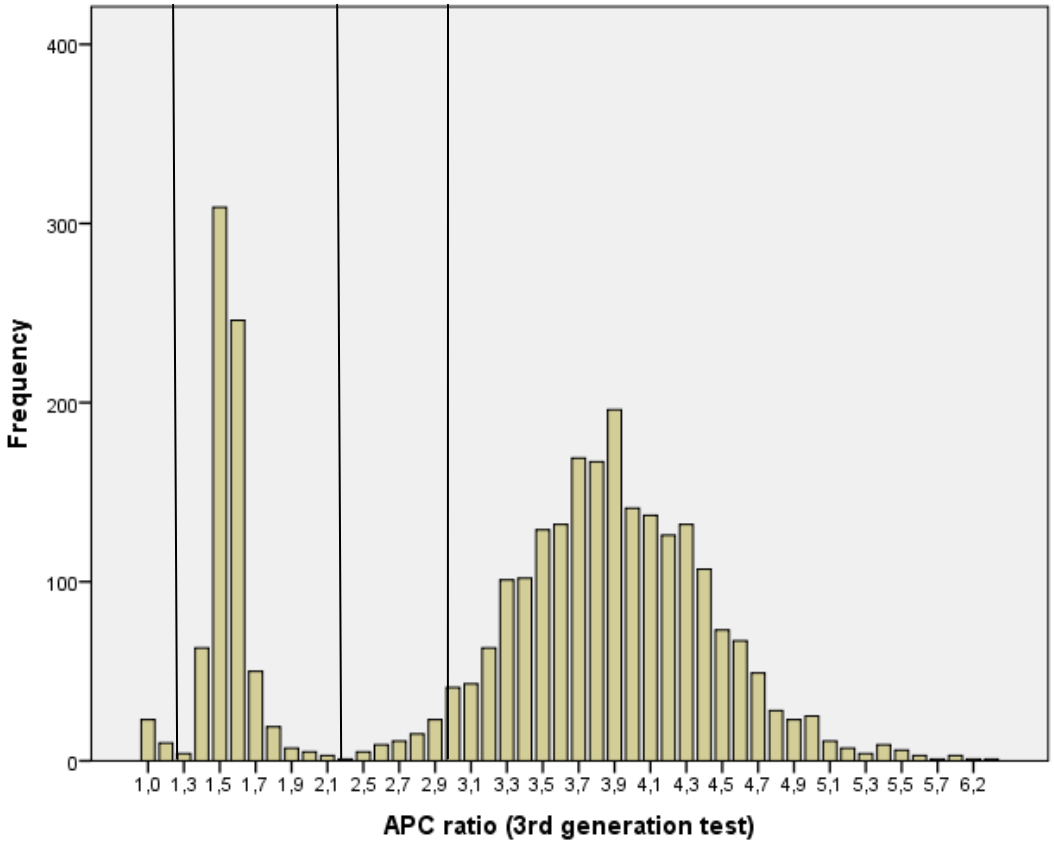

Figure 5 Frequencies of results for the APC ratio using the 3rd generation test (N=2900). Cut-off values are set at 2.2 and 1.2.

3.1.2 2ND GENERATION TEST

Patient characteristics

The 2nd generation test was performed with the COATEST® APC V resistance test. 219 patients were tested using the 2nd generation test, 156 (71.2%) were female, 63 (28.8%) male. 35 (16.0%) were tested heterozygous and 2 (0.9%) homozygous. 27 women (17.3% of females) were classified as being heterozygous, 1 female patient (0.6% of females) was classified as being homozygous. Out of the male individuals, 8 (13% of males) were classified as heterozygous, 1 (1.6%) as homozygous.

The mean age of probands was 15.75 (±11.2), with a mean age of 15.74 (±9.3) for females and 15.79 (±14.98) for males.

195 were tested for the prothrombin variant, 9 (4.6%) of whom were heterozygous. Off 199 tested for protein C activity, 11 (5.5%) were protein C deficient. 15 (7.4%) of 204 tested individuals were protein S deficient.

2 individuals were tested for both the G20210A variant and heterozygosity in FV^{LEIDEN}. The patient homozygous for ^{FVLEIDEN} was also determined as being heterozygous in the G20210A variant. 1 patient was determined deficient for protein C and protein S. 6 individuals with protein c deficiency were also genotyped as being heterozygous in FV^{LEIDEN}.

Cut-off limits

The cut-off limits for the discrimination between wild-type FV carriers and heterozygous and homozygous FV^{LEIDEN} carriers was determined by ROC analysis (see **Table 8** Cut-off **limits for the 2nd generation APC resistance test**

APC ratio	**< 1.4**	**1.4 – 2.0**	**> 2.0**
FV Leiden	homozygous	heterozygous	wild-type

Table 8 Cut-off limits for the 2nd generation APC resistance test

Test discrimination

Of the 182 individuals being negative for the FV^{LEIDEN} mutation (wild-type configuration), 180 were correctly classified by the functional test using the described cut-off levels. Of the 35 heterozygous individuals, 34 were correctly classified. 1 heterozygous patient was misclassified as being wild-type and 2 wild-type carriers were misclassified as being heterozygous (see **Table 9**). The discrimination between heterozygous and homozygous individuals was perfect, however the number of indivuals was low (see **Table 10**). The diagnostic values for the 2nd generation test are shown in **Table 11**. Mean values, standard deviation and range of results are depicted in **Table 12**.The distribution of the APC ratios for the 2nd generation test is shown in **Figure 6.**

	2nd gen. test **neg.(wt)**	2nd gen. test **pos.(het.+hom)**
FVL **negative** (wt)	180 (TN)	2 (FP)
FVL **positive (het+hom)**	1 (FN)	36 (TP)

Table 9 2nd generation test discrimination between negatives (wild-type; wt) for FV^{LEIDEN} and positives (heterozygotes and homozygotes) for the FV^{LEIDEN}; TN true negatives, FN false negatives, FP false positives, TP true positives

	2nd gen test **heterozygous**	2nd gen.test **homozygous**
FVL **pos.het**.	34 (TN)	0 (FP)
FVL **pos.hom.**	0 (FN)	2(TP)

Table 10 2nd generation test discrimination between positive heterozygotes (pos.het.) and positive homozygotes (pos.hom.) in FV^{LEIDEN}; TN true negatives, FN false negatives, FP false positives, TP true positives

	Neg.versus Pos.(het.+hom.)	**Pos.Het. versus Pos.hom.**
Sensitivity	0.973	1.0
Specificity	0.989	1.0
PPV	0.947	1.0
NPV	0.994	1.0

Table 11 2nd generation test diagnostic values. PPV positive predictive value; NPV negative predictive value

	Mean	SD	Range
homozygous	1.25	0.07	1.2-1.3
heterozygous	1.74	0.12	1.5-2.1
Wild-type	2.68	0.24	1.9-3.4

Table 12 Mean values, standard deviation and range of results of the 2nd generation test

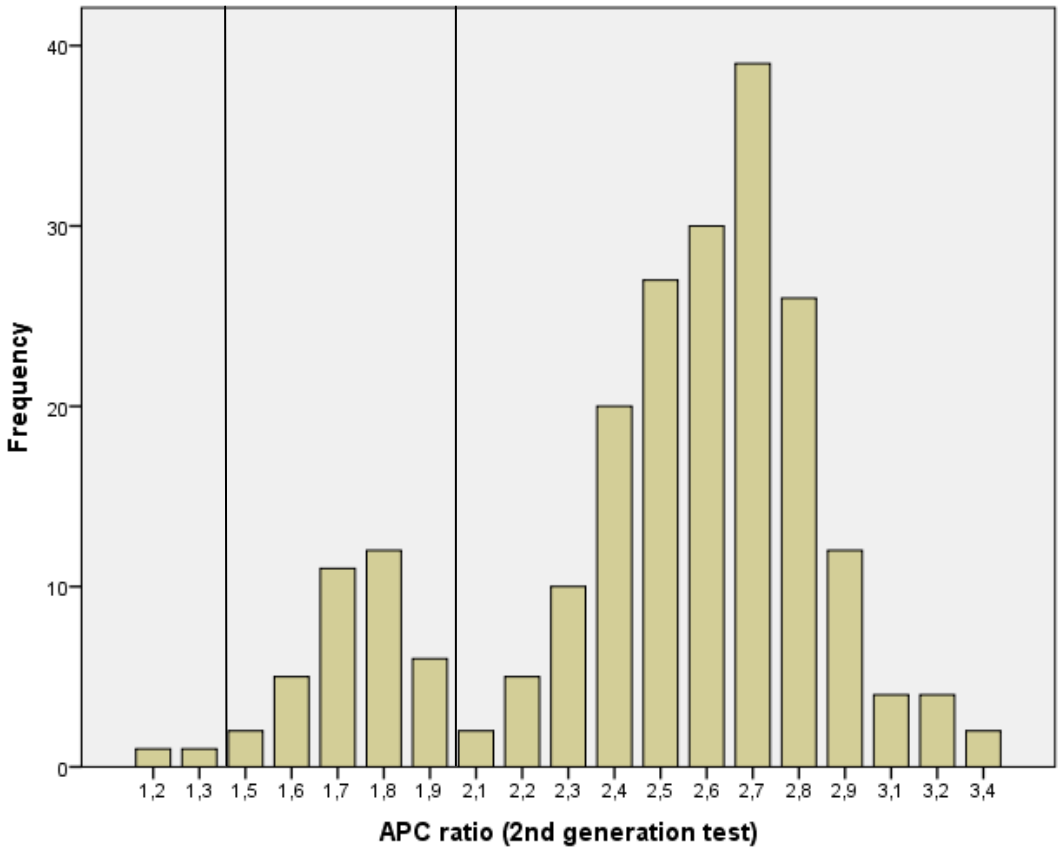

Figure 6 Frequencies of results for the APC ratio using the 2nd generation test (N=219). Cut-off values are set at 2.0 and 1.4.

3.1.3 2ND AND 3RD GENERATION TEST

In 33 individuals both the 2nd and 3rd generation test was performed, enabling direct comparison of test results. The heterozygous genotype was present in 13 of

26 tested women and in 3 out of 7 male individuals. 2 individuals beared the prothrombin G20210A variant, 2 patients were protein C deficient and 4 patients deficient in protein S.

A comparison of the mean values between the 2nd and 3rd generation APC resistance test is shown in **Table 13**.

	3rd gen. test APC ratio	**2nd gen. test APC ratio**
Mean Negative (wt)	3.97	2.72
Range	2.5-6.3	2.2-3.4
Mean Positive (het)	1.65	1.75
Range	1.4-1.9	1.5-2.1

Table 13 Mean values for 2nd and 3rd generation test for patients tested with both assays (N=33)

4 DISCUSSION

The primary aim of the study was the evaluation of two functional APC resistance assays regarding their diagnostic power for the FV^{LEIDEN} mutation compared to the PCR method for direct genetic testing.

The APTT-based 2nd generation test showed a sensitivity of 0.973 in the detection of indivuals with the mutation. The specificity was 0.989. These data suggest a better discriminatory power of the test compared to a previous study (93), with sensitivity and specificity of 0.931 and 0.93, respectively. Nevertheless, the 3rd generation test showed even better discrimination between wild-type FV and FV^{LEIDEN} carriers, with sensitivity and specificity of 0.997 and 1.0, respectively. Furthermore, the 3rd generation test yielded a clear cut off between normal and FV^{LEIDEN} individuals, with the APC ratios 2.2 and 2.3 not even occurring (see **Figure 5**). The cut off value for the 2nd generation test was somewhat lower (APC ratio 2.0) and there was an overlap between heterozygous and wild type individuals in this area of values (see **Figure 6**). Moreover, the APC ratios spread over a wider range at the 3rd generation test. The mean value for heterozygous individuals was lower at the 3rd generation test compared to the 2nd generation test (1.56 versus 1.74). Contrariwise, the 3rd generation test yielded a higher mean value at the wild type individuals (3.90 versus 2.68). Those findings are in line with the data from comparable studies (93)(94)(95)(96)(97). The differences in mean values are also demonstrated in **Table 12**. In 33 patients the APC ratio was determined with both the 2nd and 3rd generation assay. The obtained results are fairly in line with those of the larger single test groups.

In contrast to other studies (93)(94)(95)(96)(97), which all reported perfect discrimination (sensitivity and specificity of 1.0, respectively) using the third generation test, the present study yielded two heterozygous patients being misclassified as wild-type. In one of these patients, a 16 year old female with an APC ratio of 3.3

and a heterozygous FV^{LEIDEN} state, an additional mutation on the factor V gene was found. It is located within the A_3 domain of the factor V gene on the same chromosome bearing the FV^{LEIDEN} mutation. Functionally, it seems to reduce the activity of factor V, thus decreasing the effect of the FV^{LEIDEN} mutation located on the same chromosome. This constellation explains the discrepancy between the functional and genetic test. Although genetically being heterozygous, functionally speaking the thrombotic risk seems to be similar to those of wild-type individuals as indicated by the result of the functional test. (100) One further patient was genetically determined as heterozygous for FV^{LEIDEN}, despite an APC ratio forecasting a wild type genotype. The APC ratio of this 46 year old woman was 2.5, thus ranging in the lowest area of wild-types. Possibly, a mechanism similar to that of the patient described above is involved in this case as well. For a more detailed genetic characterization of this patient further investigation would be necessary.

Evaluating the test discrimination between heterozygous and homozygous individuals is reasonably difficult. Mostly, there are not enough homozygous patients available for a significant interpretation of results. However, owing to the high number of patient plasmas tested with the 3rd generation test in this study (2900 tested, 713 heterozygous, 29 homozygous), the calculation of meaningful diagnostic values was possible. The 3rd generation test showed a sensitivity of 1.0, a specificity of 0.994 and a positive predictive value (PPV) of 0.875 in discriminating between heterozygotes and homozygotes. Since only two homozygous patients were tested with the 2nd generation test, the obtained diagnostic values are lacking of statistical relevance. The cut off value between heterozygotes and homozygotes was 1.2 in the 3rd generation assay, and 1.4 in the 2nd generation assay. The mean values for homozygotes was 1.024 at the 3rd generation test and 1.25 (N=2) at the 2nd generation test. There was a clear distinction between heterozygous and homozygous individuals, although it was somewhat narrower than the gap between heterozygotes and wild-types. Compared to previous publications (93)(95) the results are similar, but in virtue of the higher number of samples the data obtained in this study is of better statistical power.

In four patients functionally tested as homozygotes a discrepancy between their APC ratio and their genotype was found. Although the functional test yielded results of 1.0 or 1.1, in the respective patients, they were all classified as being heterozygous for the FV^{LEIDEN} mutation in the genetic evaluation. These findings may refer to a phenomenon called pseudohomozygosity. It is caused by a compound heterozygous state in the FV gene: One allele bears the FV^{LEIDEN} mutation, whereas the other allele contains a null mutation. Accordingly, the FV activity and the FV antigen level of these patients are reduced and, more importantly, there is only FV^{LEIDEN} left in the plasma (101). Clinically, the pseudohomozygous state may confer a phenotypic state similar to that of true homozygotes for the FV^{LEIDEN} mutation. As proposed by Simioni et al (102), the thrombosis free survival of pseudohomozygotes is similar to that of homozygous patients (27 vs. 31 years), plus significantly shorter than in heterozygotes (49 years). Furthermore, the pseudohomozygous genotype may favor superficial vein thrombosis (e.g. thrombophlebitis) as first thromboembolic event (102).

In three of the four patients in this study suspected to be pseudohomozygous medical data was available. Two female patients, aged 15 and 21, respectively, haven't yet developed a thromboembolic event. Since the thrombosis free survival for pseudohomozygotes is about 27 years, as mentioned above, this finding is coherent. The third patient was a 50 year old male with an APC ratio of 1.1 and a heterozygous genotype. He showed a history of recurrent thrombophlebitis. At the age of 49 the first event, a thrombophlebitis of the left lower leg, occurred, followed by further episodes. In conjunction with a venous line thrombophlebitis of the right cubita occurred. The preference of superficial venous thrombosis in this patient goes in line with the above-mentioned, albeit the age of first manifestation is higher than to be expectable for pseudohomozygotes.

Genetic testing for the FV^{LEIDEN} mutation using PCR techniques is still the gold standard in the evaluation of APC. However, owing to both higher costs and work effort compared to functional APC resistance assays, a reduction of patients

needed to be genetically tested is to be sought. To achieve this goal functional testing systems with clear cut off levels and profound discriminatory power are necessary. In the present study two functional APC resistance assays were evaluated in the critical context to the genetic test method for FV^{LEIDEN}. Primarily, the evaluation of the 3rd generation test (PEFAKIT® APC-R Factor V Leiden) was of special interest, because it was implemented at the KIMCL in 2006. Nevertheless, the 2nd generation test (COATEST® APC V resistance test) has still been used in some cases. Hence, a comparison between those two functional assays regarding their diagnostic power was also included in the study. It showed preponderance of the 3rd generation test against the 2nd generation test in all respects. The 3rd generation test yielded convincing values for sensitivity, specificity as well as for positive and negative predictive values. The cut-off limits were clear and broad, especially for the discrimination between factor V wild-type and heterozygous individuals. Misclassifications were attributable to either pseudohomozygous genotypes or to another, previously unrecognized, mutational states. Only one patient, being heterozygous for FV^{LEIDEN} and misclassified as wild-type, has not been assessed on possible underlying causes so far. Based on the data a diagnostic algorithm may be proposed. It includes functional testing of patients in first line. Depending on the result of the functional test, consideration for a confirmatory genetic test may be conducted. The cut-off limit between wild-types and heterozygotes established in this study was 2.2, which is considerably lower than indicated by the manufacturer and used at the Institute so far (about 3.0). Accordingly, to enhance diagnostic security all patients ranging in an APC ratio borderline area may be tested genetically. This may be between 2.5 and 2.8. In the present study 41 patients are ranging in this area. In addition, patients ranging in the borderline area between homozygosity and heterozygosity (APC ratio between 1.2 and 1.4) may be also tested genetically.

Since the FV^{LEIDEN} mutation is reasonably frequent among Caucasians, and venous thromboembolism accounts for a considerable degree of morbidity and mortality among the affected population, security in testing is of utmost importance. The predominant part of tested patients is female. This reflects the circumstances in which the FV^{LEIDEN} mutation comes in the field of clinical vision. There are some periods in a woman's life in which the thrombotic risk increases, namely pregnancy

and puerperium. Moreover, the combination of heterozygosity in FV^{LEIDEN} and use of oral contraceptives yields a more than 30-fold increased risk for a thrombotic event. Thus, precise functional testing assays should achieve both goals, security in detecting FV^{LEIDEN} positives and economic advantage over the genetic test.

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