

# Evaluation of two functional assays in detection of factor V Leiden carriers

S. Valverde<sup>a</sup>, E. Trabuio<sup>a</sup>, F. Antico<sup>a</sup>, F. Manoni<sup>b</sup>, G. Gessoni<sup>a,c</sup>

<sup>a</sup>Servizio di Medicina di Laboratorio, Azienda ULS 14, Chioggia (VE)

<sup>b</sup>Servizio di Medicina di Laboratorio, Azienda ULS 17, Monselice (PD)

<sup>c</sup>Servizio Trasfusionale, Azienda ULS 14, Chioggia (VE)

## Summary

**Background.** In Clinical Pathology Laboratories the approach to diagnosis of Factor V Leiden is based upon functional tests that allowed the revelation of an activated protein C resistance (APCr) phenotype by using a PTT based assay. In this study the authors evaluated efficiency of two functional assays in comparison with to genetic diagnosis.

**Materials and Methods.** We considered 292 patients after an episode of deep venous thrombosis. These subjects were evaluated by using routine protocol adopted in our Laboratory. Moreover all these samples were tested by using beside the routine PTT based assay, with a new PT based test for revelation of APCr.

**Results.** Among the considered 292 patients the genetic study identified 124 (42.5%) FVL carriers: 111 heterozygous and 13 homozygotes. In discrimination between normal subjects and FVL carrier the aPTT based assay had a SE 1.00 and SP

of 0.99, in discrimination between homozygous the performance was less satisfactory (SE=0.93, SP=0.96). In discrimination between normal subjects and FVL carrier the PT based assay had a SE 1.00 and SP of 1.00, in discrimination between homozygous the performance also very good (SE=1.00, SP=0.99).

**Conclusions.** The PTT based test showed a satisfactory analytical performance to discriminate normal subjects from FVL carriers but are less useful to discriminate between heterozygous and homozygous patients. In our experience the PT based APC assay provides improved discrimination between normal subjects and FVL carriers compared with classical PTT based methods, moreover this assay allowed a good discrimination between homozygous and heterozygous FVL patients.

**Key-words:** Thrombophilia, Factor V Leiden, APC resistance, Functional tests.

## Introduction

Activated protein C (APC) degrades factor Va (FVa) and factor VIIIa (FVIIIa) by cleavage at specific arginine residues. In factor V Leiden (FVL), a single point mutation in position 1691 from guanine to adenine in FV genes, replaces in position 506 an arginine to a glutamine residue. This results in a ten-fold decrease in the rate of FVa inactivation by APC and an APC resistance (APCr) phenotype<sup>1</sup>. The great majority of APCr phenotype is due to presence of FVL and this mutation leads to the most common inherited form of venous thrombophilia. Heterozygosis for FVL occurs

in 3-8%, homozygosis is around 1/5,000 of the general population in Italy<sup>2</sup>. The odds ratio for venous thrombosis is 3-10 for heterozygous and 40-80 for homozygous individuals over the baseline for thrombosis in the general population<sup>2</sup>.

In clinical laboratories the diagnosis of FVL is approached by using a functional screening test (APCr) to identify the phenotype followed by a genetic assay to identify the genotype. The APCr test consists in an activated partial thromboplastin time (PTT) performed in presence and absence of exogenous APC. In normal subjects APC degrades the patient's FVa (and FVI-

IIa) and prolongs PTT. In FVL carriers APC degrades slightly the patients FVa and PTT does not become prolonged. The ratio of PTT with and PTT without added APC is calculated. Usually normal subjects have a ratio >2.0 however there is a considerable overlap between normal subjects and heterozygous carriers<sup>3,4</sup>.

The aim of this study is a comparison between two commercial functional assays designed to detect APCr phenotype. In a group of Italian patients with previous venous thrombosis the authors compared the analytical performance of a functional prothrombin time (PT) based APCr test with an aPTT based APCr test. Particular attention was reserved to correlation with genetic assay and efficiency in discrimination between homozygous and heterozygous carriers.

## Materials and Methods

**Patients.** In this study we considered plasma samples stored at -80 °C obtained from 292 consecutive patients observed from May 2004 until June 2007, to evaluate the presence of a hypercoagulable status, after an episode of venous thrombosis (VTE). All these patients are of Italian ancestry with age from 16 to 89 years (mean 49 years), 186 (64%) were female and 106 (36%) were males.

**Antiphospholipids antibodies.** To investigate the presence of lupus anticoagulant (LAC) we adopted two commercial methods: PTT Lupus and DRVVT Screening supplied by Biopool, Dublin Ireland. To investigate the presence of anti phospholipids autoantibodies we adopted commercial ELISA methods supplied by Instrumentation Laboratories, Milan, Italy: anti-β2 Glycoprotein 1 antibodies (anti-b2GP1) IgG and IgM, anti-cardiolipin antibodies (aCL) IgG and IgM, anti-prothrombin antibodies (anti-PT) IgG and IgM.

**Research of Gene Mutation.** For genetic tests we adopted a commercial automated analyser LightCycler with software version 3.5, and commercial kits: Factor V Leiden Kit for detection of FV Leiden G1691A mutation, Factor II Prothrombin G20210 Kit for detection of Prothrombin G20210A mutation, MTHFR C677T for detection of MTHFR C677T mutation. All those kits adopted a real-time PCR with LightCycler Red 640 FRET probes. Analyser and assay methods were supplied by Roche, Milan, Italy.

**Diagen PCA-Ratio test.** (Diagen APC-R) is a functional PTT based assay. This test was performed by using Sysmex CA 7000 following the manufacturer's instructions. Briefly 10 microliters of patient plasma were diluted with 40 microliters of FV deficient plasma. The diluted sample was placed in a cuvette and, after an incubation period (60 seconds at 37° C), 50 microliters of a PTT reagent containing a PC activator were added. After a second incubation period (300 seconds at 37 °C) 50 microliters of CaCl 0.025 M in saline solution were added and the coagulation time (aPTT) was detected. A second determination is performed

under identical condition with the exception that no PC activator is added to the second reagent. Thus the aPTT is determined with and without inactivation of FVa by APC and the ratio between APC (+) and APC (-) analysis is calculated: ratios under 2.2 are associated with APCr phenotype<sup>5</sup>. Reagents and analyser were supplied by Dasit, Milan, Italy.

**Pefakit APC-R FVL test.** (Pefakit APC-R) (Pentapharm Ltd, Basel, Switzerland) is a functional prothrombin time based assay. This test was performed by using Sysmex CA 7000 following the manufacturer's instructions. Briefly, in this test two aliquots of 30 microliters of patient plasma were diluted with 20 microliters of a reagent containing FV depleted plasma. The diluted samples were placed in a couple of cuvettes with 50 microliters of a reagent (with or without APC) containing a snake venom specifically activating the FV in the plasma samples (RVV-V, Russel viper venom from *Daboia russelli*) and polybrene. During an incubation period (180 seconds at 37°C) activated FV is hydrolysed by the added APC. The velocity of the FVa inactivation depends on their binding kinetics to APC and is decelerated significantly in presence of FVL mutation. Subsequently 50 microliters of a second reagent containing an FVa dependent prothrombin activator (Noscarin venom from *Notechis scutatus*) and EDTA is added. The PTA converts prothrombin in thrombin and leads to clotting of the sample and the clotting times were recorded. If the FVa molecules have been eliminated during the incubation step the velocity of prothrombin activation is slower compared to an incomplete FVa elimination due to a FVL mutation. In this second situation the velocity of prothrombin activation is higher and the clotting time (PT) is shorter. A second determination is performed under identical condition with the exception that no APC is added to the first reagent. Thus the PT is determined with and without inactivation of FVa by APC and the ratio between APC (+) and APC (-) analysis is calculated (Pefakit APC-R) (Pentapharm Ltd, Basel, Switzerland) is a functional PT based assay<sup>6-8</sup>.

**Statistical analysis.** We considered the following parameters: mean (ME) with 95% confidence interval (IC), median (MD) with 95% confidence interval (IC), standard deviation (SD), interquartile range (IQR), percentile (PER). Moreover, after classification as true positive (TP) false positive (FP) false negative (FN) and true positive (TN), sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), correctly classified incidence (CCI) was evaluated. Statistical analysis was performed with the Analyse.it software, version 2.03 (Analyse.it Software Ltd, Leeds UK).

## Results

By using the genetic test to demonstrate G1691A mutation we observed, among the 292 considered patients, 168 (57.5%) normal subjects and 124 (42.5%)

**Table I.** Statistical analysis of observed ratio's values.

Genotype	N°	Mean	SD	Median	Min	Max	2.5°per	97.5°per
<b>Pefakit APC-R</b>								
Norm.	168	4.88	0.95	4.87	3.14	9.63	3.31	7.23
Hetero.	111	1.49	0.10	1.51	1.00	1.78	1.30	1.68
Homo.	13	1.01	0.01	1.01	1.00	1.02	ND	ND
<b>Diagen APC-R</b>								
Norm.	168	3.69	0.73	3.61	1.98	6.60	2.45	5.00
Hetero.	111	1.71	0.16	1.70	1.03	2.10	1.35	1.98
Homo.	13	1.18	0.07	1.20	1.06	1.26	ND	ND

Norm. = normal subjects, Hetero = heterozygous subjects, Homo = homozygous subjects for FVL.

SD = standard deviation, Min = minimal observed value, Max = maximal observed value, 2.5°per = 2.2° percentiles, 97.5°per = 97.5° percentiles.

FV Leiden carriers: 13 (4.5%) homozygous and 111 (38.0%) heterozygous.

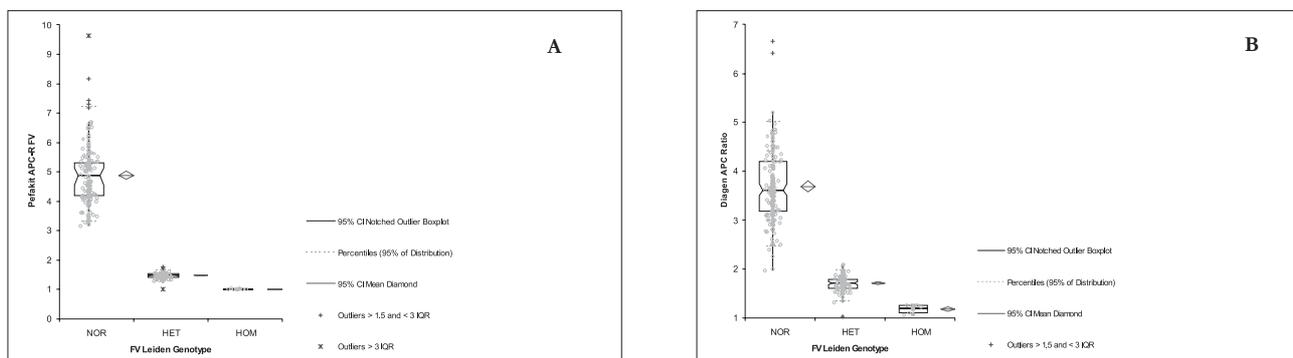
By using the Diagen APC-R assay we observed a mean aPTT ratio of 3.69 (CI 95% from 3.60 to 3.80) among normal subjects; 1.71 (CI 95% from 1.67 to 1.74) among heterozygous carriers; 1.18 (CI 95% from 1.13 to 1.23) among homozygous patients. By using the Pefakit APC-R assay we observed a mean PT ratio of 4.88 (CI 95% from 4.79 to 5.03) among normal subjects; 1.49 (CI 95% from 1.47 to 1.51) among heterozygous carriers; 1.01 (CI 95% from 1.00 to 1.01) among homozygous patients. These results were reported in Table I.

Distribution of the values observed in normal subjects, heterozygous and homozygous patients with Diagen APC-R test and Pefakit APC-R is shown in Figure 1.

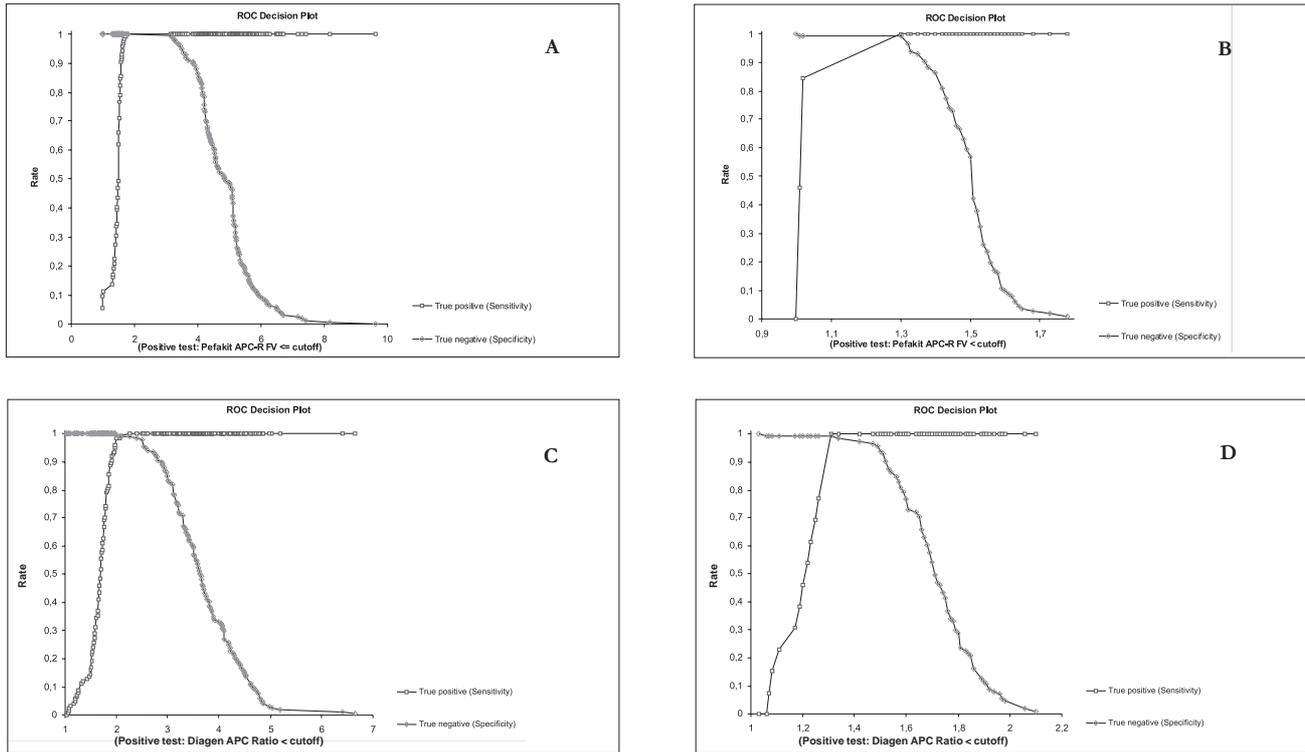
By elaboration of ROC Curves we established experimental cut-off to discriminate normal subjects from FLV carriers and among this group between heterozygous and homozygous patients. These data are reported in Figure 2 in which are reported the experimental

ROC decision plots.

To discriminate normal subjects from FVL carriers the cut-off value adopted for Pefakit APC-R assay was 2.50 (each value between 1.80 and 3.10 gave equivalent results). This cut-off value allowed a correct classification all the considered subjects with 124 TP and 168 TN. To discriminate normal subjects from FVL carriers the cut-off value adopted for Diagen APC-R assay was 2.26. This cut-off value allowed identification of 124 TP, 2 FP and 166 TN subjects. To discriminate between heterozygous and homozygous FVL patients the cut-off value adopted for Pefakit APC-R was 1.10 (each value between 1.03 and 1.3 gave equivalent result). This cut-off value allowed identification of 13 TP, 1 FP and 110 FN subjects. To discriminate between heterozygous and homozygous FVL patients the cut-off value adopted for Diagen APC-R was 1.30. This cut-off value allowed identification of 12 TP, 4 FP, 1 FN and 106 TN subjects. In Table II were showed the characteristics of SE, SP, PPV and NPV on Pefakit APC-R and Diagen APC-R assays in both these applications.

**Figure 1.** Ratio values and presence of FVL mutations.

A) distribution of ratio values obtained by using Pefakit APC-R assay in comparison with the presence of FVL mutations. B) distribution of ratio values obtained by using Diagen APC-R assay in comparison with the presence of FVL mutations. The considered patients were subdivided in three groups: normal subjects (NOR), heterozygous (HET) and homozygous (HOM) FVL carriers. For each group were reported the ratio values: PT ratio for Pefakit APC-R assay and PTT ratio for Diagen APC-R assay. The Blue diamonds report the mean values with 95% CI. The black boxes report median values with 95% CI and limits for 2.5 and 97.5 percentiles.



**Figure 2.** ROC curves.

A) ROC Decision Plot for Pefakit APC-R FV. Normal subjects versus FLV carriers. B) ROC Decision Plot for Pefakit APC-R FV in FVL carriers. Heterozygous versus homozygous. C) ROC Decision Plot for Diagen APC-R FV. Normal subjects versus FLV carriers. D) ROC Decision Plot for Diagen APC Ratio in FVL carriers. Heterozygous versus homozygous.

**Discussion**

By using genetic tests as standard, in the considered population the overall prevalence of FVL carriers was 42.5%, the prevalence of homozygous patients was 4.5%. These results were higher than the prevalence reported in literature in patients with VTE of around 15-18%<sup>9-12</sup>.

In our experience both the considered assay were able to well discriminate between normal subjects and FVL carriers. In this application Pefakit APC-R assay showed a perfect correlation with the genetic test allowing a correct identification of 124 FVL carriers (13 homozygous and 111 heterozygous) and of 168 normal subjects; as consequence SE and SP in this applica-

tion was 1.00. By using the Diagen APC-R assay, in this application we observed 2 FP subject, these subjects were positive (at high titre) for antibodies directed against phospholipids (aPL); as consequence SE was 1.00 and SP was 0.99. More critical appeared the use of these functional assays in discrimination between heterozygous and homozygous FVL carriers. In this application, by using Pefakit APC-R assay, we observed 13 TP, 1 FP and 110 TN; as consequence SE was 1.00 and SP was 0.99. By using Diagen APC.R assay we observed 12 TP, 4 FP, 1 FN and 106 TN: as consequence SE was 0.93 and SP was 0.96. Among samples with a false positive results, two were positive (at high titre) for antibodies directed against phospholipids.

**Table II.** Analytical performance of Pefakit APC-R and of Pefakit APC-R.

Group Comparison	SE	SP	PPV	NPV
Pefakit APC-R				
Normal vs FV carriers	1.00	1.00	1.00	1.00
Hetero vs Homo	1.00	0.99	0.93	1.00
Diagen APC-R				
Normal vs F V carriers	1.00	0.99	1.00	0.98
Hetero vs Homo	0.93	0.96	0.76	0.99

SE=sensitivity, SP= specificity, PPV=positive predictive value, NPV=negative predictive value.

By using both the considered assays one heterozygous subjects was classified as homozygous. In this patient we observed a very low FV activity (45%). Thus we classified this patient as pseudo-homozygous<sup>13,14</sup>.

aPL are an heterogeneous group of antibodies directed against phospholipids. In our study we evaluated the presence of LAC, anti-PT IgG and IgM, anti- $\beta$ 2GP1 IgG and IgM, aCL IgG and IgM. It is well known the ability of aPL to induce APC resistance maintaining the APC response at low level and preventing the interactions of coagulation proteins on the phospholipids surface<sup>15,16</sup>. By using classical aPTT based APC resistance assays the presence of aPL is considered a major interfering factor and in the past years it was often considered a reason to avoid the determination of APCr in these patients. To minimise these interference it was suggested, for more recent tests, to dilute plasma samples before the assay, but usually these patients should be evaluated directly with a genetic assay<sup>17</sup>. In this study, we observed these interference only for the aPTT based assay and not for the PT based one. This fact should be explained by considering the constructive characteristics of Pefakit APC-R test that is performed in the absence of calcium ions: the citrated plasma sample is not recalcified and additional EDTA is added. So no phospholipids are present in the reagent because the formation of phospholipids based complexes need the presence of free calcium ions<sup>18</sup>.

### Acknowledgements

We thank Aurelio Pacioni BioScD and Vincenzo Scala BioScD Marketing Scientific Managers of Dasit SpA, Italy, for their expert technical assistance and helpful discussion.

### References

1. Tripodi A, Mannucci P. Laboratory investigation of thrombophilia. *Clin Chem* 2001; 47:1597-606.
2. Somma J, Sussman I, Rand J. An evaluation of thrombophilia screening in an urban tertiary care medical center: a "real world" experience. *Am J Clin Pathol* 2006; 126:1-8.
3. League S, Hooper W. Molecular diagnosis of inherited thrombosis. *Clin Lab Sci* 2005; 18:271-9.
4. Moll S. Thrombophilias – practical implications and testing caveat. *J Thromb Thrombolysis* 2006; 21:7-15.
5. Wilmer M, Stocker C, Buhler B, Conell B, Calatzis A. Improved distinction of factor V wild-type and Factor V Leiden using a novel prothrombin based activated Protein C resistance assay. *Am J Clin Pathol* 2004; 122:836-42.
6. Schoni R. The use of snake venom derived compounds for new functional test kits in the field of haemostasis. *Pathophysiol Haemost Thromb* 2005; 34:234-40.
7. Valverde S, Gessoni G, Canistro R, Basso D, Navaglia F. Valutazione di un esame funzionale basato sul tempo di protrombina nello studio della resistenza alla proteina C attivata. *Bioc Clin* 2007; 31:567-72.
8. Gessoni G, Valverde S. Clinical evaluation of a functional prothrombin time based assay for identification of factor V Leiden carriers in a group of Italian patients with venous thrombosis. *Blood Coagul Fibrinolysis* 2007; 18: 603-10.
9. De Stefano V, Rossi E, Paciaroni K, Leone G. Screening for inherited thrombophilia: indications and therapeutic implications. *Haematologica* 2002; 87:1095-108.
10. Paschoa A, Guillaumon A. Impact of screening on thrombophilia for patients with venous thrombosis. *Int Angiol* 2006; 25:52-9.
11. Franchini M, Veneri D, Salvagno G, Manzato F, Lippi G. Inherited thrombophilia. *Crit Rev Clin Lab Sci* 2006; 43:249-50.
12. Vig S, Chitolie A, Bevan D, Dormandy J, Thompson M, Halliday A. The prevalence of thrombophilia in patients with symptomatic peripheral vascular disease. *Br J Surg* 2006; 93:577-81.
13. Brugge J, Simioni P, Bernardi F, Tormene D, Lunghi B, Tans G, et al. Expression of the normal factor V allele modulates the APC resistance phenotype in heterozygous carriers of the factor V Leiden mutation. *J Thromb Haemost* 2005; 3:2695-702.
14. Castoldi E, Kalafatis M, Lunghi B, Simioni P, Ioannou P, Petio M, et al. Molecular bases of pseudo homozygous APC resistance: the compound heterozygosity for FV R506Q and a FV null mutation results in the exclusive presence of FV Leiden molecules in plasma. *Thromb Haemost* 1998; 80:403-6.
15. Nojima J, Kuratsume H, Suehisa E, Iwarani Y, Kanakura Y. Acquired activated protein C resistance associated with IgG antibodies against beta2-glycoprotein 1 and Prothrombin is a strong risk factor for venous thromboembolism. *Clin Chem* 2005; 51:545-52.
16. Gardiner C, Cohen H, Jenkins A, Machin S, Mackie I. Detection of acquired resistance to activated protein C associated with antiphospholipid antibodies using a novel clotting assay. *Blood Coag Fibrinolysis* 2006; 17: 477-83.
17. Tripodi A. Issues concerning the laboratory investigations of inherited thrombophilia. *Mol Diagn* 2005; 9:181-6.
18. Schoni R, Quehenberger P, Wu J, Wilmer M. Clinical evaluation of a new functional test for detection of activated protein C resistance Pefakit APC-R Factor V Leiden at two centers in Europe and the USA. *Thromb Res* 2007; 119: 17-26.