Activated protein C resistance, data from a population-based case-control study

Rosendaal, F.R.

British Journal of Haematology, 1996, 93, 694–699

Activated protein C resistance: a comparison between two clotting assays and their relationship to the presence of the factor V Leiden mutation

C. LEGNANI,[1] G. PALARETI,[1] R. BIAGI,[1] S. COCCHERI,[1] F. BERNARDI,[2] F. R. ROSENDAAL,[3] P. H. REITSMA,[4] H. DE RONDE[4] AND R. M. BERTINA[4] [1]*Department of Angiology and Blood Coagulation, University Hospital S. Orsola, Bologna, Italy,* [2]*Centro Studi Biochimici delle Patologie del Genoma Umano-Istituto di Chimica Biologica, University of Ferrara, Italy,* [3]*Department of Clinical Epidemiology and* [4]*Haemostasis and Thrombosis Research Centre, University Hospital Leiden, The Netherlands*

Received 18 September 1995; accepted for publication 28 February 1996

Summary. Resistance to the anticoagulant effect of activated protein C (APC resistance), a frequent abnormality in patients with a history of venous thrombosis, is known to be due, in the large majority of cases, to the presence of an abnormal factor V: the factor V Leiden. It is reasonable to surmise that screening for this abnormality should be performed with a clotting method for APC resistance, before submitting the patients with abnormal results to DNA analysis. The present study was performed on 216 individuals enrolled at the Bologna centre, of which 189 were unrelated patients with a history of juvenile venous thromboembolism and 27 were relatives with or without thrombosis. APC resistance was first measured in Bologna by a standard commercial method and then, in Leiden, by an in-house method; DNA analysis was performed in those cases in which at least one of the clotting methods was abnormal. The data obtained confirm the good performance and the optimal positive predictive value for the Leiden mutation (100%) of the Leiden in-house clotting method. Performance of the commercial method was less satisfactory but markedly improved by expressing the data in relation to the values simultaneously obtained with a normal plasma pool. Even with optimal data expression, however, the positive predictive value of the commercial method, versus DNA analysis, did not exceed 88%.

It is concluded that further standardization of the commercial method here evaluated is necessary before it can be widely adopted for the screening of APC resistance and prediction of the presence of factor V Leiden.

Keywords: APC resistance, factor V Leiden mutation, thrombophilia.

A previously unrecognized mechanism for thrombophilia, characterized by a poor anticoagulant response to activated protein C (APC), was recently described by Dahlbäck *et al* (1993) in several families with an inherited tendency to thrombosis. This poor response to APC was then shown to be due in the large majority of cases to a selective defect in factor V (R506Q or factor V Leiden mutation), a factor that not only expresses procoagulant properties but also plays a role in the anticoagulant system as an important target of APC (Bertina *et al*, 1994; Dahlbäck & Hildebrand, 1994). The so-called 'APC resistance' has been found to be the most frequent alteration in previously undiagnosed thrombotic patients with a prevalence ranging from 20% to 65% in different studies (Faioni *et al*, 1993; Griffin *et al*, 1993; Koster *et al*, 1993; Cadroy *et al*, 1994; Cushman *et al*, 1994; Halbmayer *et al*, 1994; Legnani *et al*, 1994; Svensson & Dahlbäck, 1994). On the basis of these results, APC resistance seems far from being a rarity; it has therefore been suggested that all thrombotic patients should be tested for this abnormality (Koster *et al*, 1993).

In a series of patients suffering from juvenile thromboembolic episodes recruited in the Bologna centre, a poor anticoagulant response to APC was found in about 20% of cases (Legnani *et al*, 1994). However, a very high variability of results was observed when the patients were re-tested, the diagnosis being confirmed in only about half the patients with a first abnormal test. On this basis, it may be surmised that some methodological problems could be, at least in part,

Correspondence: Dr Cristina Legnani, Department of Angiology and Blood Coagulation, University Hospital S. Orsola, Via Massarenti 9, 40138 Bologna, Italy.

responsible for these inconsistencies. Difficulties in standardization of clotting APC resistance assays could also explain some of the differences in the prevalence of APC resistance observed in different series of thrombophilic patients, albeit recruited with different criteria (Faioni *et al*, 1993; Griffin *et al*, 1993; Koster *et al*, 1993; Cadroy *et al*, 1994; Cushman *et al*, 1994; Halbmayer *et al*, 1994; Legnani *et al*, 1994; Svensson & Dahlbäck, 1994).

The aim of this study was to compare the results of two different APC-resistance tests in a series of young thrombophilic patients by using: (1) the commercial method customarily used in the Bologna laboratory, and (2) the in-house method developed at the Leiden laboratory, which proved to have a very high sensitivity and specificity in detecting the factor V Leiden mutation (Bertina *et al*, 1994; de Ronde & Bertina, 1994). The results of these two clotting methods were then compared with those of DNA analysis performed to identify the presence of the factor V Leiden mutation.

CLINICAL MATERIAL AND METHODS

Samples. APC resistance tests were performed on plasma of 216 individuals: 189 unrelated patients (88 males, 12–73 years) whose first or only venous thromboembolic episode (VTE) occurred before 45 years, and 27 relatives (with or without previous thrombotic episodes, 13 males) of 15 propositi. A second blood sample taken from 37 of the 189 above-mentioned patients was also tested to assess reproducibility of results.

Blood was sampled at least 3 months after the last or single venous thromboembolic episode and 3 weeks after withdrawal of any antithrombotic treatment. Samples from patients with evidence of lupus-like anticoagulant or prolonged aPTT for any causes were excluded.

Venous blood samples were collected from a forearm vein, anticoagulated with trisodium citrate (0·129 M, 1/10), kept in melting ice until centrifugation at 3000 *g* at room temperature for 30 min, and immediately snap frozen. Both tests were performed on plasma samples, stored at −70°C for a period ranging from 1 week to 3 years. Plasma samples (one coded aliquot for each patient) were shipped on dry ice to the laboratory in Leiden and were received in good condition.

Commercial method (Bologna). The anticoagulant response of plasmas to APC was evaluated in Bologna by a commercial method (Chromogenix, Mölndal, Sweden), using an ACL-300 (Instrumentation Laboratory, Milan, Italy) with the research programme. The results were expressed as follows: (1) as APC ratio (APC-SR), obtained by dividing the aPTT plus APC by the aPTT minus APC; (2) as normalized APC-SR (n-APC-SR), obtained by dividing the APC-SR of the patient sample by the APC-SR of the normal plasma pool; (3) as per cent APC sensitivity (APC-Sens%), calculated as $(\text{aPTT plus APC} - \text{aPTT minus APC})_{\text{sample}} / (\text{aPTT plus APC} - \text{aPTT minus APC})_{\text{pool}} \times 100\%$.

Normal plasma pool was prepared from donations of a large number (>100) of apparently healthy subjects, separated in aliquots and stored at −70°C.

Five different batches of the APC resistance kit were used (lot nos.: X0548, X0705, X0725, X0727, X0922) and the normal plasma pool was tested in each working session. The inter-assay coefficients (CV%) of variation were calculated on the basis of the results of a commercial lyophilized normal plasma (Citrol, Baxter), and were 9·8%, 9·7% and 9·3% for the APC-SR, the n-APC-SR and the APC-Sens% values, respectively.

The normal values were obtained from 70 healthy age-matched subjects (APC-SR: mean 3·06, SD 0·73, range 1·99–4·95; n-APC-SR: mean 1·06, SD 0·19, range 0·68–1·54; APC-Sens%: mean 122%, SD 31%, range 70–196%); none of these subjects gave results outside 3 SD above or below the mean, the minimum value (APC-SR 1·99; n-APC-SR 0·68; APC-Sens% 70%) being selected as the lower limit of the normal range.

In-house method (Leiden). The anticoagulant response of plasmas to APC was also measured in Leiden using the in-house assay, following the procedure described in details elsewhere (de Ronde & Bertina, 1994). Results were expressed as n-APC-SR, and a value ⩽0·70 was considered to be abnormal.

DNA analysis. DNA analysis was performed in Leiden as described elsewhere (Bertina *et al*, 1994). Briefly, the presence of the factor V Leiden mutation was shown by *MnlI* digestion of amplified factor V DNA and visualization of the cleavage products on ethidium-bromide-stained agarose gels. In a few cases DNA analysis was performed in the Ferrara laboratory (Centro Studi Biochimici delle Patologie del Genoma Umano–Istituto di Chimica Biologica, University of Ferrara, Italy), following the same procedure as mentioned above. Patients were selected for DNA analysis on the basis of abnormal results obtained with both or at least one of the clotting methods. The data obtained with the commercial method were considered abnormal when either APC-SR or n-APC-SR or APC-Sens% were equal or below the lower limit of normal.

Statistical analysis. In all 216 samples we used both clotting tests, and so we could compare the prevalence of abnormal results and the number of concordant and discordant results with the two tests. The availability of a second sample in 37 of these patients made it possible to assess the consistency of both tests, by comparing the results on the first and second sample.

We recalled patients who had an abnormal result in either or both (Bologna/Leiden) of the tests, and performed DNA analysis for factor V Leiden as a 'gold standard'. First, this enabled us to calculate the positive predictive value of each of the tests, which is defined as the proportion among those with an abnormal test result who have the disease the test is aimed at (in this instance, carriers of the mutation among those with a positive clotting test). Since the positive predictive value is defined among those with a positive (clotting) test, and since we performed DNA analysis in all individuals with positive result, the estimates of the predictive values for both tests are unbiased. Confidence intervals were based on the normal approximation to the binomial distribution.

The sensitivity (proportion of positive clotting tests among

Table I. Number (%) of concordant (normal and abnormal) and discordant results of commercial versus in-house method for the identification of APC resistance in 216 patients. Results of the commercial method are expressed as APC-SR, n-APC-SR and APC-Sens%.

In-house method			
Result expression, n-APC-SR cut-off limit, ⩽0·70			
Abnormal results, n = 43 (20%)			
Commercial method			
Result expression, cut-off limit	APC-SR ⩽1·99	n-APC-SR ⩽0·68	APC-Sens% ⩽70%
Abnormal results	n = 45 (21%)	n = 22 (10%)	n = 47 (22%)
Concordant normal results	152 (70·4%)	170 (78·7%)	163 (75·5%)
Concordant abnormal results	24 (11·1%)	19 (8·8%)	37 (17·1%)
Discordant results	40 (18·5%)	27 (12·5%)	16 (7·4%)

gene carriers) and specificity (proportion of negative clotting test among wildtype individuals) cannot be estimated without bias in our design, since we did not perform DNA tests in all individuals. However, the ratio of the observed sensitivities is identical to the ratios of the true sensitivities (see Appendix).

RESULTS

The laboratory criteria for APC resistance were fulfilled in 43/216 (20%) cases by the Leiden in-house method and in 45 (21%), 22 (10%) and 47 (22%) cases by the commercial method employed in Bologna, using APC-SR, n-APC-SR and APC-Sens%, respectively (see Table I). The number of concordant (normal and abnormal) and discordant results between the two methods are reported in Table I. The percentage of discordant results obtained by the commercial versus the in-house method differed greatly depending on the way results were expressed. The disagreement was greater (18·5%) when the results were expressed as APC-SR; in contrast, discordance was effectively reduced if values were presented so as to take account of the results of a normal plasma pool obtained in the same test run (n-APC-SR 12·5%, APC-Sens% 7·4%). To assess whether the prolonged period of storage of some samples (up to 3 years) could have contributed to the large number of discordant results, samples were evaluated separately according to the year of freezing; concordance of results, however, did not improve (data not reported). Though samples with the lupus anticoagulant (LAC) phenomenon were excluded from the study, anticardiolipin levels (IgG and IgM) were measured in all examined samples; the results, however, were always within the normal ranges in those samples whose APC resistance results were discordant with the two methods (data not shown).

Consistency of results of the two methods was investigated by examining the 37 patients sampled at two different occasions. As can be seen in Table II, the commercial method gave a higher inconsistency compared to the in-house method. The percentage of inconsistent results obtained with the commercial method was largely affected by the way results were expressed, being higher for APC-SR (43·3%) and lower for n-APC-SR (16·2%). The in-house method proved inconsistent in only 3/37 (8·1%) cases.

Subjects with either concordant abnormal or discordant results with the two methods (66 patients) were invited for further blood sampling to perform DNA analysis; 51 subjects presented. The factor V Leiden mutation was identified in 39/49 patients (all heterozygous carriers). The individual results of the two clotting methods (presence or absence of factor V Leiden mutation) are plotted in Fig 1. In the 37 patients tested twice, the lower value obtained was used.

The sensitivity ratio of the commercial versus the in-house method differed greatly according to the ways of expressing the results of the former, the ratio being 0·59, 0·56 and 0·92 using APC-SR, n-APC-SR and APC-Sens% values respectively. Table III reports the positive predictive values of the two tests vs the presence of factor V Leiden mutation.

Table II. Number (%) of consistent (normal and abnormal) and inconsistent results obtained by the commercial and in-house methods for the identification of APC resistance in 37 patients, sampled on two different occasions. Results of the commercial method are expressed as APC-SR, n-APC-SR and APC-Sens%.

	Commercial method			In-house method
	APC-SR ⩽1·99	n-APC-SR ⩽0·68	APC-Sens% ⩽70%	n-APC-SR ⩽0·70
Consistent normal results	14 (37·8%)	30 (81·1%)	19 (51·4%)	22 (59·5%)
Consistent abnormal results	7 (18·9%)	1 (2·7%)	9 (24·3%)	12 (32·4%)
Inconsistent results	16 (43·3%)	6 (16·2%)	9 (24·3%)	3 (8·1%)

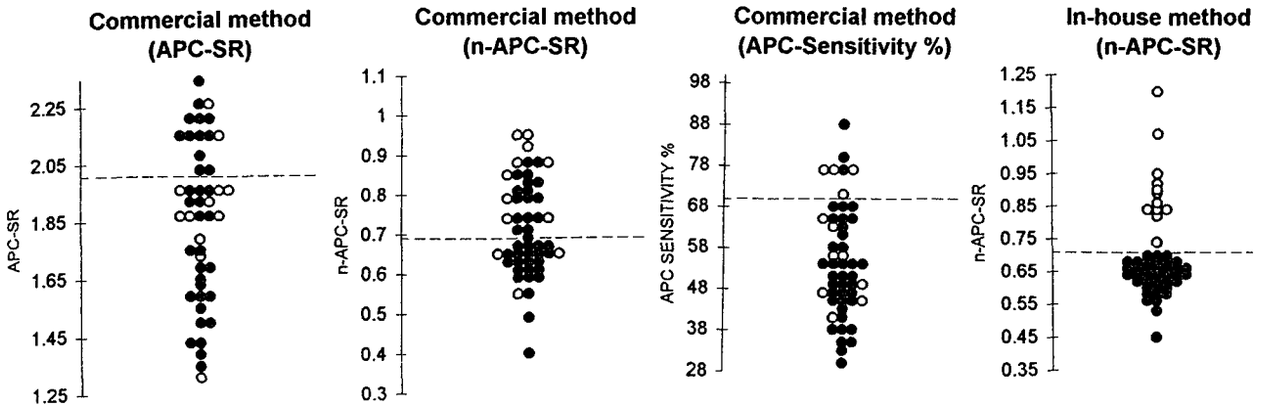

Fig 1. Individual results of the commercial and in-house methods for identification of APC-resistance phenomenon versus those of DNA analysis performed to identify the presence (closed circles) or absence (open circles) of the factor V Leiden mutation. Results of the commercial method are separately plotted as APC-SR, N-APC-SR and APC-Sens%. For 37 patients tested on two different occasions the lower values obtained have been used. Dotted line = lower normal limit. Commercial method cut-off limits: APC-SR ⩽ 1·99; n-APC-SR ⩽ 0·68; APC-Sens% ⩽ 70%; in-house method cut-off limit: n-APC-SR ⩽ 0·70.

Table III. Results (95% confidence interval [95% CI]) of positive predictive value (PV+) of the commercial and in-house methods for the identification of APC resistance, calculated on the basis of results of DNA analysis performed to identify the presence of factor V Leiden mutation. Results of the commercial method are separately calculated for the three different ways of expressing results. For 37 patients tested on two different occasions, the lower values obtained have been used.

	Commercial method			In-house method
	APC-SR ⩽1·99	n-APC-SR ⩽0·68	APC-Sens% ⩽70%	n-APC-SR ⩽0·70
PV+ (95% CI)	69·7% (54–85%)	88·8% (75–100%)	84·1% (73–95%)	100% (91–100%)

DISCUSSION

APC resistance, in the majority of cases due to factor V Leiden mutation, has been reported as being common in the general population, with a prevalence of 3–7% of subjects in different countries (Koster *et al*, 1993; Beauchamp *et al*, 1994; Bertina *et al*, 1994; Svensson & Dahlbäck 1994). This alteration – to date mainly diagnosed by clotting methods only – has been quoted as the single most frequent abnormality in patients suffering from thrombotic episodes (Faioni *et al*, 1993; Griffin *et al*, 1993; Koster *et al*, 1993; Cadroy *et al*, 1994; Cushman *et al*, 1994; Halbmayer *et al*, 1994; Legnani *et al*, 1994; Svensson & Dahlbäck, 1994). However, its prevalence in thrombotic patients has in different studies, shown great variability (20–65%) only partially attributable to the different criteria used for patient selection (Faioni *et al*, 1993; Griffin *et al*, 1993; Koster *et al*, 1993; Cadroy *et al*, 1994; Cushman *et al*, 1994; Legnani *et al*, 1994; Svensson & Dahlbäck, 1994). In the experience of the Bologna Centre, a poor response of plasmas to APC was the most frequent alteration detectable in patients with juvenile thromboembolism (21% of cases; Legnani *et al*, 1994).

On the basis of these figures, it has been suggested that all thrombotic patients should be tested for APC resistance. The question has also been raised whether it is worthwhile to screen for this abnormality subjects before surgery, during pregnancy and before oral contraception (Cook *et al*, 1994; Vandenbroucke *et al*, 1994). It is therefore necessary to investigate large groups of subjects for this abnormality.

Clotting methods for APC resistance are straightforward and may also be performed in non-specialized laboratories; they are rapid and can be completely automated. Furthermore, they might allow the identification of APC resistance phenomena caused by conditions other than the one to date identified (factor V Leiden). Zöller *et al* (1994) have in fact reported a limited number of patients with APC resistance without factor V mutation, suggesting that other, as yet unknown, alterations may cause a reduced response to activated protein C. Since the APC-resistance test is based on an aPTT system it is not reliable in patients receiving oral anticoagulants, though modifications of the test have recently been proposed to overcome this limitation (Jorquera *et al*, 1994; Trossaërt *et al*, 1994).

On the other hand, DNA analysis, which provides 100% sensitivity and specificity for factor V mutation, could be used to diagnose this condition. However, the genetic test

requires molecular biology expertise, is time-consuming and expensive, and cannot identify APC resistance phenomenon due to other causes.

Clotting assays for APC resistance seem therefore to be a first choice for the screening of this condition in large groups of patients; it is necessary, however, that their diagnostic sensitivity and specificity for factor V mutation prove good enough, since this mutation has already been shown a risk factor for thrombosis (Bertina *et al*, 1995; Rosendaal *et al*, 1995; Zöller *et al*, 1994). However, as previously mentioned, discrepancies in the results of APC-resistance tests have been reported (Alhenc-Gelas *et al*, 1994; Baker *et al*, 1994; Voorberg *et al*, 1994), and in the Bologna experience abnormal results were confirmed only in about 40% of the patients sampled twice (Legnani *et al*, 1994). Several assay conditions (such as aPTT reagent, APC concentration and source, expression of results, instruments used) may account for these discrepancies and proper standardization is highly recommended.

The commercial clotting method from Chromogenix, customarily used in the Bologna laboratory, has recently been evaluated in a multicentre study (Rosen *et al*, 1994) showing good reproducibility. Unfortunately this study only included samples from apparently healthy subjects (not tested for the presence of the factor V Leiden mutation). No information on its diagnostic sensitivity and specificity could therefore be provided.

The two functional methods examined in the present study were not performed in the same laboratory. Though the handling of samples was performed in only one laboratory and the instruments used were of the same type, it cannot be excluded that observed discrepancies in results may be due, at least in part, to the effects of different technologists and ambient conditions. The results obtained in the present study confirm the previously reported good performance of the Leiden method (Bertina *et al*, 1994; de Ronde & Bertina, 1994). Performance of the commercial method seems to be greatly influenced by data expression; in fact, both its positive predictive value and the ratio of its sensitivity versus the sensitivity of the Leiden method markedly improve when the expression of results includes consideration of the value of a normal plasma pool obtained simultaneously (n-APC-SR or APC-Sens instead of APC-SR). Concordance of results of the commercial method versus the Leiden in-house method was greater when they were expressed as APC-Sens than in the other modes (discordant results in 7·4% of cases with APC-Sens, and 18·5% and 12·5% with APC-SR and n-APC-SR respectively). Consistency of results when patients were examined on two different occasions was also greater with the in-house versus commercial method, though the consistency of the latter improved when results were expressed as APC-Sens or n-APC-SR instead of as APC-SR. Since the between assay variability of the commercial method proved to be acceptable (about 9%), the inconsistency of results when patients were sampled twice may be due, at least in part, to a greater sensitivity of the test to some factors involved in the handling of samples (centrifugation, temperature, presence of platelets or platelet debris), as pointed out in the recent collaborative study on the performance of this method (Rosen *et al*, 1994).

The conclusions of our study are therefore: (a) adoption of a clotting test for APC-resistance screening is feasible and realistic as proved by the optimal performance of the in-house Leiden method; (b) the performance of the commercial method is highly influenced by the mode of result expression; (c) since the principle of the two clotting methods examined in the study is the same (as were the instruments used in the two laboratories), further standardization of the commercial method reagents and analytic protocols is necessary.

APPENDIX

Our design resembles what has been called 'work-up bias' (Ransohoff *et al*, 1978), when a definite test in clinical practice is applied only to those with a positive first screening test. Because of the selection of individuals with a high probability of disease, estimates of sensitivities will be unduly optimistic, and estimates of specificity unduly pessimistic.

Methods for dealing with work-up bias have been developed for single diagnostic tests followed by a definite test, and require additional information, e.g. on the prevalence of the disorder (Choi *et al*, 1992). Our study is different, since we applied two diagnostic tests to all individuals. Although sensitivity and specificity estimates will be biased, it is now possible to arrive at an unbiased estimate of the ratio of the two sensitivities.

The observed sensitivity (S') of test A, when the final 2×2 tables contain only information on DNA status for those positive on one or both of the tests A and B, and S' is calculated in the classic way from these tables, is a function of the unbiased sensitivity (S) of tests A and B:

$$S'(A) = \frac{S(A)}{S(A) + \{1 - S(A)\}.S(B)} = \frac{S(A)}{S(A) + S(B) - S(A).S(B)}$$

in which the numerator reflects those rightly detected by test A, whereas the denominator consists of all gene carriers, either detected by test A (S(A)), or, when test A failed ($1 - S(A)$), by test B (S(B)).

Since S(A) and S(B) will both be less than 1, $S'(A)$ will be greater than S(A), and is an overestimate of the true sensitivity.

Similarly, we will observe for test B:

$$S'(B) = \frac{S(B)}{S(A) + S(B) - S(A).S(B)}$$

From these it follows that:

$$\frac{S'(A)}{S'(B)} = \frac{S(A)}{S(B)}$$

Therefore, although $S'(A)$ and $S'(B)$ are themselves biased estimates of S(A) and S(B), the ratio of the observed sensitivities is identical to the ratios of the true sensitivities. A ratio < 1 indicates a higher sensitivity for test B than for A and vice versa.

ACKNOWLEDGMENTS

This work was supported by funds of CNR (1994) and of the

Emilia-Romagna Region to Professor Coccheri. F.B. acknowledges the financial support of Telethon Italy (Grant NE 125). We thank Stephen Jewkes for revising the manuscript.

REFERENCES

Alhenc-Gelas, M., Gandrille, S., Aubry, M.L., Emmerich, J., Fiessinger, J.N. & Aiach, M. (1994) Unexplained thrombosis and factor V Leiden mutation. *Lancet*, **344**, 555–556.

Baker, R., Thom, J. & van Bockxmeer, F. (1994) Diagnosis of activated protein C resistance (factor V Leiden). *Lancet*, **344**, 1162.

Beauchamp, N.J., Daly, M.E., Hampton, K.K., Cooper, P.C., Preston, F.E. & Peake, I.R. (1994) High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. *British Journal of Haematology*, **88**, 219–222.

Bertina, R.M., Koeleman, B.P.C., Koster, T., Rosendaal, F.R., Dirven, R.J., de Ronde, H., van der Velden, P.A. & Reitsma, P.H. (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*, **369**, 64–67.

Bertina, R.M., Reitsma, P.H., Rosendaal, F.R. & Vandenbroucke, J.P. (1995) Resistance to activated protein C and factor V Leiden as risk factors for venous thrombosis. *Thrombosis and Haemostasis*, **74**, 449–453.

Cadroy, Y., Sie, P. & Boneu, B. (1994) Frequency of a defective response to activated protein C in patients with history of venous thrombosis. *Blood*, **83**, 2008–2009.

Choi, B.C.K. (1992) Sensitivity and specificity of a single diagnostic test in the presence of work-up bias. *Journal of Clinical Epidemiology*, **45**, 581–586.

Cook, G., Walker, I.D., McCall, F., Conkie, J.A. & Greer, I.A. (1994) Familial thrombophilia and activated protein C resistance: thrombotic risk in pregnancy? *British Journal of Haematology*, **87**, 873–875.

Cushman, M., Bhushan, F., Bovill, E. & Tracy, R. (1994) Plasma resistance to activated protein C in venous and arterial thrombosis. *Thrombosis and Haemostasis*, **72**, 643–651.

Dahlbäck, B., Carlsson, M. & Svensson, P.J. (1993) Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 1004–1008.

Dahlbäck, B. & Hildebrand, B. (1994) Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a properties of factor V. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 1396–1400.

de Ronde, H. & Bertina, R.M. (1994) Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. *Thrombosis and Haemostasis*, **72**, 880–886.

Faioni, E.M., Franchi, F., Asti, D., Sacchi, E., Bernardi, F. & Mannucci, P.M. (1993) Resistance to activated protein C in nine thrombophilic families: interference in a protein S functional assay. *Thrombosis and Haemostasis*, **70**, 1067–1071.

Griffin, J.H., Evat, B., Wideman, C. & Fernandez, J.A. (1993) Anticoagulant protein C pathway defective in majority of thrombophilic patients. *Blood*, **82**, 1989–1993.

Halbmayer, W.M., Haushofer, A., Schön, R. & Fischer, M. (1994) Prevalence of poor anticoagulant response to activated protein C (APC resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects. *Blood Coagulation and Fibrinolysis*, **5**, 51–57.

Jorquera, J.I., Montoro, J.M., Fernandez, M.A., Aznar, J.A. & Aznar, J. (1994) Modified test for activated APC resistance. *Lancet*, **344**, 1162–1163.

Koster, T., Rosendaal, F.R., de Ronde, H., Briët, E., Vandenbroucke, J.P. & Bertina, R.M. (1993) Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet*, **342**, 1503–1506.

Legnani, C., Palareti, G., Biagi, R. & Coccheri, S. (1994) Activated protein C resistance in deep-vein thrombosis. *Lancet*, **343**, 541–542.

Ransohoff, D.F. & Feinstein, A.R. (1978) Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. *New England Journal of Medicine*, **17**, 926–930.

Rosen, S., Johansson, K., Lindberg, K. & Dahlbäck, B., for the APC Resistance Study Group (1994) Multicentric evaluation of a kit for activated protein C resistance on various coagulation instruments using plasmas from healthy individuals. *Thrombosis and Haemostasis*, **72**, 255–260.

Rosendaal, F.R., Koster, T., Vandenbroucke, J.P. & Reitsma, P.H. (1995) High risk of thrombosis in patients homozygous for factor V Leiden. *Blood*, **85**,1504–1508.

Svensson, P.J. & Dahlbäck, B. (1994) Resistance to activated protein C as a basis for venous thrombosis. *New England Journal of Medicine*, **330**, 517–522.

Trossaërt, M., Conard, J., Horellou, M.H., Samama, M.M., Ireland, H., Bayston, T.A. & Lane, D.A. (1994) Modified APC resistance assay for patients on oral anticoagulants. *Lancet*, **344**, 1709.

Vandenbroucke, J.P., Koster, T., Briët, E., Reitsma, P.H., Bertina, R.M. & Rosendaal, F.R. (1994) Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation. *Lancet*, **344**, 1453–1457.

Voorberg, J., Roelse, J., Koopman, R., Büller, H., Berends, F., ten Cate, J.W., Mertens, K. & van Mourik, J.A. (1994) Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. *Lancet*, **343**, 1535–1536.

Zöller, B., Svensson, P.J., He, X. & Dahlbäck, B. (1994) Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. *Journal of Clinical Investigation*, **94**, 2521–2524.