# Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study

Rosendaal, F.R.

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# Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study

*Ted Koster, Frits R Rosendaal, Hans de Ronde, Ernest Briët, Jan P Vandenbroucke, Rogier M Bertina*


## Summary

We undertook a population-based case-control study to test the clinical importance of a hereditary abnormality in the coagulation system, characterised by poor anticoagulant response to activated protein C (APC), which is associated with familial thrombophilia.

The abnormality was detected in 64 (21%) of 301 unselected consecutive patients younger than 70 years, with a first, objectively confirmed episode of deep-vein thrombosis and without underlying malignant disease. Among 301 healthy control subjects matched for age and sex, the frequency was 5% (14 subjects). Thus, there is a seven-fold increase in risk of deep-vein thrombosis in subjects with a poor response to APC (matched odds ratio 6 6 [95% CI 3 6–12 0]). In addition, there was a clear inverse relation between the degree of response to APC and thrombosis risk. In the families of the patients an autosomal dominant mode of transmission of the abnormality was confirmed. 9 of 10 thrombosis patients with a poor response to APC had 1 parent with a similar poor response, whereas 9 of 10 patients with normal tests had parents with equally normal tests. The abnormality was found in both parents of 1 patient with an extremely poor response to APC; this patient is probably homozygous for the abnormality.

We conclude that the poor response to APC is the most important hereditary cause of venous thrombosis. Its high prevalence in a series of unselected patients will make testing of all thrombosis patients for this abnormality worth while.


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**Department of Clinical Epidemiology** (T Koster MD, F R Rosendaal MD, Prof J P Vandenbroucke MD) **and the Hemostasis and Thrombosis Research Center** (F R Rosendaal, H de Ronde, Prof E Briet MD, Prof R M Bertina PhD), **University Hospital, Leiden, Netherlands**

**Correspondence to:** Dr T Koster, Department of Clinical Epidemiology, Building 1, CO-P, University Hospital, PO Box 9600, 2300 RC Leiden, Netherlands

## Introduction

Deep-vein thrombosis is a common disease. Well-established risk factors include recent surgery, malignant disorders, pregnancy and labour, long-term immobilisation, and deficiency of one of the main inhibitors of the clotting system—protein C, protein S, or antithrombin III.[1] However, the causes of deep-vein thrombosis in many patients are unclear. Dahlbäck et al[2] reported earlier this year a poor anticoagulant response to activated protein C (APC) in several families with a hereditary tendency to venous thrombosis.

The anticoagulant property of APC lies in its capacity to inactivate the activated cofactors Va and VIIIa by limited proteolysis.[3] It has been generally accepted that this reaction proceeds optimally only in the presence of calcium ions, phospholipids, and the APC co-factor, protein S.[4] However, this view has been challenged by the finding that in systems of purified proteins, protein S has hardly any co-factor activity to APC.[5,6] A possible explanation for the discrepancy between in-vivo observations (thrombotic tendency in hereditary protein S deficiency) and the findings in vitro may be provided by Dahlbäck and colleagues' findings.[2] In their thrombotic patients, addition of APC to plasma did not result in the expected prolongation of the activated partial thromboplastin time (APTT). After ruling out several other theoretical possibilities that could have provoked the poor anticoagulant response to APC, Dahlbäck et al postulated deficiency in these patients of a hitherto unknown co-factor to APC.

Studies within families suggest that the poor response to APC is inherited as an autosomal dominant trait.[2,7] Among patients referred to a coagulation unit because of unexplained thrombosis, this abnormality was an important cause of thrombophilia, with a prevalence of about 40%.[8,9]

We have investigated the clinical importance of the poor response to APC in 301 unselected patients with a first, objectively confirmed episode of deep-vein thrombosis and without underlying malignant disease, by comparing their sensitivity to APC with that of matched healthy controls. The study was part of a population-based case-control study on hereditary venous thrombosis, the Leiden Thrombophilia Study (LETS).

## Patients and methods

Patients were selected from the files of thrombosis centres in Leiden, Amsterdam, and Rotterdam. In the Netherlands, thrombosis centres monitor coumarin treatment in virtually all patients with venous thrombosis, each for patients in a defined geographical area.[10] Since all our patients originated from one of

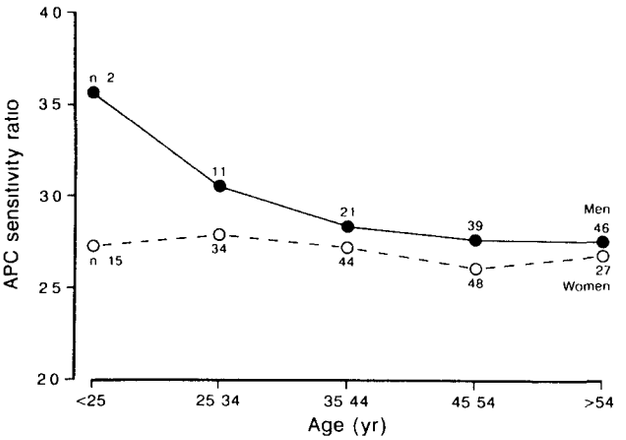


Figure 1 **Mean APC-sensitivity ratio according to sex and age group among controls (n = 287)**

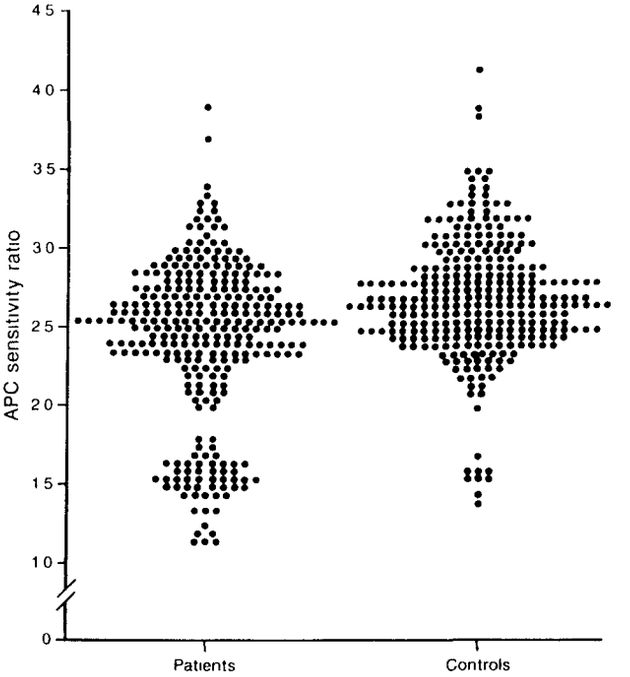


Figure 2 **APC-sensitivity ratios of thrombosis patients and controls**

these areas, geography was the only criterion for entry We included the first 345 consecutive outpatients younger than 70 years, who were referred for anticoagulant treatment because of a first, objectively confirmed episode of deep-vein thrombosis that occurred between Jan 1, 1988, and Dec 31, 1992 Patients with known malignant disorders were excluded Information about the inclusion and exclusion criteria was obtained from general practitioners and from hospital discharge records Patients were seen only after anticoagulant treatment had been discontinued for at least 3 months The median time between the occurrence of deep-vein thrombosis and venepuncture for this study was 18 (range 6–48) months 90% of eligible patients were willing to take part in the study

Each thrombosis patient was asked to find his or her own healthy control subject according to the following criteria same sex, the same age (plus or minus 5 years), no biological relationship, no history of venous thromboembolism, no use of coumarins for at least 3 months, and no known malignant disorders Partners of patients were also invited to serve as control subjects When a patient was unable to find a control, the first individual from the list of partners who matched for age and sex was asked to join the study, 126 (42%) control subjects were partners of patients

To confirm an autosomal dominant mode of transmission and to exclude the possibility of a post-thrombotic phenomenon, we assessed the response to APC in the parents of the first 10 eligible patients with poor responses to APC and in the parents of 10 randomly selected patients with normal responses to APC These patients were matched for sex and all came from the Leiden area We also tested the parents of 1 patient with an extremely poor response to APC (APC-sensitivity ratio 1 21)

All patients and control subjects were seen by one of us (T K) Blood was collected from the antecubital vein into 0 106 mmol/L trisodium citrate Plasma was prepared by centrifugation for 10 min at 2000 *g* at room temperature and stored at −70°C, in 1 5 mL volumes

The sensitivity of the plasma APTT to APC was measured as described by Dahlback et al,[2] with the reagents and reaction schemes developed for the protein S activity assay [11] Briefly, 50 µL undiluted plasma was incubated with 50 µL APTT reagent (Cephotest, Nycomed Pharma, Oslo, Norway) for 360 s at 37°C Clot formation was started with either 50 µL of 33 mmol/L calcium chloride, 25 mmol/L tris-HCl (pH 7 5), 50 mmol/L sodium chloride, and 0 05% ovalbumin (APTT, −APC) or 50 µL of the same reagent also containing 2 0 µg/mL human APC and 0 6% glycerol (APTT, +APC) Automated analysis was done in an ACL-300 (Instrumentation Laboratory, Milan, Italy), with the research program Results are expressed as APC-sensitivity ratios, defined as APTT (+APC) divided by the APTT (−APC) The APC-sensitivity ratio of plasma is stable for at least 2 h after thawing and is not significantly affected by two subsequent cycles of freezing and thawing Routinely, plasma was analysed within 1 h of thawing Under these conditions, the APC-sensitivity ratio for pooled normal plasma is 2 67 and the between-assay variation is 4% (n = 8) APC-sensitivity ratio is independent of protein S levels above 0 2 u/mL, and plasma completely depleted of protein S (<0 001 u/mL) still has an APC-sensitivity ratio of 2 1 Low concentrations of prothrombin, factor X, or both (<0 5 u/mL) increase the APC-sensitivity ratio, so the test cannot be used for patients receiving oral anticoagulants In a series of 98 samples we found a good correlation (Pearson correlation coefficient 0 54) between the APC-sensitivity ratios obtained with our test and those obtained with a test developed by Chromogenix (Molndal, Sweden) Additionally, in 20 samples sent to us by Chromogenix, the Pearson correlation coefficient was 0 79 APC was prepared from isolated human protein C as previously described,[12] and stored in small volumes at −30°C in a buffer containing 50 mmol/L tris-HCl (pH 7 5), 100 mmol/L sodium chloride, 0 1% ovalbumin, and 7 5% glycerol

Other coagulation assays were done according to established procedures Protein C activity was measured with Coamate (Chromogenix) on an ACL-200, total protein S by an enzyme-linked immunosorbent assay (ELISA),[13] factor VIII coagulant activity by a one-stage clotting assay with artificial FVIII-deficient plasma, and automated APTT (Organon Teknica, Durham, NC, USA) on an Electra 1000 (MLA, Pleasantville, USA) The technicians were at all times unaware of the status of the sample

42 patients were on long-term coumarin treatment, including 15 (36%) who had had recurrent thrombosis before joining the study 2 patients initially had prolonged APTT consistent with lupus anticoagulant After exclusion of these 44 subjects, our sample consisted of 301 patient-control pairs

A reference range for the APC-sensitivity ratio was derived from the 301 healthy control subjects After logarithmic transformation of the data and exclusion of 10 subjects with values outside 3 SD below or above the mean, the lower limit of normal was 2 17 (mean minus 1 96 SD)

We analysed the effect of age (continuous variable) and sex (0 women, 1 men) on the natural log-transformed APC-sensitivity ratio outcome in the healthy controls with a normal response to APC (≥2 17) by multiple linear regression techniques The regression coefficient obtained shows the increase or decrease in the logarithm of the APC-sensitivity ratio outcome per unit increase in the factor studied, adjusted for the effect of other variables in the model

| Age group (yr) | No of patients | No (%) poor responders |
|---|---|---|
| <25 | 19 | 8 (42) |
| 25–34 | 52 | 11 (21) |
| 35–44 | 58 | 11 (19) |
| 45–54 | 87 | 14 (16) |
| >54 | 85 | 20 (24) |

Table 1: **Age-specific prevalence of poor response to APC in 301 thrombosis patients**

We calculated crude matched odds ratios as estimates of the relative risk by simple cross tabulation. We used Miettinen's test-based 95% CI.[14] The odds ratio reflects the thrombosis risk when the APC-sensitivity ratio is too low in comparison with the risk when the ratio is within the normal range, adjusted for age and sex. We also sought a dose-response relation, by calculating odds ratios over several strata of APC-sensitivity ratio. We used a conditional logistic model (Egret software), which allows adjustment for several factors (eg, factor VIII:C, fibrinogen, protein C, and protein S) simultaneously.

## Results

The male/female ratio among patients and controls was 1/1·5 and mean age was 46 years (range 17–70 patients, 17–73 controls).

Among the controls, at all ages, men had a more pronounced anticoagulant response to APC than women (regression coefficient for natural logarithm of APC-sensitivity ratio 0·05 [95% CI 0·03–0·08]). Age also seemed to be a determinant of the APC-sensitivity ratio (regression coefficient −0·002 [−0·001 to −0·003], figure 1).

None of the 602 subjects had an APC-sensitivity ratio between 1·8 and 2·0 (figure 2). This gap cannot be explained by digit preference, since the result is given by automated computer output. We take it to be evidence of strongly bimodal distribution. 64 (21%) of the patients showed a poor response to APC (APC-sensitivity ratio below 2·17). Only 14 (5%) of the 301 healthy control subjects had a poor response. There were 68 discordant patient-control pairs, in 59 of which the patient had an abnormal APC-sensitivity ratio and the control subject did not. The abnormality was evenly distributed among age groups (table 1).

The crude (matched) odds ratio for a poor response to APC was 6·6 (95% CI 3·6–12·0)—ie, an almost seven-fold increase in risk of thrombosis associated with a poor response to APC. Table 2 gives the odds ratios for the strata of APC-sensitivity ratios. It shows a relation between the risk of thrombosis and the degree of response. Adjustment for factor VIII:C, protein C, protein S, or fibrinogen concentrations did not change the reported odds ratios. Among the subjects with a poor response to APC there was no one with protein S deficiency, and only 1 individual with a possible protein C deficiency.

We have summarised the results of the family studies in figure 3. For 9 of the 10 patients with a normal response to APC, both parents had normal responses too. 1 patient with a normal response to APC (sensitivity ratio 2·38) had one parent with a low APC-sensitivity ratio (1·64). With a prevalence of 5% in control subjects, we expected to find one parent with a poor response to APC. Among the patients with poor responses, all but 1 had one parent with a poor response to APC and one parent with a normal response. Both parents of the remaining patient had a normal test outcome. This pattern could be the result of a new mutation, laboratory errors, a non-biological parent, or the presence of an overlap-area in which true heterozygotes and normal individuals cannot be separated on the APC-sensitivity ratio. The subject's APC-sensitivity ratio was only slightly low (2·10), whereas all the other patients in this subgroup had APC-sensitivity ratios between 1·46 and 1·76. Figure 1 shows that when the APC-sensitivity ratio is 1·9 or less, there is little doubt that a person is a poor responder. We found 6 patients who had very low APC-sensitivity ratios (<1·25). The parents of only 1 of these patients could be tested; both had a poor response to APC. It is likely that this patient was homozygous or double heterozygous for the abnormality, and that this state is associated with an even greater risk of thrombosis than is the heterozygous state (table 2).

| APC-sensitivity | Patients | Controls | Odds ratio (95% CI)* |
|---|---|---|---|
| ≥2·5 | 163 | 220 | 1 |
| 2·0–2·5 | 84 | 72 | 1·6 (1·1–2·4) |
| 1·5–2·0 | 36 | 7 | 7·4 (3·0–18·0) |
| <1·5 | 18 | 2 | 12·0 (2·7–56·0) |

* Matched (crude) odds ratio, adjustment for factor VIII:C, protein C, or protein S concentrations or fibrinogen did not affect these results. Test for trend, p<0·001

Table 2: **Thrombosis risk for strata of APC-sensitivity ratios**

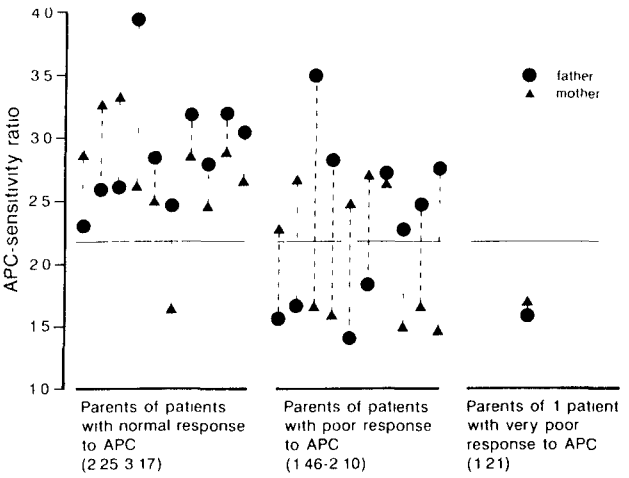


Figure 3: **APC-sensitivity ratios of parents of thrombosis patients by response to APC**

Pairs of parents indicated by broken lines. Horizontal solid line represents lower limit of normal APC-sensitivity ratio. Fisher's exact test of offspring response by parent response p=0·001.

## Discussion

The 21% prevalence of a poor response to APC among thrombosis patients and the odds ratio for thrombosis of 6·6 lead to the conclusion that a poor response to APC is a common and strong risk factor for deep-vein thrombosis. Both age and sex are determinants of the APC-sensitivity ratio in normal individuals, although the differences are quite small.

The pattern in the parents of the patients was compatible with the reported dominant heredity of the abnormality.[2,7] We speculate that subjects with APC-sensitivity ratios around 1·10 are homozygous or double heterozygous, whereas subjects with APC-sensitivity ratios around 1·50 are heterozygous for the abnormality. This notion could explain the apparent "dose-response" shown in table 2, and our finding that the APC-sensitivity ratio of a 1/1 mixture of pooled normal plasma and plasma of a presumed homozygote is 1·60.

An important point in the assessment of case-control studies is the selection of patients and control subjects.

Since we were interested in the risk of a first episode of deep-vein thrombosis, we opted for incident cases. 42 patients were excluded because they were receiving long-term coumarin treatment; a substantial percentage of them had had recurrent thrombosis before the study. If these subjects have a higher chance of having a poor response to APC, our estimates may be conservative. Svensson et al[8] found a much higher prevalence—40% of selected patients with deep-vein thrombosis. We believe that this difference is due to the selection of the patients. The subjects in Svensson's study were referred from the south of Sweden with unexplained thrombosis.[8,9] The control subjects were selected by our patients. We do not think that this practice can have biased our findings on this new abnormality in the coagulation system.

The prevalence of the abnormality was 5% among the healthy control subjects. Because distribution was clearly bimodal, we believe these subjects really did have abnormal responses to APC rather than low values within a normal range. The relation between risk of thrombosis and the response to APC seems therefore not to follow the model of a simple single-gene deficiency. Because the abnormality is so prevalent in healthy subjects, it is unlikely that the defect in itself is sufficient to cause thrombosis, as is true also for protein C deficiency.[15,16] Other causal factors seem to be required for the development of thrombosis; these may be acquired factors or as yet unknown genetic defects or variations. However, when other causal factors are present, poor APC response strongly increases the risk of thrombosis.

The underlying defect of the poor response to APC remains unclear, even though a deficiency of a co-factor to APC with autosomal dominant inheritance has been postulated.[7] Although a poor response to APC appears to be about 5–10 times more frequent than deficiencies of protein C, protein S or antithrombin III, it confers a similar relative risk of thrombosis.[17,18] It may well be worth while to test all patients with venous thrombosis for this abnormality.

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