

## THE RISK OF RECURRENT VENOUS THROMBOEMBOLISM IN PATIENTS WITH AN Arg<sup>506</sup>→Gln MUTATION IN THE GENE FOR FACTOR V (FACTOR V LEIDEN)

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### ABSTRACT

**Background** A recently discovered mutation in coagulation factor V (Arg<sup>506</sup>→Gln, referred to as factor V Leiden), which results in resistance to activated protein C, is found in approximately one fifth of patients with venous thromboembolism. However, the risk of recurrent thromboembolism in heterozygous carriers of this genetic abnormality is unknown.

**Methods** We searched for factor V Leiden in 251 unselected patients with a first episode of symptomatic deep-vein thrombosis diagnosed by venography. The patients were followed prospectively for a mean of 3.9 years to determine the frequency of recurrent venous thrombosis and pulmonary embolism.

**Results** Factor V Leiden was found in 41 of the patients (16.3 percent; 95 percent confidence interval, 11.8 to 20.9 percent). The cumulative incidence of recurrent venous thromboembolism after follow-up of up to eight years was 39.7 percent (95 percent confidence interval, 22.8 to 56.5 percent) among carriers of the mutation, as compared with 18.3 percent (95 percent confidence interval, 12.3 to 24.3 percent) among patients without the mutation (hazard ratio, 2.4; 95 percent confidence interval, 1.3 to 4.5;  $P < 0.01$ ).

**Conclusions** The risk of recurrent thromboembolic events is significantly higher in carriers of factor V Leiden than in patients without this abnormality. Large trials assessing the risk-benefit ratio of long-term anticoagulation in carriers of the mutation who have had a first episode of venous thromboembolism are indicated. (N Engl J Med 1997;336:399-403.)

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PATIENTS who have symptomatic deep-vein thrombosis of the legs remain at risk for recurrent venous thromboembolism despite adequate treatment with heparin and warfarin.<sup>1</sup> In a recent long-term follow-up study of a consecutive series of patients with a first episode of symptomatic deep-vein thrombosis, the cumulative incidence of recurrent venous thrombosis or pulmonary embolism exceeded 30 percent over a period of eight years.<sup>2</sup>

Venous thromboembolism can occur without apparent cause, after surgical procedures or trauma, and in the presence of cancer or molecular defects in several hemostatic components (antithrombin, protein C, protein S, plasminogen, and fibrinogen). The prevalence of these protein abnormalities among patients with venous thromboembolism, however, is only 5 to 10 percent.<sup>3,4</sup>

Recently, resistance to activated protein C, a newly discovered hereditary trait potentially accounting for a far greater proportion of patients with thromboembolic disorders, has been described.<sup>5,6</sup> This abnormality is caused by the substitution of a single amino acid — glutamine for arginine — at position 506 (Arg<sup>506</sup>→Gln, also referred to as factor V Leiden), in the coagulation factor V molecule.<sup>7-9</sup> In its activated form protein C is a natural anticoagulant that cleaves two activated coagulant factors, factor VIIIa and factor Va, thereby inhibiting the conversion of factor X to factor Xa and of prothrombin to thrombin. The mutation in the factor V molecule renders factor Va resistant to proteolysis by activated protein C. Venous thromboembolism develops in up to 40 percent of patients with resistance to activated protein C.<sup>10-12</sup>

Although it is important to know the risk of recurrent venous thromboembolic disease in order to make decisions about therapy in carriers of the factor V Leiden mutation, the few studies of the risk have yielded conflicting results.<sup>13,14</sup> Therefore, we studied the prevalence of this mutation in a large cohort of unselected patients with a first episode of symptomatic deep-vein thrombosis who underwent long-term, prospective follow-up for recurrent thromboembolic events.

### METHODS

#### Identification of Study Cohort

All outpatients who were referred to the thrombosis unit of the University of Padua between January 1986 and June 1994 because of a first episode of deep-vein thrombosis diagnosed by venography and who underwent long-term follow-up were potentially eligible for the study.<sup>2</sup> Patients were excluded from the study if they had malignant disease or confirmed abnormalities in the coagulation or fibrinolytic system (defects of antithrombin, protein C, protein S, fibrinogen, or plasminogen or the presence of lupus-like anticoagulants), or if they had received warfarin therapy for more than six months for reasons other than recurrent thromboembolic events. For all remaining patients, resistance to activated protein C was assessed.

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Surviving patients who met the eligibility criteria and who provided informed consent were asked to come to the thrombosis center for assessment of factor V Leiden status. For all patients in whom the mutation was confirmed, family members were tested to determine whether the defect was familial. Laboratory tests were performed by technicians unaware of the identity of the subjects. The study protocol was approved by the ethics board of the University of Padua.

### Collection of Blood Samples and Routine Coagulation Tests

Blood samples were collected by venipuncture with 21-gauge butterfly infusion sets connected to plastic tubes containing 3.8 percent sodium citrate in a ratio of 0.1:0.9 (vol/vol, anticoagulant to blood). Platelet-poor plasma was obtained by centrifugation at  $2000 \times g$  for 20 minutes and stored at  $-80^{\circ}\text{C}$  until it was analyzed.

The laboratory investigations included the measurement of the activated partial-thromboplastin time and prothrombin time. Assays for fibrinogen, plasminogen, antithrombin, protein C, protein S, and antiphospholipid antibodies were performed as previously described.<sup>15,16</sup> A reference pool of normal plasma for all these assays was obtained from 40 healthy subjects of both sexes (age, 20 to 60 years). Normal values for each test were determined in 80 healthy subjects of both sexes (age, 20 to 70 years).

### Assay for Resistance to Activated Protein C

The assay for resistance to activated protein C was performed as described previously.<sup>7</sup> Briefly, 50  $\mu\text{l}$  of undiluted plasma was incubated with 50  $\mu\text{l}$  of NAPPT reagent (Cephotest, Immuno, Pisa, Italy) for 360 seconds at  $37^{\circ}\text{C}$ . Clot formation was started by the addition of either 50  $\mu\text{l}$  of 33 mM calcium chloride, 25 mM TRIS-hydrochloric acid (pH 7.5), 50 mM sodium chloride, and 0.05 percent ovalbumin (to measure the activated partial-thromboplastin time in the absence of activated protein C) or 50  $\mu\text{l}$  of the same reagent containing 1.0  $\mu\text{l}$  of human activated protein C per milliliter (final concentration; Enzyme Research Laboratory, South Bend, Ind.) (to measure the activated partial-thromboplastin time in the presence of activated protein C). A normalized ratio of sensitivity to activated protein C was then calculated. Resistance to activated protein C was defined as a normalized ratio of less than 0.84.<sup>7</sup>

### DNA Analysis for Factor V Leiden

High-molecular-weight DNA was extracted from 5  $\mu\text{l}$  of peripheral blood with a binding matrix (Bio-Rad Laboratories, Hercules, Calif.). DNA analysis was performed as previously described with minor modifications.<sup>7,9</sup> Briefly, the 220-bp fragment of factor V exon 10-intron 10 was amplified by the polymerase chain reaction, with 5'TGCCAGTGCTTAACAAGACCA3' as the forward primer and 5'CTTGAAGGAAATGCCCCATTA3' as the reverse primer. Amplification involved 36 cycles consisting of denaturation at  $91^{\circ}\text{C}$  for 40 seconds, annealing at  $55^{\circ}\text{C}$  for 40 seconds, and extension at  $71^{\circ}\text{C}$  for 2 minutes in the presence of 2 U of *Taq* polymerase. Subsequently, the 220-bp fragment was digested by 0.4 U of *MnII* at  $37^{\circ}\text{C}$  over a 16-hour period. *MnII* digests the 220-bp fragment of the normal factor V allele into three fragments of 37, 67, and 116 bp each. The factor V Leiden allele was cleaved in only two fragments of 67 and 153 bp. Finally, the digestion products were separated by electrophoresis on 2 percent agarose gels stained with ethidium bromide for 30 minutes at 150 V.

### Treatment

Patients were admitted to the hospital and treated with an initial course of high-dose intravenous standard heparin (a bolus of 5000 U was followed by a continuous infusion of 30,000 U per day, with the dose subsequently adjusted to maintain an activated partial-thromboplastin time between 1.5 and 2.5 times the normal value) or subcutaneous low-molecular-weight heparin (90 U

of antifactor Xa per kilogram of body weight twice daily). Therapy with oral anticoagulant agents (warfarin) was started on day 5, 6, or 7 of treatment and continued for a period of three or six months; the dose was adjusted daily to maintain an international normalized ratio between 2.0 and 3.0. Treatment with low-molecular-weight heparin was discontinued on day 10 or later if the international normalized ratio was less than 2.0. All patients were instructed to wear elastic graduated-compression stockings (providing 40 mm Hg of pressure at the ankle) for at least two years.

### Diagnosis of Recurrent Venous Thromboembolism

Recurrent deep-vein thrombosis was identified by venography or compression ultrasonography, according to standard methods. If the results of venography were not diagnostic, recurrent venous thrombosis was diagnosed on the basis of abnormal results on scanning of the legs with fibrinogen I 125 or on the basis of a change in the results of noninvasive tests from normal to abnormal.<sup>17-20</sup> Patients thought to have pulmonary embolism underwent lung scanning or venography if they had concurrent symptoms of thromboembolism in the legs. Patients with lung scans indicating a low or intermediate probability of pulmonary embolism underwent pulmonary angiography. Lung scanning and pulmonary angiography were performed and interpreted according to standard procedures.<sup>21,22</sup> A diagnosis of fatal pulmonary embolism was based on the findings at autopsy or the opinion of a physician who was not associated with the study. Recurrent venous thromboembolic events were assessed by a committee that was unaware of further clinical details (including factor V Leiden status) of the patients.

### Statistical Analysis

The cumulative incidence of recurrent thromboembolic events among patients with and those without factor V Leiden was calculated according to the method of Kaplan and Meier. The Cox proportional-hazards model was used to assess the significance of the difference between groups and to determine the hazard ratios and 95 percent confidence intervals. All P values of less than 0.05 were considered to indicate statistical significance.

## RESULTS

### Patients

Of the 517 patients who were referred for a first episode of deep-vein thrombosis, 170 were excluded from the study for the following reasons: 101 had malignant disease at the time of the initial diagnosis, 51 had documented abnormalities in the coagulation or fibrinolytic system, and 18 had been taking oral anticoagulants for more than six months for reasons other than venous thromboembolism. Of the remaining 347 patients, 85 were not available for genetic testing. Resistance to activated protein C was found in 13 of these 85 patients (15.3 percent). Of the remaining 262 patients, 251 gave their informed consent and were enrolled in the study.

### Demographic and Clinical Characteristics

The demographic and clinical characteristics of the study patients are presented in Table 1, according to their factor V Leiden status. Additional risk factors that may contribute to the development of deep-vein thrombosis (recent trauma or fracture, recent surgery, the use of oral contraceptive drugs, and pregnancy or childbirth) were equally distributed between the groups.

A total of 112 patients (44.6 percent) completed five years of follow-up, and 45 (17.9 percent) completed eight years of follow-up. The mean duration of follow-up was 3.9 years.

**Prevalence of Factor V Leiden**

Factor V Leiden was found in 41 patients (16.3 percent; 95 percent confidence interval, 11.8 to 20.9 percent). The prevalence of this abnormality in patients with idiopathic deep-vein thrombosis (28 of 145, 19.3 percent) did not differ from that in patients whose thrombotic episode was associated with a well-recognized risk factor (13 of 106, 12.3 percent). All carriers of the factor V gene were heterozygous for the mutation. The hereditary nature of the defect was confirmed in all 41 patients by the identification of at least one first-degree relative who carried the mutation.

**Recurrent Venous Thromboembolism in Patients with and Those without Factor V Leiden**

Of the 251 patients, 49 had had one or more documented recurrent venous thromboembolic events. Fourteen patients with factor V Leiden (10 of the 28 patients with idiopathic deep-vein thrombosis [35.7 percent] and 4 of the 13 patients with risk factors for deep-vein thrombosis [30.7 percent]) had recurrent thromboembolic events. Thirty-five patients without the mutation had recurrences (29 of the 128 patients with idiopathic deep-vein thrombosis [22.7 percent] and 6 of the 82 patients with secondary deep-vein thrombosis [7.3 percent]). Of the 49 first recurrences, 25 (51.0 percent) were in the leg that was involved in the initial episode, 17 (34.7 percent) were in the contralateral leg, and 7 (14.3 percent) were pulmonary embolisms. Forty-four of the recurrences were not associated with any apparent risk factor, and 5 were associated with a (new) risk factor; 2 of these 5 recurrences were in carriers of factor V Leiden.

The cumulative incidence of recurrent thromboembolism among patients with factor V Leiden after eight years of follow-up was 39.7 percent (95 percent confidence interval, 22.8 to 56.5 percent), as compared with 18.3 percent (95 percent confidence interval, 12.3 to 24.3 percent) among patients without this mutation (Fig. 1). The hazard ratio for recurrent venous thromboembolism among patients with the mutation, as compared with patients without the defect, was 2.4 (95 percent confidence interval, 1.3 to 4.5;  $P < 0.01$ ). The hazard ratio for patients with secondary deep-vein thrombosis, as compared with those with idiopathic deep-vein thrombosis, was 0.41 (95 percent confidence interval, 0.20 to 0.82). A test for an interaction between factor V Leiden status and the type of deep-vein thrombosis was negative ( $P > 0.15$ ).

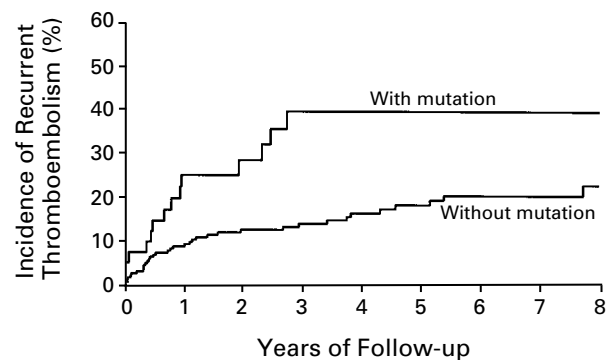
Seventeen of the 85 patients who were not avail-

**TABLE 1. DEMOGRAPHIC, CLINICAL, AND TREATMENT-RELATED CHARACTERISTICS OF PATIENTS WITH AND THOSE WITHOUT FACTOR V LEIDEN.\***

CHARACTERISTIC	PATIENTS WITH FACTOR V LEIDEN (N=41)	PATIENTS WITHOUT FACTOR V LEIDEN (N=210)
Age — yr		
Median	63	62
Range	23–84	28–80
Sex — M/F	25/16	106/104
Interval between onset of symptoms of venous thrombosis and treatment — days		
Median	8	7
Range	1–30	1–30
Risk factors for venous thrombosis — no. of patients (%)		
Recent surgery	6 (14.6)	47 (22.4)
Recent trauma or fracture	5 (12.2)	46 (21.9)
Oral contraceptive therapy	4 (9.8)	14 (6.7)
Pregnancy or childbirth	4 (9.8)	7 (3.3)
Extent of deep-vein thrombosis — no. of patients (%)		
Calf vein	3 (7.3)	12 (5.7)
Calf and popliteal veins	5 (12.2)	22 (10.5)
Calf, popliteal, and femoral veins	17 (41.5)	75 (35.7)
All proximal veins	13 (31.7)	83 (39.5)
Femoral or iliac vein or both	3 (7.3)	18 (8.6)
Initial treatment — no. of patients (%)		
Unfractionated heparin	28 (68.3)	144 (68.6)
Low-molecular-weight heparin	10 (24.4)	55 (26.2)
Other†	3 (7.3)	11 (5.2)
Duration of anticoagulant treatment — no. of patients (%)		
3 mo	38 (92.7)	201 (95.7)
6 mo	3 (7.3)	9 (4.3)

\*Because of rounding not all percentages total 100.

†Other treatments consisted of oral anticoagulant therapy alone, thrombolytic therapy, and caval-vein filter.



**Figure 1. Cumulative Incidence of Recurrent Venous Thromboembolism after a First Episode of Symptomatic Deep-Vein Thrombosis in Patients with and Those without Factor V Leiden.**

able for genetic testing had recurrent thromboembolic events: 5 of the 13 patients with resistance to activated protein C and 12 of the 72 patients without this abnormality (relative risk of a recurrence among patients with resistance to activated protein C, 2.4).

Six of the patients without the mutation had recurrent thromboembolic episodes while receiving anticoagulant therapy (2.9 percent), all within the first three months, and four of the patients with the mutation had recurrences (9.8 percent), three within the first three months.

## DISCUSSION

Hereditary abnormalities in the coagulation or fibrinolytic system are well-recognized but uncommon conditions predisposing patients to venous thrombosis. Recently, it was demonstrated that a large proportion of patients with venous thromboembolic disease have factor V Leiden.<sup>10-12</sup> The activated form of factor V Leiden is resistant to proteolytic cleavage by the natural anticoagulant activated protein C. The results of our study in a large cohort of consecutive patients with venous thrombosis confirm that the prevalence of the genetic mutation responsible for resistance to activated protein C is higher (16.3 percent) than that of all the previously described inherited defects (5 to 10 percent). Furthermore, it is equally distributed in patients with idiopathic or secondary thrombosis.

Once factor V Leiden has been demonstrated to be associated with venous thromboembolism, it is important to know the risk of recurrent thrombotic events in carriers of this mutation to aid in decisions about widespread screening and therapeutic management in identified carriers. Studies in small and selected groups of patients have yielded conflicting results.<sup>13,14</sup> Ridker et al. found a higher incidence of recurrent thrombosis in carriers of the mutation,<sup>13</sup> whereas Rintelen et al. did not.<sup>14</sup>

Our study clearly shows that the risk of recurrent thromboembolic events is strongly and significantly higher in carriers of factor V Leiden than in patients without this abnormality (relative risk, 2.4), with a cumulative incidence of almost 40 percent after eight years. These results are similar to those in patients with hereditary defects of antithrombin, protein C, and protein S.<sup>2</sup> Although in our study all the recurrent thrombotic events in patients with factor V Leiden occurred during the first three years of follow-up, this finding could be due to the small number of patients who were followed for more than three years.

We believe that our estimate of the risk of recurrence associated with the presence of factor V Leiden is accurate. Only patients referred with a first episode of objectively documented deep-vein thrombosis who did not have conditions confounding the risk of recurrence (i.e., cancer, known thrombophilic states,

and other conditions requiring long-term treatment with oral anticoagulants) were enrolled. Also, although 15 percent of potentially eligible patients died during follow-up and could not be tested for the mutation, only a small minority (about 10 percent) of these patients died of pulmonary embolism.<sup>2</sup> Furthermore, patients who declined to participate constituted a negligible proportion (4.2 percent) of the cohort. Finally, interpretation bias was avoided by having observers with no knowledge of the patients assess recurrent events and the factor V Leiden status. The relative risk of recurrent venous thromboembolic episodes associated with resistance to activated protein C in the 85 patients in whom a genetic analysis could not be performed was 2.4. This value is similar to the hazard ratio for the patients who underwent genetic testing. Therefore, it is unlikely that an important bias influenced our results.

Although recurrent venous thromboembolism is more frequent in patients with factor V Leiden, such a statistic does not automatically imply that anticoagulant treatment should be prolonged in all these patients. Because the duration of treatment is also a major determinant of the risk of hemorrhage, it is essential to balance the protective effect of these agents against their risk of inducing bleeding. A large trial evaluating the use of coumarins for more than the currently recommended three-month period in carriers of this genetic mutation is clearly warranted. This trial should include both patients with idiopathic thrombosis and those with secondary thrombosis.

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