

Prediction of Recurrent Venous Thromboembolism by Endogenous Thrombin Potential and D-Dimer

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BACKGROUND: Increased thrombin generation is associated with an increased risk of recurrent venous thromboembolism. We investigated the relation between endogenous thrombin potential (ETP) and risk of recurrent venous thromboembolism and evaluated whether prediction of recurrence can be improved by a combined analysis of ETP and D-dimer.

METHODS: We followed 861 patients with first spontaneous venous thromboembolism and determined ETP and D-dimer after discontinuation of anticoagulation. Patients with natural inhibitor deficiency, lupus anticoagulant, or cancer were excluded. The study endpoint was symptomatic recurrent venous thromboembolism.

RESULTS: One hundred thirty patients (15.1%) had recurrence. High ETP ($\geq 100\%$) conferred a 1.6-fold increased risk of recurrence (95% CI 1.1–2.3) after adjustment for age, sex, factor V Leiden, factor II G20210A, and duration of anticoagulation. After adjustment for D-dimer, risk of recurrence remained significantly higher among patients with high ETP [hazard ratio 1.6 (95% CI 1.01–2.4)]. After adjustment for ETP, high D-dimer (≥ 0.5 mg/L) conferred a 1.8-fold (95% CI 1.1–2.8) increased risk of recurrence. Compared with patients with low ETP and low D-dimer, risk of recurrence was 2.8-fold (95% CI 1.5–5.3) higher among patients with both high ETP and high D-dimer after adjustment for potential confounders.

CONCLUSIONS: ETP and D-dimer are independent predictors of recurrent venous thromboembolism. Assessing risk of recurrence can be optimized by combining these indicators of thrombin generation.

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Venous thromboembolism is a common disease with an annual incidence of 1% to 2% (1). A serious complication of venous thromboembolism is recurrence, which is fatal in approximately 5% of patients (2). Recurrence can be prevented by antithrombotic therapy, albeit at the price of severe or sometimes even fatal bleeding. The annual incidence of life-threatening bleeding during oral anticoagulation ranges between 0.1% and 0.5% (3).

We have come to understand venous thromboembolism as a multifactorial disease. Risk of recurrence is strongly influenced by the number and severity of risk factors in an individual patient at a given time point. Several risk factors—including multiple episodes of venous thromboembolism, cancer, a deficiency of a natural inhibitor, presence of the lupus anticoagulant, hyperhomocysteinemia, increased coagulation factors, homozygous or combined clotting defects—confer an increased risk of recurrence (4, 5). Conversely, recurrence risk is low among women and patients with secondary or distal leg vein thrombosis. Stratification of patients according to their recurrence risk is of utmost clinical importance since patients at high risk might benefit from long-term secondary thromboprophylaxis, whereas a short course of anticoagulation might be sufficient in low-risk patients.

A novel approach to stratify patients according to their risk of recurrence is use of global markers of coagulation that measure multifactorial thrombophilia. Patients with first venous thromboembolism and low D-dimer, an *ex vivo* indicator of thrombin generation, after discontinuation of vitamin K antagonists have a significantly lower risk of recurrence than those with high D-dimer (6, 7). Evidence is increasing that the capacity of *in vitro* thrombin generation is related to risk of (recurrent) venous thromboembolism (8–10). Thrombin generation can be registered continuously by measuring cleavage of a chromogenic or fluorescent

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substrate (11). Consequently, a thrombin generation curve is obtained. From this curve, important parameters can be determined, including peak thrombin and the area under the thrombin generation curve, which is called the endogenous thrombin potential (ETP).² We previously showed that patients with a first spontaneous venous thromboembolism can be stratified according to their risk of recurrence by use of a fluorescent substrate and the amount of peak thrombin as a measure of *in vitro* thrombin generation (9).

In this study, we investigated the relation between *in vitro* thrombin generation measured by use of a chromogenic assay and the ETP as readout variable and the risk of recurrent venous thromboembolism. We also evaluated whether prediction of venous thromboembolism recurrence can be improved by a combined analysis of indicators of *in vitro* and *ex vivo* thrombin generation, *i.e.*, ETP and D-dimer.

Materials and Methods

PATIENTS

The study is part of the Austrian Study on Recurrent Venous Thromboembolism (AUREC), an ongoing multicenter cohort study, aiming to identify incidence and risk factors of recurrent venous thromboembolism (12). Between July 1992 and July 2005, 2833 patients were enrolled; all patients were >18 years old with objectively confirmed venous thromboembolism who had been treated with vitamin K antagonists for at least 3 months. Deep vein thrombosis was established by venography, ultrasonography (in case of proximal vein thrombosis), and pulmonary embolism by ventilation-perfusion scanning or spiral computed tomography. A total of 1972 patients were excluded because of requirement of long-term antithrombotic treatment for reasons other than venous thromboembolism (451); cancer (458); surgery, trauma, or pregnancy within 3 months of venous thromboembolism (384); >1 previous venous thromboembolism (369); deficiency of protein C, protein S, or antithrombin (72); or presence of the lupus anticoagulant (75). Twenty-six patients with high factor VIII concentrations were excluded because they participated in a prospective trial investigating the effect of long-term anticoagulation. In 137 patients, material for laboratory testing was not available. In total, 861 patients with an unprovoked first episode of VTE were included.

The study was approved by the local ethics committee, and written informed consent was obtained

from all patients before inclusion. Patients entered the study at the time of withdrawal of vitamin K antagonists and were seen at the investigating center at 3-month intervals during the first year and every 6 months thereafter. They were instructed to report symptoms suggestive of recurrent venous thromboembolism. All women were advised to refrain from female hormone intake.

STUDY ENDPOINTS

The study endpoints were recurrent symptomatic deep vein thrombosis or recurrent pulmonary embolism. Deep vein thrombosis was considered to have recurred if the patient had a thrombus in the leg or arm not affected by the previous thromboembolic event, a thrombus in another deep vein in the leg or arm affected by the previous event, or a thrombus in the same venous system affected in the previous event, with proximal extension of the thrombus (if the upper limit of the original thrombus had been visible) or with a constant-filling defect surrounded by contrast medium (if the original thrombus had not been visible). The diagnosis had to be established by venography or ultrasonography. Recurrent pulmonary embolism had to be confirmed by ventilation-perfusion scanning or spiral computed tomography.

Recurrent events were adjudicated blindly by investigators and clinicians who were unaware of D-dimer and ETP results.

BLOOD SAMPLING AND LABORATORY ANALYSIS

Venous blood was collected into a 1:10 dilution of 0.11 mmol/L trisodium citrate and was centrifuged for 20 min at 2000g. Plasma was stored at -80°C for a median of 62 months (range 46–96 months) until time of assay. DNA was isolated from leukocytes using standard methods. For measurement of protein C, protein S, antithrombin, the lupus anticoagulant, factor VIII, factor V Leiden, and factor II G20210A venous blood was collected 3 weeks after discontinuation of vitamin K antagonists after an overnight fast. For measurement of ETP and D-dimer, blood was collected at a median of 13 months (interquartile range 5–25 months).

We measured antithrombin activity, protein C activity, protein S free antigen and activity, factor VIII and IX; screened for factor V Leiden and for factor II G20210A; and assessed the presence of the lupus anticoagulant as described (12).

MEASUREMENT OF ETP AND D-DIMER

ETP was measured in platelet-poor plasma using a commercially available assay (for research use only; Dade Behring). Coagulation activation was initiated by incubation of plasma with phospholipids, human recombinant tissue factor (Innovin; Dade Behring), and

² Nonstandard abbreviations: ETP, endogenous thrombin potential; HR, hazard ratio.

Characteristics	ETP <100%	ETP ≥100%	P
n	418	443	
Mean age at first venous thromboembolism, years (SD)	47 (17)	48 (14)	0.2
Men, n (%)	177 (42)	206 (47)	0.2
Site of thrombosis, n (%)			0.2
Proximal deep vein thrombosis	126 (30)	163 (37)	
Distal deep vein thrombosis	82 (20)	70 (16)	
Arm vein thrombosis	8 (2)	6 (1)	
Pulmonary embolism ±	202 (48)	204 (46)	
Deep vein thrombosis			
Mean duration of anticoagulation, months (SD)	8 ± 10	8 ± 11	0.2
Factor V Leiden, n (%)	127 (31)	111 (25)	0.08
Factor II G20210A, n (%)	2 (1)	52 (12)	<0.001
High factor VIII, n (%) ^a	39 (9)	43 (10)	0.9
High factor IX, n (%) ^b	88 (21)	128 (29)	0.008

^a Factor VIII concentration >2210 IU/L (90th percentile of patient cohort).
^b Factor IX concentration ≥1340 IU/L (75th percentile of patient cohort).

calcium ions in the absence of thrombomodulin. The concentration of phospholipids and tissue factor is confidential to the manufacturer. Thrombin generation and subsequent inactivation was recorded by monitoring conversion of a specific slow reacting chromogenic substrate at a wavelength of 405 nm over time. A mathematical algorithm was applied to correct the substrate conversion curve for the activity of α_2 -macroglobulin-bound thrombin, which has no known biological activity but is still capable of cleaving small chromogenic substrates. The ETP value was calculated as the area under the thrombin generation curve. Evaluation of reaction curves as well as computer-assisted calculation of thrombin generation over time used the curve evaluation software Curves version 1.0 with specification 3.2 (for research use only; Dade Behring).

ETP values are given as percent of normal. Standardization was performed by measuring the ETP standard (Dade Behring) daily in parallel to the patient samples. Value assignment of the ETP standard (percent of normal) was performed against a normal plasma pool of 80 healthy donors which was defined to have 100% ETP.

We measured D-dimer plasma concentrations in a subset of 734 patients using immunoturbidimetric assay (Innovance D-Dimer; Dade Behring).

STATISTICAL ANALYSIS

We analyzed time to recurrence (possibly censored) according to survival methods (13) and estimated the probability of recurrence according to the method of

Kaplan and Meier (14). To test for homogeneity between strata, we applied the log-rank test. We analyzed ETP was analyzed in Cox proportional-hazard models as a continuous variable and as a dichotomized variable (when appropriate) to compare the relative risks of recurrence associated with different levels of the respective parameter. We adjusted data for age, sex, factor V Leiden, factor II G20210A mutation, and duration of anticoagulation. We checked continuous data for homogeneity using nonparametric tests (Mann-Whitney *U*-test) and categorical data using contingency-table analyses (χ^2 -test). SPSS 15.0.1 was used for statistical analysis. All data are given as mean (SD) unless otherwise indicated.

Results

PATIENTS

Characteristics of the 861 patients are shown in Table 1. During follow-up, 20 patients (2%) left the study because of cancer, 134 (16%) because they required long-term antithrombotic therapy (including acetylsalicylic acid) for reasons other than venous thromboembolism, and 42 (5%) because of pregnancy; 26 (3%) were lost to follow-up. Nineteen patients (2%) died, 3 of recurrent venous thromboembolism. All patients were followed until they left the study or died, when data were censored.

Of the 861 patients, 130 (15.1%, 2.9 events/100 patient years) had recurrent venous thromboembolism (75 deep vein thrombosis, 55 pulmonary embolism)

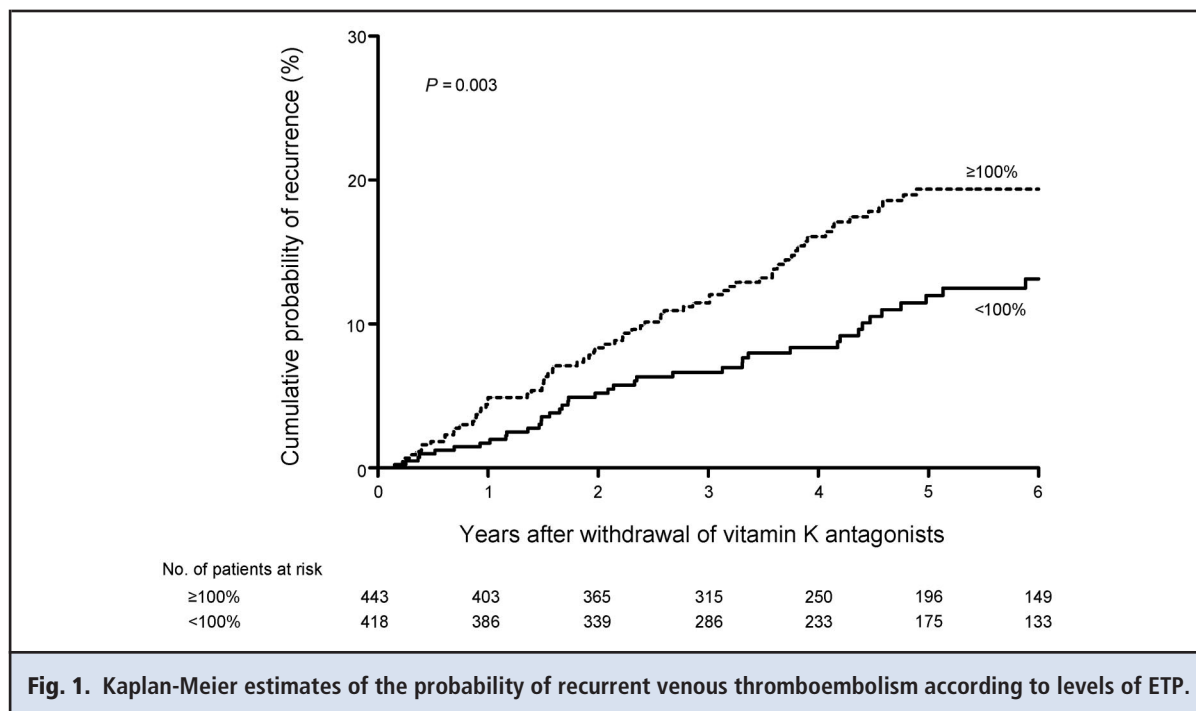


Fig. 1. Kaplan-Meier estimates of the probability of recurrent venous thromboembolism according to levels of ETP.

during an average follow-up of 62 months. In 12 patients, recurrence was provoked by surgery or trauma. Patients with recurrence were slightly older (50 vs 47 years, $P = 0.06$), were predominantly male (87 men vs 43 women, $P < 0.001$), and had higher clotting factor VIII and IX concentrations [1750 (430) vs 1640 (450) IU/L, $P = 0.03$, and 1240 (290) vs 1180 (260) IU/L, $P = 0.04$, respectively]. Duration of anticoagulation did not differ significantly between patients with and without recurrence [8 (8) vs 10 (19) months, $P = 0.6$].

ETP AND RISK OF RECURRENT VENOUS THROMBOEMBOLISM

ETP was significantly higher among patients with recurrence than among those without recurrence [104.8% (15.6%) vs 101.7% (14.2%), $P = 0.02$].

To analyze the relationship between ETP and risk of recurrent venous thromboembolism, we entered ETP as a continuous variable in a Cox proportional-hazard model. The hazard ratio (HR) of recurrence was 1.011 (95% CI 1.0–1.02; $P = 0.06$) for each 1% increase of ETP and 1.014 (95% CI 1.0–1.03; $P = 0.06$) after adjustment for age, sex, factor V Leiden, factor II G210210A mutation, and duration of anticoagulation. To distinguish patients with and without recurrence by ETP, we dichotomized patients into 2 groups using an arbitrary, not-predefined cutoff of 100%. Eighty-four of 443 patients with high ETP (19% or 3.7/100 patient years) and 46 of 418 patients with low ETP (11% or 2.6/100 patient years) had recurrence. Patients with ETP $\geq 100\%$ had a 1.7-fold (95% CI 1.2–2.4; $P =$

0.004) higher risk of recurrence than patients with lower levels. After adjustment for age, sex, factor V Leiden, factor II G20210A, and duration of anticoagulation, HR was 1.6 (95% CI 1.1–2.3; $P = 0.01$). According to Kaplan-Meier analysis, the cumulative probability of recurrence after 5 years was 19.4% (95% CI 15.2%–23.5%) among patients with ETP $\geq 100\%$ and 12.0% (95% CI 8.3%–15.7%) among those with lower levels ($P = 0.003$) (Fig. 1).

ETP was significantly higher in heterozygous carriers of factor II G20210A than in patients with wild-type factor II [130% (17%) vs 100% (12%), $P < 0.001$]. Patients with factor II G20210A mutation were predominant among those with ETP $\geq 100\%$. Carriers of factor V Leiden were more frequent among patients with low ETP (Table 1), and ETP was slightly lower in carriers of factor V Leiden than in noncarriers [100 (13) vs 103 (15), $P = 0.03$].

COMBINING ETP AND D-DIMER TO ASSESS THE RISK OF RECURRENT VENOUS THROMBOEMBOLISM

We next investigated whether ETP and D-dimer are independently associated with risk of recurrent venous thromboembolism. We therefore adjusted HR of recurrence associated with ETP for D-dimer and vice versa. After adjustment for D-dimer, risk of recurrence remained significantly higher among patients with high ETP [HR 1.6 (95% CI 1.01–2.4, $P < 0.05$)]. When ETP was entered in the regression model for D-dimer, HR of recurrence was 1.8 (95% CI 1.1–2.8;

Table 2. Risk of recurrence according to categories of ETP and D-Dimer levels.^a

ETP	D-Dimer	Patients, n	Recurrences, n	Unadjusted HR (95% CI)	Adjusted HR (95% CI) ^b
Low	Low	241	17	1.0	1.0
High	Low	216	29	1.9 (1.0–3.5)	1.8 (1.0–3.2)
Low	High	123	17	2.5 (1.3–4.9)	2.1 (1.0–4.2)
High	High	154	30	3.0 (1.7–5.4)	2.8 (1.5–5.3)

^a Low ETP was defined as <100% and low D-dimer as <0.5 mg/L. D-dimer concentrations were available in 734 patients.

^b Adjusted for age, sex, factor V Leiden, factor II G20210A, and duration of anticoagulation.

$P = 0.01$) for patients with D-dimer concentrations ≥ 0.5 mg/L compared to those with lower concentrations.

Table 2 shows adjusted and unadjusted HR of recurrent venous thromboembolism for patients with combinations of high and low ETP and D-dimer levels, respectively. Compared with patients with low ETP and low D-dimer, HR of recurrence increased among patients with either high ETP [1.8 (95% CI 1.0–3.2)] or high D-dimer [2.1 (95% CI 1.0–4.2)] and was highest among patients with both high ETP and high D-dimer [2.8 (95% CI 1.5–5.3)] after adjustment for age, sex, factor V Leiden, factor II G20210A, and duration of anticoagulation. After 5 years, the cumulative proba-

bility of recurrence was 7.3% (95% CI 3.4%–11.2%) in patients with low ETP and low D-dimer and was 19.1% (95% CI 11.9%–26.2%) in those with high ETP and high D-dimer levels ($P < 0.001$) (Fig. 2).

Discussion

Our prospective cohort study shows that patients with first spontaneous venous thromboembolism can be stratified according to their risk of recurrence by in vitro and ex vivo indicators of thrombin generation. Patients with high ETP ($\geq 100\%$ of normal) after discontinuation of vitamin K antagonists had a 1.7-fold higher risk of recurrence than those with lower levels.

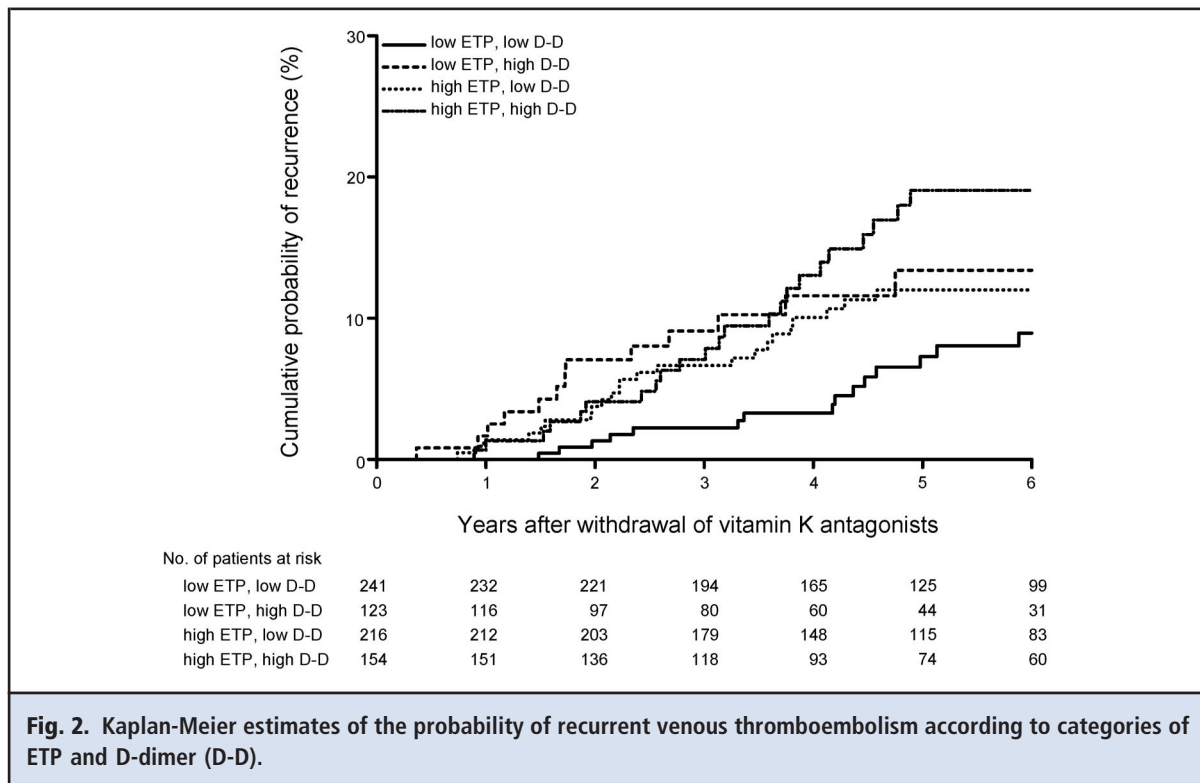


Fig. 2. Kaplan-Meier estimates of the probability of recurrent venous thromboembolism according to categories of ETP and D-dimer (D-D).

This increased risk was independent of other potential risk factors of thrombosis including age, sex, factor V Leiden, and factor II G20210A. Likelihood of recurrence at 5 years after anticoagulation was 19.4% among patients with high ETP and 12.0% among those with lower levels. These results are in good agreement with our previous finding that increased in vitro thrombin generation predicts risk of recurrent venous thromboembolism (9). In the present study, thrombin generation was determined by use of a chromogenic assay and by ETP as read-out variable, whereas in the other study we used a fluorescent assay and the amount of peak thrombin as a measure of thrombin generation. These results are in contrast with those of a Dutch study that did not show a significant association between ETP as measured by a fluorescent assay and risk of recurrent venous thromboembolism (10). That study, however, also included a large proportion of patients with initial secondary thrombosis, who have a known low risk of recurrence. Moreover, the studies differ with regard to concentrations of tissue factor and phospholipids used to trigger coagulation activation as well as addition of thrombomodulin in the Dutch study.

In contrast to one of our earlier studies (6), we determined D-dimer by use of an immunoturbidimetric assay rather than by enzyme-linked immunoassay and applied a higher cutoff concentration (0.5 mg/L instead of 0.25 mg/L). Patients with first venous thromboembolism and abnormal D-dimer concentrations after discontinuation of anticoagulation had a significantly higher risk of recurrence than those with normal concentrations (16.9% vs 9.6% at 5 years after anticoagulation). It has been shown by several large prospective studies that discrimination of patients into groups of high and low risk of recurrence is possible by measuring D-dimer concentrations (6, 7, 15, 16). These studies used different assays for determination of D-dimer including enzyme-linked immunoassays, immunoturbidimetric assays, and a quantitative assay, and all provided precise and reliable estimates of the risk of recurrent venous thromboembolism.

The most important finding of our study was that ETP and D-dimer were independently associated with the risk of recurrence. The decision of how long to treat a patient with anticoagulants after venous thromboembolism entails balancing the risk of recurrence (with a case fatality rate of about 5% (2)) and the risk of bleeding (with an annual 0.2% risk of fatal bleeding (3)) associated with anticoagulation. In patients with a first venous thromboembolism and low ETP and low D-dimer, the rate of fatal bleeding after 5 years will be 1%, which is twice the risk of dying from pulmonary embolism in case of recurrence (0.55% based on 11% probability of recurrence). Thus, despite the lack of

interventional trials, it is conceivable that these patients will not benefit from extended anticoagulation. Patients with high ETP and/or high D-dimer may benefit from prolonged anticoagulation, however.

The independent association of ETP and D-dimer with risk of recurrence provides clinical evidence that markers of in vitro and ex vivo thrombin generation are indicators of different processes within the hemostatic system. ETP quantifies the amount of thrombin that can potentially be generated upon activation of the coagulation system, whereas D-dimer is a measure of ongoing thrombin generation.

Some limitations of our study need to be considered. Patients at high risk of recurrence including those with antithrombin, protein C, or protein S deficiency; the lupus anticoagulant; or cancer were excluded from the study because they are already candidates for prolonged anticoagulation after their first venous thromboembolism. Patients with venous thromboembolism provoked by surgery or trauma were also excluded because their known low risk of recurrence justifies thromboprophylaxis for a short period of time. The results of the study can therefore not be applied to these patient groups. Plasma for determination of ETP and D-dimer was obtained after discontinuation of anticoagulation, so stratification of patients according to the risk of recurrence by ETP or D-dimer during anticoagulation is not possible. Samples were drawn at a median of 13 months after anticoagulation. In 137 patients, samples for measurement of D-dimer and ETP were unavailable; 49 of these patients had recurrence, many of them shortly after discontinuation of anticoagulation. We can therefore not comment on the relevance of ETP and D-dimer measurement for prediction of early recurrence. The course of ETP and D-dimer levels over time is unknown. Levels of ETP or D-dimer collected at different time points did not significantly differ (data not shown). Studies are currently underway to investigate whether these indicators of thrombin generation become normal in patients who initially have high levels or, conversely, increase in patients with initially normal levels, and whether such fluctuations over time are related to risk of recurrent venous thromboembolism.

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