Thrombin generation in first-degree relatives of patients with venous thromboembolism who have factor V Leiden

A pilot study

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Summary

The thrombin generation test appears to be a highly sensitive and specific test in the detection of thrombophilia in patients with venous thromboembolism. We aimed to determine the accuracy of the thrombin generation test to detect factor V Leiden and/or other prothrombotic states in first-degree relatives of patients with venous thromboembolism and factor V Leiden. Sixty-two first-degree relatives of 21 index cases were tested for factor V Leiden, the G20210A prothrombin gene mutation and thrombin generation. Information about oestrogen therapy and previous VTE was also collected. The normalized Thrombomodulin sensitivity ratio (n-TMsr) was defined as the ratio of endogenous thrombin potential determined in the presence and absence of thrombomodulin which was normalized against the same ratio determined in normal control plasma. The mean n-TMsr was 1.37 (± 0.33) in the 45 relatives with one or more prothrombotic state (factor V Leiden, G20210A prothrombin mutation, oestrogen therapy or hormonal therapy) and 1.02 (± 0.34) in the 17 relatives without prothrombotic state (p = 0.001). The positive predictive value was 90.3 (95%CI, 73.1 – 97.4). In relatives with an abnormal n-TMsr, the adjusted odds ratio for having a prothrombotic state was 8.3 (95%CI, 1.9 – 36.9) and the adjusted odds ratio for having the factor V Leiden was 14.3 (95%CI, 2.9 – 71.2). An abnormal thrombin generation test appears highly predictive for having factor V Leiden and/or other prothrombotic states in first-degree relatives of patients with venous thromboembolism and factor V Leiden.

Keywords

Venous thromboembolism, thrombophilia, thrombin generation, family cohort

Thromb Haemost 2008; 99: 223–228

Introduction

In patients with acute venous thromboembolism, the detection of a prothrombotic state may have two major implications: i) to extend the duration of anticoagulation after a first episode of venous thromboembolism (1, 2); and ii), to screen first degree relatives of patients with venous thromboembolism and inherited thrombophilia in order to detect relatives with a high risk for a first episode of venous thromboembolism and to provide counseling for the prevention of venous thromboembolism (3–5). However, there is strong evidence that common thrombophilia, such as factor V Leiden or the prothrombin gene mutation, is not associated with an increased risk of recurrent venous thromboembolism, particularly in patients with idiopathic venous thrombosis (6, 7), suggesting that patients with a high risk of recurrence and without detectable thrombophilia may have an underlying prothrombotic state that has yet to be discovered. In this setting, it would be useful to have a biochemical test that explores all the components of the coagulation pathway.

The thrombin generation test is a global test of the coagulation (8). Most publications have addressed the accuracy of this test in patients with acute venous thromboembolism in order to establish a correlation between an abnormal thrombin generation test and the presence of thrombophilia, and to determine if an abnormal test would result in detecting patients with a high risk of a first or recurrent venous thromboembolism (9–13). There is no standardization of this test and the reported accuracy test remains variable, particularly if thrombin generation is

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Financial Support:
This study is granted by a national source "Programme Hospitalier de Recherche Clinique" 1995 and 1996.

Received August 22, 2007
Accepted after major revision November 15, 2007

Prepublished online December 5, 2007
doi:10.1160/TH07-08-0515

measured after the addition of activated protein C (APC) or not (9–11). No study evaluated the thrombin generation test in first-degree relatives of patients with venous thromboembolism and inherited thrombophilia to detect high risk thrombosis first-degree relatives. Such an approach is important, as some first degree relatives of patients with venous thromboembolism and inherited thrombophilia may have a high risk of thrombosis because they have other or additional prothombotic abnormalities (e.g. oral contraception, elevated factor VIII), and as a number of patients have venous thromboembolism without detectable thrombophilia and a strong family history of venous thromboembolism.

The aim of this pilot study was to determine the accuracy of thrombin generation measurement with and without thrombomodulin in the detection of a prothrombotic state in first degree relatives of patients with venous thromboembolism in association with factor V Leiden.

Methods

Population

The study subjects were first-degree relatives (i.e. parents, siblings, children) of patients (index cases) who had been diagnosed with an acute episode of venous thromboembolism (first or recurrent) at the hospital in Brest, France, and who had factor V Leiden. The recruitment of index cases has been previously described (5). Briefly, all of the following eligibility criteria had to be met: age 16 years and above; acute venous thromboembolism documented by objective testing (see diagnostic criteria below); presence of at least one living first-degree relative, written informed consent to participate in the study, as well as permission for one or more of their first-degree relatives to be approached for the study. In addition to the present study, index cases had to have at least one symptomatic first-degree relative with a frozen blood sample available to perform a thrombin generation test. First-degree relatives had to meet the following eligibility criteria: age 16 years and above, frozen blood sample available, no ongoing anticoagulant therapy, and willingness to provide written informed consent. The study was approved by the Research Ethics Committee of Brest Hospital.

Diagnosis of venous thromboembolism

Venous thromboembolism in index cases or first-degree relatives was defined as acute deep vein thrombosis and/or pulmonary embolism. Deep vein thrombosis of the proximal and/or distal deep veins was diagnosed by a lack of full compression on compression ultrasonography, or an intraluminal filling defect on venography. Pulmonary embolism was diagnosed by a "high probability" ventilation-perfusion lung scan, an intraluminal filling defect in a segmental or more proximal pulmonary artery on CT pulmonary angiography, an intraluminal filling defect on pulmonary angiography, or diagnostic criteria for deep vein thrombosis in patients with suspected pulmonary embolism who had a non-high probability lung scan (14–16). If the results of previous testing were not available, first-degree relatives were considered as having had previous venous thromboembolism if the relatives were treated for deep vein thrombosis or pulmonary embolism with anticoagulant therapy for more than two months.

Study personal who were not aware if the first-degree relatives had factor V Leiden or not recorded all the data in a standardized manner. In addition, first-degree relatives did not know if they had factor V Leiden when they were assessed for the presence of previous venous thromboembolism.

Period of observation

The period of observation for first-degree relatives was from 16 years of age to the date of the first venous thromboembolism or, in the absence of venous thromboembolism, to the date of inclusion in the study.

Definition of biochemical prothrombotic states

First degree relatives were classified as having one or more biochemical prothrombotic states if they had: factor V Leiden, the G20210A prothrombin gene mutation, oestrogen contraception and/or hormonal replacement therapy.

Genetic testing for factor V Leiden and the prothrombin gene mutation

Blood from index cases and first-degree relatives was collected in 0.05 M EDTA for DNA analysis. DNA was extracted according to standard procedures and analysed for factor V Leiden and the Prothrombin G20210A mutation as previously described (17); the results were classified as normal, heterozygous, or homozygous, for each mutation.

Thrombin generation measurement

Plasma and reagents

Blood samples were obtained after informed consent using a peripheral vein catheter. Blood (9 volumes) was collected in 5 ml tubes (Vacutainer, Becton-Dickinson, ((add city etc. nn)) containing 0.105 M trisodium citrate (1 volume). For all samples, we removed the first 10 ml of waste blood. All blood samples were kept frozen at minus 80°C until the thrombin generation test was performed.

Recombinant relipidated human tissue factor (TF) (Innovinâ) was obtained from Dade Behring ((City etc.nn)). The concentration of TF determined by activity measurement was about 5000 pM. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). In order to approach the phospholipid distribution at the surface of activated platelets (18), phospholipids (PL) vesicles were prepared as previously described (19) in order to obtain a (PE + PS / PC) ratio close to 1.39 i.e. 45% PE, 13% PS and 42% PC. Rabbit lung thrombomodulin was purchased from American Diagnostica (Stamford, CT, USA) and treated as previously described (20). Mixing buffer contained 20 mM Hepes (Sigma, Saint Louis, MO, USA), 140 mM NaCl and 5 mg/ml bovine serum albumin (BSA) (Sigma), pH 7.35. In order to eliminate the possible influence of heparin, we added 30 mg/ml polybrene (Sigma nn) to the mixing buffer. Fluorogenic substrate I-1140 (Z-Gly-Gly-Arg-AMC) was obtained from Bachem AG (Bubendorf, Switzerland). The mixture of fluorogenic substrate 2.5 mM and $CaCl_2$ 0.1 M was prepared using buffer containing 20 mM Hepes and 60 mg/ml BSA, pH 7.35. The calibrator for thrombin generation with an activity of 680 nM human thrombin was obtained from Biodis (Signes, France).

Table 1: Characteristics of the 62 first-degree relatives.

		Prothrombotic states					
	No prothrombotic state*	**FVL**	**PGM**	**OCHRT**	**FVL and PGM**	**FVL and OC**	**Total**
Age (mean ± SD)	47.4±18.9	48,5±18.2	35.0±15.6	34.7±17.3	58.3±14.2	36.5±8.3	45.9±18.0
Sex (men/women)	8/9	15/14	1/1	0/7	2/1	0/4	26/36
FVL (+ / -)	0/17	29/0	0/2	0/7	3/0	4/0	36/26
VTE (+ / -)	3/14	8/21	0/2	3/4	1/2	1/3	16/46
Sampling time-period (mean±SD)**	13.3±13.6	6.8±9.5	-	1.7±5.1	15.0	-	7.1±9.6
n-TMsr	1.01 ± 0.34	1.36 ± 0.32¶	1.23 ± 0.01	1.31 ± 0.42	1.56 ± 0.07¶¶	1.44 ± 0.51	1.27 ± 0.05

FVL: factor V Leiden ; PGM : G20210A prothrombin gene mutation ; OCHRT : oral contraceptives and Hormonal Replacement Therapy; *No detectable prothrombotic state; **Time-period between the collection of blood sample and the generation thrombin test which corresponds to lengths of time, in years, when blood samples were frozen. ¶ p=0.04 with relatives without prothrombotic states; ¶¶ p<0.001 with relatives without prothrombotic states.

Thrombin generation

Thrombin generation was measured according to the method described by Hemker et al. (9), in a Fluoroscan Ascent fluorometer (Thermolabsystems OY, Helsinki, Finland) equipped with a dispenser. Briefly, 80 µl of plasma were dispensed into 96 round-bottom well microtiter plates. Twenty µl of a mixture containing TF and PL were added to the plasma sample to obtain a final concentration of 5 pM TF and 4 µM PL with or without 10 nM TM (final concentration). Finally, 20 µl of starting reagent containing fluorogenic substrate and $CaCl_2$ were added. The molar amount of thrombin present in clotting plasma was calculated using the Thrombinoscope™ software version 3.0.0.29 (Synapse B.V., Maastricht, The Netherlands). The measurement lasted 60 minutes and the read interval was 30 seconds. Three wells were needed for each experiment: one well to measure thrombin generation of a plasma sample in the presence of TM, one well to measure thrombin generation of a plasma sample in the absence of TM, and another for calibration. All of the experiments were carried out in triplicate and the mean value was reported. For each experiment, the effect of TM on thrombin generation was expressed as the normalized TM sensitivity ratio (n-TMsr). The n-TMsr was defined as the ratio of endogenous thrombin potential (ETP, i.e. area under the curve) determined in the presence and absence of TM which was normalized against the same ratio determined in a normal control plasma.

Statistical analysis

The proportions and means were compared using the Chi-square test and Student t test as required. A p-value < 0.05 was considered statistically significant. Ninety-five percent confidence intervals (CI) were calculated according to the normal approximation of the binomial distribution. The relative risks and the influence of a number of predefined variables were calculated using logistical regression. ROC curves and statistical analysis were performed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA).

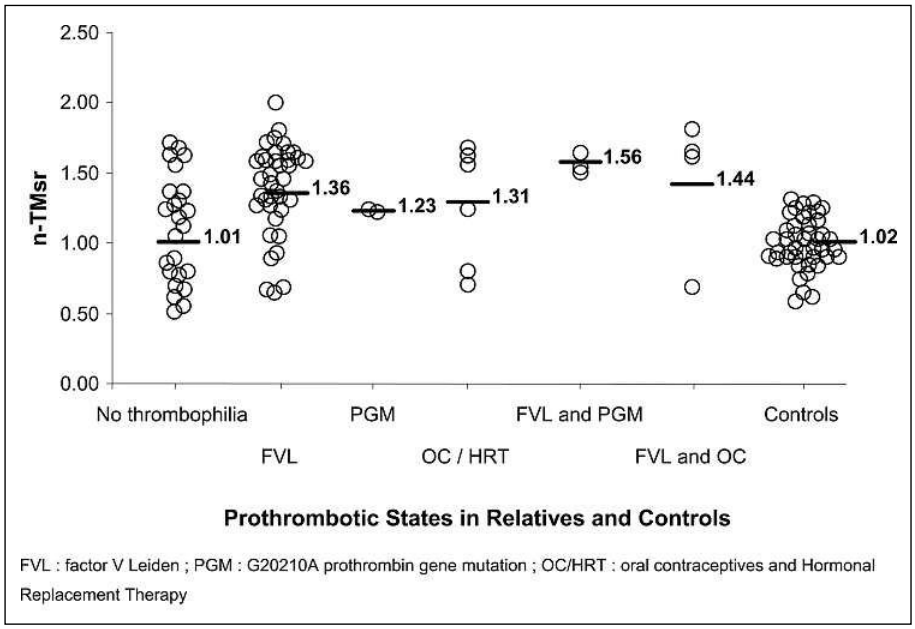

Figure 1: Mean values of n-TMsr in 62 relatives (with and without a prothrombotic state) and in 43 controls.

Accuracy of n-TMsr*	Detection of a prothrombotic state	Detection of factor V Leiden
Sensitivity, [95%CI]	62.2 [46.5 – 75.8]	66.6 [48.9 – 80.9]
Specificity, [95%CI]	82.4 [55.8 – 95.3]	73.1 [51.9 – 87.6]
Positive predictive value, [95%CI]	90.3 [73.1 – 97.4]	77.4 [58.4 – 89.7]
Negative predictive value, [95%CI]	45.2 [27.8 – 63.7]	61 [42.3 – 77.6]

*using 95th percentile for normal value.

Table 2: Accuracy of the thrombin generation test in detecting a prothrombotic state and the factor V Leiden.

Results

Between September 1993 and September 2000, 161 patients were eligible as index cases and gave consent to participate (5); 45 of these patients had at least one symptomatic first-degree relative with previous venous thromboembolism and frozen blood samples were available in 21 patients. Finally, 62 first degree relatives of 21 index cases were included in the study. The characteristics of the first-degree relatives are shown in Table 1. Sixteen first degree relatives had a previous episode of venous thromboembolism. Seventeen first-degree relatives had no prothrombotic state, 38 had only one biochemical prothrombotic state (factor V Leiden in 29, or prothrombin gene mutation in two or estrogen therapy in seven), and seven patients had two or more prothrombotic states (factor V Leiden and prothrombin gene mutation in three, factor V Leiden and estrogen therapy in four).

Results of the thrombin generation test according to the presence or absence of a prothrombotic state (Table 1)

The mean n-TMsr was 1.02 (± 0.34) in the first-degree relatives without prothrombotic state and 1.37 (± 0.33) in the first degree relatives with one or more prothrombotic states (p = 0.001). There was no correlation between the n-TMsr and either the age of the first-degree relatives or the time-period between the date of inclusion and the date of the thrombin generation test (i.e. lengths of time, in years, when the blood samples were frozen). There was also no correlation between the n-TMsr and the time-period between the date of a previous venous thromboembolism and the date of the thrombin generation test. In women, the mean n-TMsr was 1.32 (± 0.38) whereas in men, the mean n-TMsr was 1.20 (± 0.34), (p = 0.18). In first-degree relatives with previous venous thromboembolism, the mean n-TMsr was 1.42 (± 0.37) and in those without venous thromboembolism, the mean n-TMsr was 1.22 (± 0.35), (p = 0.05). In the 16 first-degree relatives with previous venous thromboembolism, three had no prothrombotic state (mean n-TMsr of 0.89 ± 0.32) and 13 had at least one prothrombotic state (mean n-TMsr of 1.54 ± 0.26, p = 0.002). In the 46 first-degree relatives without previous venous thromboembolism, 14 had no detectable prothrombotic state (mean n-TMsr of 1.04 ± 0.35) and 32 had at least one prothrombotic state (mean n-TMsr of 1.29 ± 0.33, p = 0.027).

In the first-degree relatives with at least one prothrombotic state, the mean n-TMsr was 1.34 (± 0.33) in those with only one prothrombotic state and 1.49 (± 0.37) in those with two or more prothrombotic states (p = 0.28), a non significant difference that was also not affected by the presence or absence of previous venous thromboembolism.

Accuracy of the thrombin generation test in the detection of a prothrombotic state

Normal n-TMsr was determined from 43 control subjects without a personal or a familial past history of venous thromboembolism using the 95th percentile. Mean n-TMsr of relatives and controls are shown in Figure 1. In controls, mean n-TMsr was 1.02 and the 95th percentile was 1.30. The ROC curves showed that the ability of the test to discriminate between relatives with one or more prothrombotic states and relatives without a prothrombotic state was 0.77 (0.64 – 0.90 and p = 0.001). The diagnostic accuracy of the thrombin generation test for the detection of a prothrombotic state and the factor V Leiden is shown in Table 2. The thrombin generation test was found to be highly predictive of the presence of a prothrombotic state. Regarding the detection of a prothrombotic state, the unadjusted likelihood ratio for an abnormal n-TMsr was 9.3 (95%CI, 6.4 – 13.5), whereas for the detection of factor V Leiden, the unad-

	Risk of having a prothrombotic state		Risk of having factor V Leiden	
	OR	p	OR	p
Age	**1.0 [0.96 – 1.03]**	**0.89**	1.03 [0.99 – 1.07]	0.11
Sampling Time-period*	**1.29 [0.76 – 2.17]**	**0.34**	1.47 [0.87 – 2.46]	0.15
Gender	**0.64 [0.17 – 2.48]**	**0.52**	0.15 [0.03 – 0.74]	0.02
Previous VTE	**1.33 [0.27 – 6.62]**	**0.74**	1.23 [0.28 – 5.31]	0.79
nTM-sr	**8.30 [1.87 – 36.86]**	**0.005**	14.25 [2.85 – 71.15]	0.001

* Time-period between blood sampling and the generation thrombin test which corresponds to lengths of time, in years, when blood samples were frozen.

Table 3: Adjusted odds ratio for having one or more prothrombotic states in the 62 first-degree relatives.

justed likelihood ratio for an abnormal n-TMsr was 3.4 (95%CI, 2.3 – 5.1).

After adjustment on gender, age, previous venous thromboembolism and the time-period between the inclusion and the thrombin generation test, the odds ratio for having a prothrombotic state in first-degree relatives with an abnormal n-TMsr was 8.30 (95%CI, 1.87 – 36.86, p = 0.005) and the odds ratio for having factor V Leiden with an abnormal n-TMsr was 14.25 (95%CI, 2.85 – 71.15, p = 0.001) (Table 3).

Discussion

This study shows that in first-degree relatives of patients with venous thromboembolism and factor V Leiden, an abnormal thrombin generation test is highly correlated, not only with the presence of factor V Leiden, but also with the presence of any prothrombotic state, such as prothrombin gene mutation or oestrogen therapy in women.

Previous studies have shown a strong correlation between the thrombin generation test and the presence of prothrombotic states (9–13). However, the magnitude of the association differs according to the test technique. In most previous studies, the thrombin generation test was triggered by recalcification in the presence of tissue factor and phospholipid, with or without activated protein C (9–11). This test appears to be highly sensitive in detecting protein S deficiency or activated Protein C resistance related to factor V Leiden or oestrogen therapy. Conversely, the addition of activated protein C does not enable protein C deficiency to be detected. The measurement of thrombin generation with and without thrombomodulin more accurately reflects the whole coagulation system than thrombin generation with and without activated protein C (12, 20). Indeed, Dargaud et al. (12) showed that this latter technique was highly sensitive for the detection of various thrombophilias. In our study, using a thrombin generation test that was closed to that of Dargaud et al. (12), we found a high positive predictive value of this test, which is in accordance with their results. Some authors also used a thrombin generation test in order to detect patients with a low risk of recurrent venous thromboembolism after a first episode of venous thromboembolism. In a study by Hron et al. (13), a generation test without the adjunction of activated protein C or thrombomodulin was used. If this technique appeared to be accurate in detecting patients with a lower risk of recurrent venous thromboembolism, on the other hand, it is not sensitive in the detection of frequent thrombophilia such as factor V Leiden.

In the setting of the risk assessment for venous thromboembolism in relatives of patients with venous thromboembolism and inherited thrombophilia, our study is the first to suggest that relatives with inherited thrombophilia, as well as those with acquired prothrombotic states, may be identified using a thrombin generation test. These preliminary results need further confirmation by means of a larger cohort of relatives of patients with venous thromboembolism and inherited thrombophilia. In addition, some patients with idiopathic venous thromboembolism and no detectable inherited thrombophilia may have an inherited thrombophilia that has yet to be discovered: in the first-degree relatives of those patients with such a high risk of thrombosis, the thrombin generation test may have the potential to detect those with a high probability of having an underlying prothrombotic state.

This study has a number of potential limitations, which might have contributed to the lower accuracy of our thrombin generation test: the sample size of the study was small and it was not possible to test for elevated factor VIII, homocysteinemia and anticardiolipid antibodies. However, the blood samples and thrombin generation tests were performed by nurses and physicians who were unaware whether relatives had suffered a previous venous thromboembolism or not, or whether they had a prothrombotic state or not.

In conclusion, an abnormal thrombin generation test using an adjunction or not of thrombomodulin appears to be highly predictive of the presence of a prothrombotic state, inherited or not, in first degree relatives of patients with venous thromboembolism and inherited thrombophilia.

Acknowledgements

Dominique Mottier and Jean François Abgrall contributed equally to this study.

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