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Thromboplastin-thrombomodulin-mediated time: a new global test sensitive to protein S deficiency and increased levels of factors II, V, VII and X

MONTSERRAT BORRELL, DOLORS LLOBET, ROSA ORTÍN, ROSA FELICES, CRISTINA VALLVÉ, JOSÉ MATEO, JOAN CARLES SOUTO, JORDI FONTCUBERTA
Unitat d'Hemostàsia i Trombosi, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain

Correspondence: Montserrat Borrell, PhD, Unitat d'Hemostàsia i Trombosi, Hospital de la Santa Creu i Sant Pau, Avgda San Antoni Mª Claret 167, 08025 Barcelona, Spain.
Phone: international +34.93.2919290. Fax: international +34.93. 2919192. E-mail: mborrell@hsp.santpau.es

Background and Objectives. A new test for screening the procoagulant capacity of plasma is described and evaluated. This test is based on the coagulation of plasma initiated by thromboplastin (Tp) in the presence of thrombomodulin (TM). In a previous paper we reported that this test had a significant phenotypic and genetic correlation with thrombosis susceptibility. The present report describes the characteristics of the test and its sensitivity to the concentration of some hemostasis factors.

Design and Methods. Plasma from normal subjects, from individuals with various disorders of hemostasis and plasma with different concentrations of factors II, V, VII, VIII, X, fibrinogen, protein C and protein S were studied. The thromboplastin-thrombomodulin-mediated time (Tp-TMT) is measured after mixing 100 μL of plasma diluted 1/10 at 37°C with 100 μL of a solution composed of 2 parts of thromboplastin, 1 part of thrombomodulin at 30 U/mL and 1 part of Owren's buffer. The results are expressed as the ratio of the patient's clotting time to that of the control. Values were compared with Student's t test and the Mann-Whitney test. Differences were considered statistically significant when $p<0.05$.

Results. In the control group women showed significantly lower values than men. Raised levels of factors II, V, VII and X reduced the coagulation time obtained with Tp-TM. Elevated concentrations of fibrinogen and factor VIII did not influence the test. The Tp-TMT was sensitive to protein S deficiencies, but not to protein C deficiencies.

Interpretation and Conclusions. These results indicate that the effect of protein S on the test is through its anti-prothrombinase activity. In conclusion: Tp-TMT, which is correlated with thrombosis susceptibility, is sensitive to raised levels of factors II, V, VII and X, as well as to low levels of protein S, and may be an indicator of thrombosis risk.

Key words: procoagulant activity, screening test, coagulation factors, protein S.

Activation of coagulation *in vivo* is considered to occur via the extrinsic coagulation pathway. It is initiated by exposure of blood to tissue factor (TF) at sites of vascular injury, and by its binding to factor VII/VIIa. Subsequently, the TF/VIIa complex activates both factors IX and X, leading to the formation of thrombin. This first reaction is down-regulated by tissue factor pathway inhibitor (TFPI)[2] and takes place on the endothelial surface where thrombomodulin (TM) plays a critical role in modulating the thrombin activity generated. When thrombin binds TM, the enzyme replaces its procoagulant function by anticoagulant. It abrogates virtually all procoagulant activities such as fibrin formation and factors V, VIII, XIII and platelet activation, and it acquires a potent anticoagulant activity through protein C activation. Furthermore, the thrombin-TM complex activates the fibrinolysis inhibitor TAFI. A test that measures the coagulation time of plasma when TF, phospholipids and TM are present would thus be an indicator of the procoagulant capacity of the plasma.

Here we describe and evaluate a test based on the thromboplastin time in the presence of thrombomodulin. This test reflects the first step of coagulation initiated by TF and it depends on the level of coagulation factors, mainly factors II, V, VII, and X, as well as on protein S as a prothrombinase inhibitor.[2-3] In a previous family-based study[1] we reported that this test showed a significant phenotypic and genetic correlation with thrombosis

susceptibility, and the present report describes this global test and its sensitivity to the increased concentration of some hemostasis factors.

Design and Methods

Subjects studied

Normal subjects. A total of 96 healthy blood donors constituted the population studied. Of these, 52 were men with a mean age of 40.85 years (SD 12.65) and 44 were women with a mean age of 40.45 years (SD 12.98).

Individuals with hemostatic disorders. The population consisted of 4 individuals with factor V deficiency, 3 with factor VII deficiency, 17 with increased levels of factor VIII (>160%), 8 with factor VIII deficiency, 9 with protein C deficiency, 25 with protein S deficiency, 19 with factor V Leiden and activated protein C resistance (APCR) and 6 with anticardiolipin antibodies (one of them with lupus anticoagulant). In addition, 33 individuals who belonged to a family with the 20210 A variant of the prothrombin gene were studied: 16 had the G/A genotype and 17 the G/G genotype.

Blood sampling and plasmas studied

Blood was collected on 1/10 volume of 0.129 M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2,000 g for 20 minutes at room temperature. Plasma was used the same day or was stored at –40°C until use. Control plasma was prepared mixing plasma from 80 healthy blood donors.

Plasma with different concentrations of hemostasis proteins. Control plasma was supplemented with different concentrations of purified factors: II, V, X or fibrinogen (Diagnostica Stago, Roche), or factor VII (American Diagnostica) or factor VIII (Bayer) after which the Tp-TMT and the factor levels were evaluated. Plasma from a protein C deficient patient was supplemented with purified protein C (Diagnostica Stago, Roche), after which the Tp-TMT and the protein C level were evaluated. To obtain plasmas with different levels of protein S, normal plasma was mixed in different proportions with protein S-deficient plasma (Stago Roche). The Tp-TMT and the protein S concentration were then evaluated.

Hemostasis tests. Coagulation factors were assayed using deficient plasmas from Diagnostica Stago (Roche). Functional protein S (PS) was determined with a kit from Diagnostica Stago (Roche). APCR, antithrombin and protein C were evaluated using methods from Chromogenix (Mölndal).

Thromboplastin-thrombomodulin-mediated time (Tp-TMT) is a modified prothrombin time in the presence and in the absence of thrombomodulin. The materials used were Owren's buffer (Diagnostic Grifols, Barcelona, Spain), and thombomodulin (American Diagnostica, Greenwich, CT, USA). Thromborel S (Dade Behring, Marburg, Germany) was prepared following the manufacturer's instructions. Lubrol PX Sigma (Saint Louis, MO, USA). Solution A was composed of 2 parts of thromborel S, 1 part of thrombomodulin at 30 U/mL and 1 part of Owren's buffer. Solution B was similar to solution A except for the thrombomodulin which was replaced by its solvent: buffer 0.02M Tris, 0.1 M NaCl, pH 7.4, 0.05 % lubrol PX, and 0.02% sodium azide. The test was carried out by mixing 100 μL of plasma diluted 1/10 in Owren's buffer at 37°C with 100 μL of solution A and recording the coagulation time. Another sample of 100 μL of plasma 1/10 was mixed at 37°C with 100 μL of solution B and the coagulation time recorded. Results were expressed as R1: the ratio of the patients' clotting time to that of the control using solution A, and R2: the ratio of the patients' clotting time to that of the control using solution B. The result R2 was used as a control in order to rule out plasmas that had prolonged values because of the presence of anticoagulants, factor deficiencies or lupus anticoagulant.

Statistical analysis

Student's t-test was used to compare values from men and women in the control group, and also to compare individuals with the G/G or G/A genotype from the family with the 20210 A mutation on the prothrombin gene. Mann-Whitney's test was used to compare values from individuals with hemostasis disorders and controls. The differences were considered statistically significant when $p<0.05$.

Results

Normal values. Table 1 shows values of Tp-TMT in normal individuals. Women had significantly lower R1 values than men. There were no statistically significant differences between individuals of different ages (older or younger than 46 years) in either men or women. The inter-assay coefficient of variation was 6.13%.

Influence of hemostatic disorders on Tp-TMT in different subjects. Figure 1 shows the distribution of R1 values of individuals with factor V deficiency, factor VIII deficiency, increase of factor VIII, protein C deficiency, protein S deficiency, factor V Leiden and antiphospholipid antibodies. Patients with factor VII deficiency were excluded from the figure because they had very prolonged times and therefore gave unreliable results. The Tp-TMT was prolonged in individuals with low levels of factor V, VII

Table 1. Mean and standard deviation of Tp-TMT in 96 controls, with a comparison of the results in men and women.

	no.	*R1 Mean (SD)*
All	96	1.03 (0.18)
Men	52	1.08 (0.19)*
Women	44	0.96 (0.15)*
**p*		0.001

Table 2. Influence of the 20210 A variant of the prothrombin gene on Tp-TMT.

PT 20210	*R1 mean (SD)*	*Factor II % mean (SD)*
G/G (n=17)	1.17 (0.234)	118 (13.4)
G/A (n=16)	0.89 (0.142)	140 (15.4)
p	<0.001	<0.001

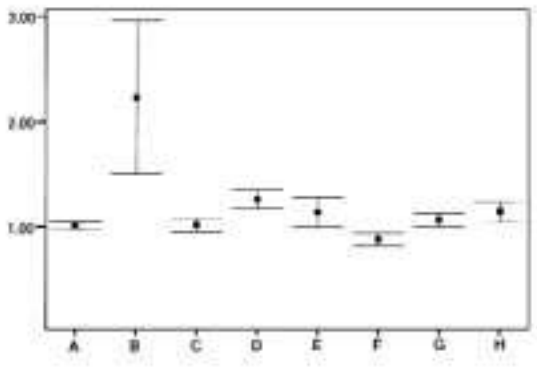

Figure 1. Tp-TMT values in individuals with hemostatic disorders. Letters indicate groups of individuals. A: controls; B: factor V deficiency; C: elevated levels of factor VIII; D: factor VIII deficiency; E: protein C deficiency; F: protein S deficiency; G: factor V Leiden; H: anticardiolipin antibodies. Results are expressed as the mean and the 95% confidence interval of the mean.

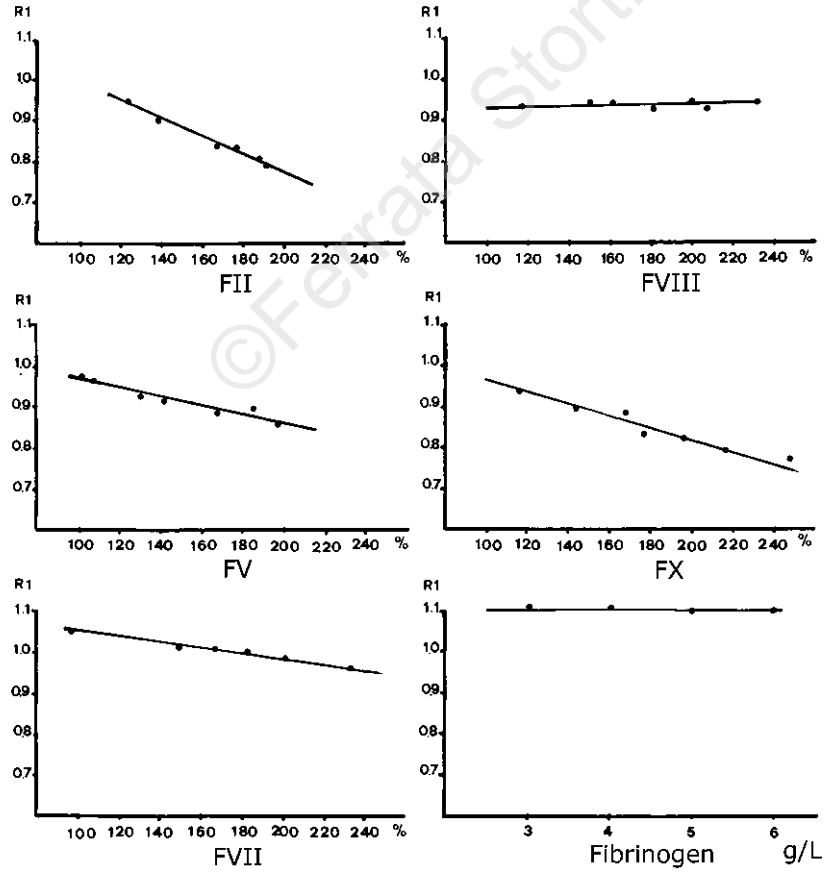

Figure 2. Relationship between factors II, V, VII, VIII, X and fibrinogen concentration and Tp-TMT. Control plasma was supplemented by different concentrations of purified factors II, V, VII, VIII, and X and fibrinogen, and Tp-TMT and the factor levels were determined.

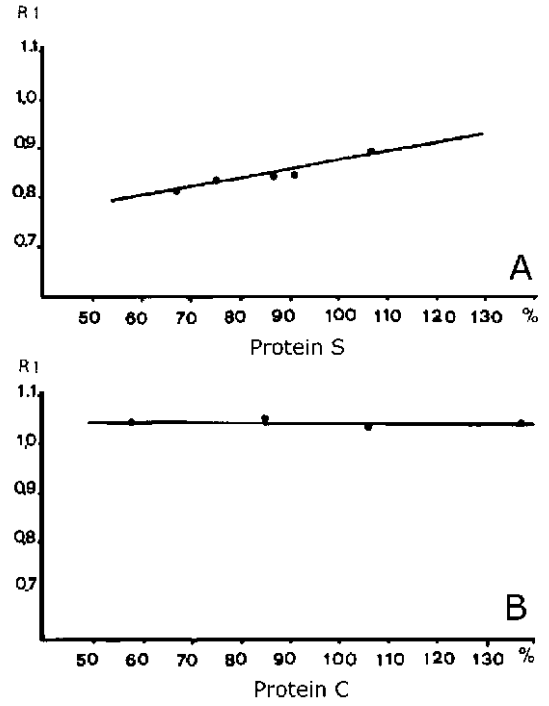

Figure 3. Relationship between protein C and protein S concentrations and Tp-TMT. A) Plasma from an individual with protein C deficiency was supplemented by different concentrations of purified protein C, and the Tp-TMT and protein C level were determined. B) Control plasma was mixed in different proportions with protein S-deficient plasma, and the Tp-TMT and protein S level were determined.

and VIII and with antiphospholipid antibodies. No differences were observed in individuals with increased levels of factor VIII, with protein C deficiency or with factor V Leiden ($p>0.05$). Individuals with protein S deficiency presented lower values than controls ($p<0.001$).

Tp-TMT in a family with the 20210 A variant of the prothrombin gene. Table 2 shows the results of Tp-TMT in a family with the 20210 A variant of the prothrombin gene. Individuals with the G/A variant had a significantly lower R1 than those with the normal G/G genotype ($p<0.001$). The G/A individuals also had significantly higher levels of factor II than did the G/G individuals (140% vs 118% $p<0.001$). Figure 2 shows the effect on Tp-TMT of the addition of purified coagulation factors to plasma. When factor II, V, VII or X was added to pooled normal plasma, there was a dose-dependent shortening of the Tp-TMT, presenting slopes of -2.17×10^{-3}, -1.1×10^{-3}, -0.7×10^{-3}, and -1.5×10^{-3} for factor II, V, VII and X, respectively. Addition of fibrinogen or factor VIII did not influence the Tp-TMT. Figure 3 shows the effect of a low concentration of protein S and protein C. A direct relationship was observed between the levels of protein S and the Tp-TMT. In contrast, protein C concentration did not influence the test.

Discussion

We describe a new global test for screening the procoagulant capacity of plasma. The test is based on the coagulation of plasma initiated by tissue factor in the presence of thrombomodulin and a low phospholipid concentration. The Tp-TMT is sensitive to different concentrations of coagulation factors from the extrinsic pathway. Raised levels of factors II, V, VII and X shorten the coagulation time obtained with the Tp-TM (Figure 2). The concentrations of fibrinogen, factor VIII and protein C do not influence the test. Of the factors studied, prothrombin is the one that shows the strongest influence, as can be deduced from the slopes of the regression lines of factor concentrations vs Tp-TMT. The influence of factor II concentration was also observed in the study of a family with the 20210 A mutation on the prothrombin gene. In this family, individuals with the G/A genotype who had significantly higher values of factor II also had a shorter Tp-TMT than subjects with the G/G genotype.

The test is also sensitive to deficiencies of protein S as was observed in protein S-deficient patients (Figure 1) and in plasma with different concentrations of protein S. In contrast, different concentrations of protein C did not influence the Tp-TMT. These results support the hypothesis that this test is not influenced by protein C pathway activation, and that the effect of protein S observed in the test is due to its anti-prothrombinase activity. Van't Veer *et al.*[3] reported that protein S interfered with the initial thrombin generation. This effect was pronounced in reactions with limited amounts of phospholipids as in our test. This condition occurs *in vivo* in physiologic amounts of quiescent platelets, on membranes or in small perturbations of the vessel walls, regulating the low basal activity of the procoagulant system in the normal circulation.

In the group of controls, a sex-related difference in Tp-TMT was observed (Table 1). Women had significantly lower values than men: 0.96 vs 1.08, $p=0.001$. This difference may be attributed to lower levels of protein S in women than in men,[4,5] and also to an increase in the level of factors observed in women taking oral contraceptives.[6] The interest of Tp-TMT was demonstrated in our previous report on a family-based study (GAIT study) in which this test showed a strong phenotypic and genetic cor-

relation with the susceptibility to thrombosis. This means that there are genes (known or unknown) that jointly influence both the thrombosis susceptibility and the physiologic variation of this test. Moreover, earlier reports[7-11] have shown that the susceptibility to thrombosis is influenced not only by abnormalities in the hemostasis system, but also by quantitative variations of these components within the normal range. The sensitivity of the Tp-TMT to increased levels of some coagulation factors and to low levels of protein S, which regulate early thrombin generation, could account for its relationship with susceptibility to thrombosis.

In summary, this test is fast and easy to carry out, screening the first step of coagulation initiated by TF. It is sensitive to elevated levels of factors II, V, VII and X as well as to low levels of protein S, and may be a useful tool for diagnosing the risk of thrombosis. In addition the test may be used to search for quantitative trait locus (QTL) in the genoma that pleiotropically affects the test results and thrombosis susceptibility.

Contribution and Acknowledgments

The first author, MB, designed the study and developed the test, and the last author, JF, supervised the study and revised the manuscript. The other authors collaborated equally: DL, RO, RF, and CV performed the laboratory assays, JM and JCS selected and recruited the patients.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received November 19, 2001; accepted March 7, 2002.

What is already known on this topic

There are not specific and sensitive tests with clinical value for investigating the first step of coagulation initiated by tissue factor.

What this study adds

A new test for screening the procoagulant capacity of plasma initiated by thromboplastin in the presence of thrombomodulin is described and evaluated.

Potential implications for clinical practice

This new test is sensitive to raised levels of factors II,V,VII and IX, as well as to low levels of protein S. The authors suggest that the assay could be a biological indicator of thrombosis risk.

Vicente Vicente, Deputy Editor