Utility of Thrombin-Generation Assay in the Screening of Factor V G1691A (Leiden) and Prothrombin G20210A Mutations and Protein S Deficiency

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Background: The thrombin-generation assay has a variety of clinical uses, including diagnosis of thromboembolism-related disease, and particular profiles are associated with thrombophilic risk factors. The aim of this study was to evaluate the use of this assay in screening and identifying patients who require specific thrombophilic testing.

Methods: We used a 2-step approach to perform specific thrombophilic testing and thrombin-generation assays on 169 consecutive patients. The first step was to identify particular profiles of thrombin generation corresponding to each type of thrombophilic risk factor and to determine the pertinent variables related to thrombin generation. We then performed ROC curve analysis for each predefined variable to determine the relevant cut-offs for identification of patients in need of further testing (negative predictive value, 100%).

Results: Suggestive profiles were seen in factor V Leiden (n = 49) and prothrombin (n = 12) mutations and in protein S deficiency (n = 12). ROC curves showed that factor V Leiden may be excluded when the difference between lag times obtained in the absence and presence of activated protein C (APC) is >1.5 min and that prothrombin G20210A may also be excluded when the peak thrombin concentration is ≤426 nmol/L. In addition, protein S deficiency may be excluded when the percentage of APC-induced endogenous thrombin potential inhibition is >63%.

Conclusion: The thrombin-generation assay represents a promising tool for screening thrombophilic risk factors, particularly in patients who are carriers of factor V Leiden or prothrombin G20210A mutations and patients with protein S deficiency.

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Venous thrombosis is a multifactorial disease that involves interactions between inherited risk factors such as factor V G1691A/R506Q (FV-Leiden) and prothrombin G20210A (FII-G20210A) single-nucleotide polymorphisms and acquired risk factors such as lupus anticoagulant (LA). Patients with venous thromboses or persons with a family history of the disease are traditionally evaluated by investigation of single components of the clotting system, such as FV-Leiden and FII-G20210A mutations, antithrombin (AT) deficiency, protein C (PC) deficiency, protein S (PS) deficiency, and LA.

Introduction of a general function test, the thrombin-generation assay, has enabled assessment of both the pro- and anticoagulant aspects of the hemostatic thrombotic system. This test has been studied in various settings, including congenital deficiencies of factors II, VII, X, and XI; hemophilia; congenital and drug-induced platelet disorders; and acquired and congenital thrombophilic

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2 Nonstandard abbreviations: FV, factor V; FII, prothrombin (factor II); AT, antithrombin; (A)PC, (activated) protein C; PS, protein S; LA, lupus anticoagulant; PRP, platelet-rich plasma; and ETP, endogenous thrombin potential.
risk factors (7–12). By enabling identification of particular profiles according to the type of thrombophilic risk factor, this assay is useful for detection of activated PC (APC) resistance attributable to inherited and acquired risk factors and for prediction of risk for venous thrombosis (11–14).

The aim of the present study was to analyze the thrombin-generation assay as a method for identifying patients requiring specific thrombophilic risk factor testing.

Patients and Methods

Patients
We investigated 169 consecutive adult outpatients referred for thrombophilic risk factor screening because of a personal or familial history of thromboembolism-related disease. In symptomatic patients, blood was sampled at least 1 month after anticoagulant treatment was discontinued. Women who were pregnant, had recently given birth, or were using oral contraceptives containing estrogens and persons undergoing anticoagulant therapy were excluded from the study. We obtained informed consent from all patients, and the study met all institutional ethics requirements.

Blood Samples and Platelet-Rich Plasma
Blood was withdrawn into Vacutainer® tubes (CTAD and EDTA; Becton Dickinson) for thrombophilic screening and by antecubital venipuncture into Monovette® tubes (0.106 mol/L citrate; Sarstedt) for the thrombin-generation assay.

Platelet-rich plasma (PRP) was obtained after centrifugation of citrated blood (190g for 12 min), adjusted to 150 × 10⁹ platelets/L in autologous poor-platelet plasma.

Thrombophilic Screening
We assessed AT concentration with an in-house amidolytic assay [S2238 substrate® (Chromogenix) and Purified Bovine Thrombin® (Diagnostica Stago)]. PS concentration was determined by chromometric (Staclot Protein S®; Diagnostica Stago) and antigenic assays (Free PS Antigen®; Roche Diagnostics) according to the manufacturers’ instructions. The PC concentration was measured with chromometric (Staclot Protein C®; Diagnostica Stago) and chromogenic (Stachrom Protein C®; Roche Diagnostics) assays. AT deficiency was defined as AT concentrations <80%, and PS and PC deficiencies were defined as PS and PC values <60%. The presence of LA was diagnosed according to the criteria of the Subcommittee on LA of the International Society on Thrombosis and Hemostasis (15). We obtained Rosner indexes by use of PTT-LA reagent (Diagnostica Stago) and performed genotyping of FV-Leiden and FII-G20210A II as described previously (16).

Thrombin-Generation Test
We used the calibrated thrombogram method (5, 17) to measure thrombin generation continuously in thawed, previously frozen PRP. Briefly, 10 μL of buffer (5 g/L bovine serum albumin, 20 mmol/L HEPEs, 140 mmol/L NaCl) or 10 μL of APC (25 pmol/L; a generous gift from Thomas Lecompte, INSERM U 734, Nancy, France), 10 μL of purified recombinant tissue factor diluted in buffer (0.5 pmol/L, a generous gift from Peter Giesen, Maastricht, The Netherlands), and 80 μL of thawed PRP were manually pipetted into polypropylene microtiter well plates (Greiner). Plates were inserted into the plate reader (Fluoroskan Ascent; Thermolab Systems) and preheated to 37 °C for 5 min. Coagulation was triggered by automated addition of 20 μL of a mixture containing the fluorescent substrate Z-Gly-Gly-Arg-AMC (2.5 mmol/L; Bachem) and calcium in buffer (0.1 mol/L CaCl₂, 20 mmol/L HEPEs, 60 g/L bovine serum albumin). The excitation and emission wavelengths were 390 and 460 nm, respectively. Fluorescence intensity was measured in real time at 37 °C. Samples were tested in duplicate. We used Thrombinoscope™ software (Synapse BV) to convert the fluorescent signal to a thrombin concentration by continuous comparison with the signal generated by a thrombin calibrator (Synpase BV) added to a separate sample of the test plasma.

The same control (frozen PRP obtained from 1 single healthy volunteer) was assayed every day in each set of experiments.

Results are expressed as lag time (minutes), peak concentration (nmol/L thrombin), and endogenous thrombin potential (ETP; nmol/L thrombin). Comparisons between ETP in the absence and presence of APC are expressed as Δlag time (lag time with APC – lag time without APC) and percentage of APC-induced ETP inhibition [ETP without APC − ETP with APC] × 100/ETP without APC.

The intraassay CV was calculated from 8 consecutive measurements of the same control. The CVs were as follows: 4% for the lag time, 4% for the ETP, 5% for the peak, 13% for the Δlag time, and 3% for the percentage of APC-induced ETP inhibition. The interassay CV was calculated from the overall results obtained for the control run daily. The CVs were as follows: 13% for the lag time, 8% for the ETP, 22% for the peak, 16% for the Δlag time, and 12% for the percentage of APC-induced ETP inhibition.

Statistical Analysis
We performed statistical comparisons with the Kruskal–Wallis and Mann–Whitney nonparametric tests and assessed the diagnostic value of the thrombin-generation test with ROC curves. Data for selected variables of the thrombin-generation assay were compared with FV-Leiden and FII-G20210A genotyping and PS concentration. For each ROC curve, the cutoff of the thrombin-generation assay variable for which sensitivity and negative
predictive value were 100% was chosen, and both specificity and positive predictive value corresponding to each chosen cutoff were reported. Statistical significance was set at $P < 0.05$.

**Results**

**Patient Characteristics**

Patient baseline characteristics are reported in Table 1. Of the 169 patients, 71% presented with a personal history of thrombosis, and the remainder were asymptomatic and were referred for familial thrombophilic risk factor testing.

Thrombophilic risk factor screening in patients detected LA ($n = 4$), AT deficiency ($n = 2$), PC deficiency ($n = 9$), PS deficiency ($n = 12$), and FV-Leiden ($n = 49$) and FII-G20210A ($n = 12$) mutations. Combined abnormalities were observed in 11 patients: 6 with the FV-Leiden mutation associated with PS deficiency, 4 with the FII-G20210A mutation associated with PS deficiency, and 1 case of PC deficiency with the FII-G20210A mutation. Of

Table 1. Characteristics of patients.

<table>
<thead>
<tr>
<th>Total no. of patients</th>
<th>169</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age, years</td>
<td>43 (16)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>61 (36.1)</td>
</tr>
<tr>
<td>Personal history of thrombosis, n (%)</td>
<td>121 (71.6)</td>
</tr>
<tr>
<td>No thrombophilic disorder, n (%)</td>
<td>92 (54.4)</td>
</tr>
<tr>
<td>FV-Leiden, n (%)</td>
<td>49 (29)</td>
</tr>
<tr>
<td>Isolated</td>
<td>10 (5.6)</td>
</tr>
<tr>
<td>Combined</td>
<td>7 (4.1)</td>
</tr>
<tr>
<td>FII-G20210A, n (%)</td>
<td>12 (7.1)</td>
</tr>
<tr>
<td>Isolated</td>
<td>5 (2.9)</td>
</tr>
<tr>
<td>Combined</td>
<td>6 (3.5)</td>
</tr>
<tr>
<td>PS deficiency (&lt;60%), n (%)</td>
<td>12 (7.1)</td>
</tr>
<tr>
<td>Isolated</td>
<td>6 (3.5)</td>
</tr>
<tr>
<td>Combined</td>
<td>9 (5.3)</td>
</tr>
<tr>
<td>PC deficiency (&lt;60%), n (%)</td>
<td>8 (4.7)</td>
</tr>
<tr>
<td>Isolated</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Combined</td>
<td>2 (1.2)</td>
</tr>
<tr>
<td>AT deficiency (&lt;80%), n (%)</td>
<td>4 (2.4)</td>
</tr>
<tr>
<td>LA (Rosner index &gt;15), n (%)</td>
<td>4 (2.4)</td>
</tr>
</tbody>
</table>

Fig. 1. Examples of thrombin-generation assay results in thrombophilic disorders.

(A) no defect; (B) FV-Leiden; (C) FII-G20210A mutation; (D) PS deficiency. Relevant variables, i.e., lag time, ETP value, peak concentration (in the absence of APC), APC-induced ETP inhibition, and $\Delta$ lag time, are indicated for each pattern.
the FV-Leiden carriers, 1 patient was found to be a homozygous carrier (A/A genotype).

No thrombophilic defect was observed in 92 patients.

**THROMBIN GENERATION IN THE ABSENCE AND PRESENCE OF APC**

The thrombin-generation data, i.e., lag time, ETP value, peak concentration, percentage of APC-induced ETP, and Δlag time, are presented in Fig. 1 and Table 2.

In patients without thrombophilic risk factors, the mean (SD) percentage of APC-induced ETP inhibition was 77 (11)% in patients without a personal history of thrombosis (n = 23) and 64 (21)% in patients with a previous thrombotic event (n = 69). The difference between these groups was significant (P < 0.05).

Data from patients with each type of thrombophilic risk factor, with the exception of PC deficiency, showed a distinct pattern compared with data from patients free of any documented thrombophilic disorder (Fig. 1). FV-Leiden was characterized by decreases in Δlag time and the percentage of APC-induced ETP inhibition (P < 0.05; Table 2). The FII-G20210A mutation was characterized by increased ETP values and peak concentrations (both in the absence of APC; P < 0.05; Table 2). Four patients were carriers of both FII-G20210A and FV-Leiden mutations, and their data patterns fit the combined criteria of FV-Leiden and FII-G20210A mutations, as demonstrated by a decrease in both APC-induced ETP inhibition and Δlag time. PS deficiency was characterized by a decrease in the percentage of APC-induced ETP inhibition and a decrease in lag time in the absence of APC (P < 0.05; Table 2). AT deficiency was characterized by a dramatic increase of ETP in the absence of APC (Table 2). The presence of LA was characterized by an increase of lag time in the absence of APC and by a decrease in the percentage of APC-induced ETP inhibition (Table 2). PC defect data did not exhibit a suggestive pattern when compared with the absence of thrombophilic risk factor (Table 2).

**DETERMINATION OF RELEVANT CUTOFF OF THROMBIN-GENERATION ASSAY**

We used ROC curves to evaluate the utility of the thrombin-generation assay as a screening test for FV-Leiden and FII-G20210A mutations and for PS deficiency. Cutoff values that allowed 100% sensitivity and a 100% negative predictive value were determined.

For FV-Leiden, the cutoffs of Δlag time and percentage of APC-induced ETP inhibition were 1.5 min and 81%, respectively (Fig. 2). With these cutoffs, Δlag time had a specificity of 96%, whereas percentage of APC-induced ETP inhibition had a lower specificity (25%).

In FII-G20210A, the cutoffs for the peak concentration and ETP value (obtained in the absence of APC) were 426 and 1762 nmol/L thrombin, respectively. Specificity was higher for the peak concentration (71%) than for the ETP value (43%).

In PS deficiency, the cutoffs for percentage of APC-induced ETP inhibition and for the lag time obtained in the absence of APC were 63% and 4.38 min, respectively. For such cutoffs, specificity was higher for the percentage of APC-induced ETP inhibition (52%) than for the lag time obtained in the absence of APC (30%).

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**Table 2. Thrombin-generation assay: Results for 5 variables according to thrombophilic disorder.**

<table>
<thead>
<tr>
<th>No thrombophilic disorder (n = 92)</th>
<th>Lag time, min</th>
<th>ETP, nmol/L thrombin</th>
<th>Peak, min</th>
<th>ETP inhibition, %</th>
<th>ΔLag time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>4.23 (0.62)</td>
<td>882 (317)</td>
<td>399 (71)</td>
<td>67 (20)</td>
<td>2.70 (0.67)</td>
</tr>
<tr>
<td>FV-Leiden (n = 49)</td>
<td></td>
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</tr>
<tr>
<td>Mean (SD)</td>
<td>4.07 (0.53)</td>
<td>1844 (299)</td>
<td>397 (57)</td>
<td>40 (22)</td>
<td>0.76 (0.36)</td>
</tr>
<tr>
<td>Range</td>
<td>3.12–5.50</td>
<td>1120–2620</td>
<td>238–523</td>
<td>6–81</td>
<td>0–1.5</td>
</tr>
<tr>
<td>FII-G20210A (n = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>4.19 (0.44)</td>
<td>2407 (364)</td>
<td>484 (52)</td>
<td>51 (20)</td>
<td>1.67 (0.78)</td>
</tr>
<tr>
<td>Range</td>
<td>3.3–4.88</td>
<td>1764–3031</td>
<td>427–591</td>
<td>11–84</td>
<td>0.63–2.87</td>
</tr>
<tr>
<td>PS deficiency (n = 12)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.56 (0.42)</td>
<td>1629 (261)</td>
<td>392 (49)</td>
<td>35 (22)</td>
<td>1.52 (0.87)</td>
</tr>
<tr>
<td>Range</td>
<td>3.00–4.36</td>
<td>1120–2040</td>
<td>319–467</td>
<td>6–63</td>
<td>0.12–3</td>
</tr>
<tr>
<td>PC deficiency (n = 9)</td>
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<tr>
<td>Mean (SD)</td>
<td>3.97 (0.63)</td>
<td>1859 (390)</td>
<td>410 (76)</td>
<td>73 (18)</td>
<td>2.94 (0.64)</td>
</tr>
<tr>
<td>AT deficiency (n = 2)</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Mean (SD)</td>
<td>4.25 (0.35)</td>
<td>2997 (928)</td>
<td>499 (6)</td>
<td>83 (2)</td>
<td>2.75 (0.70)</td>
</tr>
<tr>
<td>Range</td>
<td>6–14.6</td>
<td>1296–2164</td>
<td>149–503</td>
<td>8–67</td>
<td>0.63–4.00</td>
</tr>
</tbody>
</table>

* a Minimum–maximum.

* b P <0.05, compared with patients without thrombophilic disorder.

* c Marked difference compared with patients without thrombophilic disorder (no statistical analysis was performed because of the limited number of patients in AT deficiency and LA subgroups).
Discussion

We investigated the use of the thrombin-generation assay as a potential screening test for thrombophilic risk factors, a use of the assay that to our knowledge has not been addressed previously.

Our results showed that FV-Leiden and PS deficiency were significantly associated with a decrease in APC-induced ETP inhibition, in agreement with previous reports (11, 12). Furthermore, our results indicated that other variables may be more relevant than APC-induced ETP inhibition in the screening of FV-Leiden patients. Indeed, the lag time (the difference between lag times obtained in the absence and presence of APC) was significantly lower in FV-Leiden patients. This finding is in agreement with the fact that patients with the FV-Leiden mutation show APC resistance.

In FII-G20210A carriers, ETP values and peak concentrations were significantly different from those in patients without thrombophilic risk factors. The association of FV-Leiden with FII-G20210A was identified by the association of decreases in APC-induced ETP inhibition and lag time. These results fit with the notion that the promoter mutation 20210 is associated with enhanced factor II plasma concentrations.

A particular pattern was observed in AT deficiency, characterized by a dramatic enhancement of ETP values and peak concentrations obtained in the absence of APC. Indeed, in our experimental conditions, thrombin generation occurs in the presence of AT, and the test is therefore highly dependent on the AT functional activity. A particular pattern was also observed in patients with LA: an increase in the lag time in the absence of APC and APC resistance evidenced by the percentage of APC-induced ETP inhibition. When we used exogenous APC, we found that the thrombin-generation assay was insensitive to PC deficiency. Such profiles were in accordance with reported results (13, 18). Because of the limited number of patients in each group, no statistical analysis could be performed for LA or for AT deficiency.

The second step of analysis was to establish relevant cutoffs for each predefined risk factor in screening for FV-Leiden and FII-G20210A mutations and for PS deficiency. A cutoff that provided a sensitivity and a negative predictive value of 100% was chosen for each factor. The specificity and the positive predictive value were calculated in parallel. For FV-Leiden, lag time was the best marker, allowing screening of all patients with the FV-Leiden mutation with a high specificity (96%), whereas the APC-induced ETP inhibition had a low specificity (25%). These results for lag time were unexpected; based on lag time, the thrombin-generation assay could make FV-Leiden genotyping unnecessary in 67% of patients.

For the FII-G20210A mutation, comparisons between peak concentrations and ETP values demonstrated that the peak concentration was the most relevant variable, with a specificity of 71% vs 43% for ETP. The anticipated result for peak concentration was for FII-G20210A: genotyping should be unnecessary in 66% of tested patients. Furthermore, in the absence of APC, PS deficiency screening may be based on the percentage of APC-induced ETP inhibition rather than on the lag time. PS testing could have been avoided in 48% of patients based on the percentage of APC-induced ETP inhibition (specificity, 52%).

Taken together, these results show that (a) a lag time ≤1.5 min indicates the need for FV-Leiden genotyping; (b) a peak thrombin concentration >433 nmol/L indicates the need for FII-G20210A genotyping; and (c) APC-induced ETP inhibition ≤58% indicates the need for PS measurements. Because our current experimental conditions included the addition of exogenous APC, the thrombin-generation assay was not sensitive to PC deficiency. However, the assay may be optimized by the use of thrombomodulin, as reported previously (13).

Fig. 2. ROC curves in FV-Leiden patients, used to establish a relevant cutoff. 

δlag time is 100% sensitive for the indicated cutoff. (B), TP, true positive; TN, true negative; FP, false positive; FN, false negative; PPV, positive predictive value.

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In conclusion, the thrombin-generation assay can be used to accurately identify patients needing further specific thrombophilic risk factor testing. The potential for this assay to replace more complex and expensive tests should be investigated.

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References