Theme Issue Article

Use of calibrated automated thrombinography ± thrombomodulin to recognise the prothrombotic phenotype

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Summary
There is currently no validated method to detect a prothrombotic phenotype. The question remains, can tissue factor (TF) induced thrombin generation (TG), as measured with the calibrated automated thrombinography (CAT) technique, according to Hemker et al., recognise a prothrombotic state either as such, or when the activated protein C (APC)-system is boosted with thrombomodulin (TM)? We determined the normal range of CAT-TG ± TM in a group of 71 healthy blood donors, in 11 healthy women using oral contraceptives (OC), and in 89 patients with a history of venous thromboembolism (VTE), divided into a group of 50 in which a prothrombotic risk factor could be found (VTEprf+) and 39 others (VTEprf-). The endogenous thrombin potential (ETP) in the OC, VTEprf+ and VTEprf- group was significantly higher than for the controls. In the presence of TM, the differences were significantly higher than in its absence. The VTEprf+ group had a higher ETP, ± TM than the VTEprf- group. In conclusion, TG, measured with the CAT technique in the presence of TM is capable of detecting the prothrombotic phenotype with a high sensitivity of 0.93 (95% confidence limits 0.82–0.99).

Keywords
Thrombin generation, thrombomodulin, hypercoagulability, calibrated automated thrombogram, venous thromboembolism

Introduction
Thrombophilia may be congenital, acquired, or both (1). Current genetic screening techniques identify a risk factor in approximately 50% of patients with a single history of venous thromboembolism (VTE) (2). Acquired component(s) of thrombophilia, although important (3), tend to escape detection, hence it is hardly surprising that current thrombophilia screening approaches have a poor predictive value of the thrombotic phenotype (4). From a clinical point of view, the recognition of a thrombophilic phenotype would be of great value. Global haemostasis tests are the best candidates to provide this information as opposed to specific assays (5). However, our current clotting tests are hardly useful in this respect (6). The development of the calibrated automated TG test (CAT, 7) made it possible to measure the complete TG curve at high throughput. Because all known genetic causes of thrombophilia theoretically increase TG (8), and because all antithrombotic therapy decreases TG (8), we assumed that TG measurement could serve as a tool for assessing thrombotic risk. It has been suggested that the CAT might recognise congenital (9) and acquired hypercoagulability (10).

When TG is triggered by tissue factor (TF) the role of the protein C system (PCS) is difficult to quantify and/or standardise. Since many prothrombotic conditions implicate an abnormal function of the PCS, it is logical to trigger this system in a standardised way.

A method has been developed (9), adapted from the TG measurement method of Hemker et al. (11), in which the area under the TG curve, in the absence or presence of activated protein C (APC), is assessed from the residual amount of α2-macroglobulin-thrombin complex. This method identifies protein S (PS) deficiency, FV Leiden and acquired APC resistance (oral contraceptives), but lacks sensitivity to protein C (PC) deficiency (9, 12) and, because the results are expressed as a ratio, will not directly reflect hyperprothrombinaemia or antithrombin (AT) deficiency.

Previous work (13, 14) suggests that the addition of TM to a TG test might more closely reflect a physiological situation in
which the whole of the PCS partakes, so this test might be used to detect thrombophilic phenotypes. The aim of the present study was to investigate whether a thrombin generation test with added TM could detect prothrombotic phenotype independently of the routine thrombophilia testing.

Subjects, materials and methods

Subjects

Between November 2004 and May 2005, we obtained plasma samples from 89 consecutive outpatients (40 men and 49 women) who had suffered a first episode of proximal deep vein thrombosis (DVT) of the lower limbs (with or without pulmonary embolism). Their age was 21–70 years (43.9 years ± 14.0; mean ± SD). All of them had received oral anticoagulants for at least three months and had stopped this treatment for 30 ± 5 days when the plasma sample was taken for this study. All DVTs were objectively documented by Doppler ultrasonography and the diagnosis of pulmonary embolism was confirmed by perfusion-ventilation lung scanning or thoracic helical computed tomography. The VTE events were considered idiopathic if they could not be attributed to surgery, immobilisation, trauma, pregnancy or the use of oral contraceptives or hormone replacement therapy within three months before the VTE event. Exclusion criteria were previous VTE, venous valve implantation, surgical thrombectomy, VTE events in which the diagnosis was not objectively documented as described above, liver or renal insufficiency, evolutive cancer or malignant haematological disease, chronic inflammatory disease, known antiphospholipid syndrome, and prolonged anticoagulant therapy for other diseases.

The control population comprised 71 apparently healthy volunteer blood donors (48 men and 23 women) between 19 and 64 years (43.7 years ± 12; mean ± SD), not using drugs known to affect the coagulation system and without history of VTE or bleeding disorder. There were 36 subjects with O blood group and 35 with other blood groups. There were 14 smokers. Eleven healthy women using oral contraceptives (OC) were comparable volunteer blood donors but were excluded from the control group.

The study was approved by the local ethics committee, and informed consent was obtained for peripheral venous blood sampling.

Blood and platelet poor plasma

Peripheral venous blood was collected into Vacutainer® tubes (Becton Dickinson, Meylan, France) containing 0.129 M trisodium citrate (1 volume trisodium citrate to 9 volumes blood) from antecubital venipuncture using 21G needles. Following a double centrifugation at 2,500 ×g for 15 min, platelet poor plasma (PPP) was collected from the upper ½ volume of plasma supernatant, quick-frozen and stored at −80°C. The absence of platelet and leucocyte in PPP samples was checked with an Advia 120 (Becton Dickinson, Meylan, France) containing 0.129 M trisodium citrate (Becton Dickinson, Meylan, France) and 600 nM human thrombin was obtained from Thrombinoscope BV (Maastricht, The Netherlands). Recombinant human thrombomodulin (rh-TM) and rabbit lung thrombomodulin (RL-TM) were obtained from American Diagnostica Inc. (Stamford, CT, USA).

Routine measurements and screening

Thrombophilia tests included cell blood count, coagulation assays (activated partial thromboplastin time, prothrombin time, thrombin time, fibrinogen), antithrombin (AT), PC activity, PS activity and free PS antigen, APC resistance, lupus anticoagulant, antithrombin (AT) activity was detected by an automated amidolytic assay (Coamatic Antithrombin®, Chromogenix, Milan, Italy). PC activity was measured by two automated tests: an amidolytic assay using Coamatic Protein C® (Chromogenix, Milan, Italy) and a clotting assay (Staclot- Protein C, Diagnostica Stago, Asnières sur Seine, France). Free protein S antigen was measured by an ELISA method (Asserachrom Protein S Free, Diagnostica Stago, Asnières sur Seine, France). Protein S activity was detected by a clotting assay (Staclot Protein C, Diagnostica Stago, Asnières sur Seine, France). The first generation APC resistance assay was performed as previously described (15). The second generation APC resistance assay was performed after incubation of diluted test plasma with factor V deficient plasma (Coatest ApCTM Resistance, Chromogenix, Milan, Italy). Plasma FVIII clotting activity was measured by a one-stage clotting assay using Biomerieux deficient FVIII kit (Biomerieux, Marcy l’Etoile, France). Lupus anticoagulant assays were performed using aPTT and dilute Russell’s viper venom time (DRVVT) according to the recommendations of the International Society on Thrombosis and Haemostasis scientific subcommittee (16), Anticardiolipin antibodies (IgM and IgG) and D-dimer levels (VIDAS, Biomérieux, Marcy l’Etoile, France) were determined by ELISA methods. Plasma homocysteine levels were measured by HPLC (normal range: 4–13 μM). Factor V Leiden (G1691A) and factor II G20210A mutations were detected using a multiplex polymerase chain reaction (PCR) – directed mutagenesis protocol as previously described (17).

Reagents for the TG test

Recombinant rupophilidase tissue factor (TF) Innovin® was obtained from Dade Behring (Marburg, Germany). Phospholipid vesicles were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and prepared as previously described (18, 19). They consisted of 20 mol% phosphatidylserine, 20 mol% phosphatidylethanolamine and 60 mol% phosphatidylcholine. Heps-buffered saline contained 20 mM Hepes (Sigma Aldrich, l’Ile d’Abeau Chesnes, France), 140 mM NaCl and 5 mg/ml bovine serum albumin (BSA) (Euromedx, Souffelweyersheim, France), pH 7.35. A fresh mixture of fluorogenic substrate and CaCl₂ was prepared before each experiment. Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). The mixture of fluorogenic substrate 2.5 mM and CaCl₂ 0.1M was prepared using buffer containing Heps 20 mM and 60 mg/ml BSA, pH 7.35. The Calibrator with the activity of 600 nM human thrombin was obtained from Thrombinoscope BV (Maastricht, The Netherlands). Recombinant human thrombomodulin (rh-TM) and rabbit lung thrombomodulin (RL-TM) were obtained from American Diagnostica Inc. (Stamford, CT, USA).

Automated measurement of thrombin generation

TG was measured according to the method described by Hemker et al. (13) in a Fluoroscan Ascent® fluorometer (Thermolabsystems OY, Helsinki, Finland) equipped with a dispenser. Fluores-
cence intensity was detected at wavelengths of 390 nm (excitation filter) and 460 nm (emission filter). Briefly, 80 µl of PPP were dispensed into the wells of round-bottom 96-well microtiter plates (Greiner ref.no.65204, Poitiers, France). Twenty µl of a mixture containing TF and phospholipids was added to the plasma sample to obtain a final concentration of 5 pM and 4 µM, respectively. The starting reagent (20 µl per well) contained the fluoroegenic substrate and CaCl2. A dedicated software program, Thrombinoscope® version 3.0.0.26 (Thrombinoscope BV, Maastricht, The Netherlands), enables the calculation of thrombin activity and displays thrombin activity against time. All experiments were carried out in duplicate. The three most important parameters are the lag time, the peak of thrombin and the endogenous thrombin potential (ETP) corresponding to the area under the curve, which represents the enzymatic activity that thrombin can do during its lifetime (7, 13).

Statistics
Statistical analysis was performed using the GraphPad Instat 3.0® software package (San Diego, CA, USA). Results have been evaluated using Student t- or Mann Whitney tests. A p-value of <0.05 was considered statistically significant. ROC curves were obtained using the GraphPad Prism 4.0 software package (San Diego, CA, USA). Odd ratios (OR) were used to estimate the OR. Logistic regression was calculated using the program written by JC Pezzullo and KM Sullivan version 05.07.20.

Results
Comparison of the effect of rh-TM and RL-TM on CAT results
We compared the two types of thrombomodulin commercially available in France. In five healthy control subjects TG was measured in PPP at final concentrations 0 – 1 – 3 – 6 – 12 nM of rh-TM and 0 – 2 – 10 – 15 – 20 nM of RL-TM. Both preparations dose-dependently inhibited the ETP. Using our standard conditions, i.e. TF 5 pM and phospholipids 4 µM, 6 nM rh-TM or 15 nM RL-TM inhibited the ETP by 40 ± 13% and 48 ± 8%, respectively. Therefore, 15 nM RL-TM (final concentration) was chosen for further experiments.

Normal values of CAT in the presence and absence of TM
Table 1 shows normal values obtained under the above mentioned conditions with and without TM in a group of 71 normal donors. Our results confirmed the large inter-individual variability previously reported by Hemker et al. (13). No significant difference was observed between the ETP of women and men in the presence of TM (926 ± 284 and 807 ± 281 nM.min; p=0.10) or in its absence (1510 ± 214 and 1581 ± 214 nM.min; p=0.19). Neither was a significant difference found between the ETP of subjects with blood group O and others, either in the absence of TM (1539 ± 217 and 1575 ± 218 nM.min, respectively; p=0.49) or in the presence of TM (862 ± 308 and 852 ± 225 nM.min; p=0.88).

Thrombophilia screening
Among the 89 patients who presented with a history of VTE (58 idiopathic and 31 DVTs with transient risk factors), we found a single thrombophilia marker in 44 subjects (49.4%) and a homozygous mutation or combined risk factors in six patients (6.7%). The screening was negative in 39 subjects (43.8%). Among 50 patients with positive results, 39 had a congenital pro-thrombotic marker, e.g. 17 cases of heterozygous FV Leiden mutation, 10 patients with heterozygous FII G20210A mutation, three patients with PC deficiency, three with PS deficiency, combined thrombophilia risk factors were homozygous FV Leiden (n=1), combined FV Leiden and FII mutation (n=4) and co-inherited FII mutation and PC deficiency (n=1). Eleven patients presented a thrombophilia marker without recognised genetic cause, e.g. 10 high plasma FVIII activity >150 IU/dl (two determinations separated by at least three months with normal CRP), and one individual with isolated APC resistance without FV Leiden mutation.

Influence of TM on thrombin generating capacity of patients with a thrombophilia marker
Thrombin generating capacity was measured in all patients in the presence and absence of TM (Table 1). The ROC curves showed that the overall ability of the test to discriminate between patients positive for one of the thrombophilia markers and the others was higher in the presence of TM (0.92, 95 % CI =0.87 – 0.97 and p<0.0001) than in its absence (0.77, 95% CI =0.68 – 0.86).

Table 1: Thrombin generation assessment of 89 patients with a history of VTE with or without established thrombophilia marker and in 71 healthy blood donors in PPP using a final concentration of TF 5 pM and PL 4 µM in the presence and absence of RL-TM 15 nM (final concentrations).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>ETP – no TM (mean ± SD)</th>
<th>ETP – with TM (mean ± SD)</th>
<th>Peak – no TM (mean ± SD)</th>
<th>Peak – with TM (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>71</td>
<td>1557 ± 214</td>
<td>838 ± 271</td>
<td>398 ± 40</td>
<td>264 ± 84</td>
</tr>
<tr>
<td>No recognized VTE risk factor</td>
<td>39</td>
<td>1675 ± 295</td>
<td>1106 ± 377</td>
<td>416 ± 37</td>
<td>311 ± 76</td>
</tr>
<tr>
<td>Congenital VTE risk factor</td>
<td>39</td>
<td>1928 ± 337</td>
<td>1477 ± 295</td>
<td>445 ± 70</td>
<td>383 ± 74</td>
</tr>
<tr>
<td>High FVIII:C and isolated APC resistance group</td>
<td>11</td>
<td>1739 ± 341</td>
<td>1354 ± 339</td>
<td>406 ± 85</td>
<td>360 ± 90</td>
</tr>
<tr>
<td>Congenital VTE risk factor and high FVIII &amp; APC resistance groups</td>
<td>50</td>
<td>1886 ± 344</td>
<td>1442 ± 323</td>
<td>437 ± 74</td>
<td>378 ± 77</td>
</tr>
</tbody>
</table>

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Also, in the absence of added TM, the ETP was significantly higher in patients with a positive screening marker (1886 ± 344 nM.min) than in the others (1675 ± 295 nM.min; t-test p=0.003), but there was no statistically significant difference between the peak heights of the thrombogram (437 ± 74 nM and 416 ± 37 nM, respectively; t-test p=0.12). With the addition of TM, both the ETP (p<0.0001) and the thrombin peak (p<0.0001) were significantly higher in patients with a recognised thrombophilia marker.

TM-related inhibitions of the ETP in patients with different prothrombotic risk factors and in those without a recognised risk factor are shown in Figure 1. All conditions affecting the APC pathway (i.e. PC deficiency, PS deficiency, FV Leiden mutation, isolated APC resistance) showed a significant resistance to the inhibitory effect of TM (Figs. 1 and 2), but carriers of the G20210A allele did not (p=0.13). These patients can be recognised from their high ETP values both in the inhibited and the uninhibited thrombin generation (Fig. 2). In patients with high FVIII:C (n=10), in the presence of TM, ETP values were significantly higher (1320 ± 337 nM.min) than in the control group (p=0.0003) whilst in the absence of TM, ETPs were similar to that of the control group (p=0.12). In the group of DVT patients presenting no thrombophilia marker (n=39), the mean ETP inhibition was significantly lower in comparison with the control group (p=0.008). In the presence of TM, in healthy women with OC the ETP results were significantly higher than in the control group (1259 ± 277 and 838 ± 271, respectively; p=0.0001).

Relationship between the clinical VTE events and ETP
We investigated the relationship between high ETP values and clinical VTE using binary logistic regression. An ETP was considered abnormally high if it was above 1380 nM.min [mean±2SD for ETP values obtained in 71 healthy subjects (1=high ETP, 0=normal ETP and 1=VTE, 0=no VTE)]. We observed a high risk of VTE in subjects with high ETP results, OR = 19.4 (95% CI = 5.7 – 66.2). The VTE risk was also increased in individuals with no recognised risk factor, OR = 5.85 (95% CI=1.5 – 23.6); whilst the OR for a first episode of VTE was the highest in individuals presenting a thrombophilia marker, OR = 44 (95% IC= 12.0 – 160.8). Among those patients, in individuals with congenital prothrombotic risk marker (n=39) the OR of VTE was 40.5 (95% CI = 10.7 – 152.8).

Discussion
It is of great clinical importance to recognise prothrombotic phenotype. It has been previously shown that thrombin generation test can be used for this purpose, especially when the down regulation of prothrombinase is boosted by adding APC (13, 20). Based on the scarce amount of literature available (13, 14, 21), we hypothesized that TM can probably better serve this purpose because its addition assures the participation of the complete PCS, including protein C itself and disturbances of its activation.

In fact, all congenital thrombophilia markers and acquired thrombotic risk factors, i.e. antiphospholipid syndrome (22), and oral contraceptives (23) can be expected to show high TG in the presence of TM. This is also true for congenital conditions that do not involve the APC system, because the G20210A mutation is responsible for hyperprothrombinaemia (24) and antithrombin (AT) deficiency (25) slows down thrombin inactivation. TG measurement in the presence of TM therefore is probably the method that currently has the widest scope. This suggests that TG measurement may be a general indicator of thrombotic tendency.

As an endothelial cell membrane protein, the “physiological” concentration of thrombomodulin cannot be established (26). The optimal concentration of soluble TM to add into plasma samples is unknown. First we established the concentration of a suitable soluble TM preparation that would cause half maximum inhibition (50%) of thrombin generation in normal plasma and found that 15 nM of rabbit lung TM was a suitable reagent (14, 27, 28). Then we measured TG with and without this addition in 71 healthy individuals and 89 patients with a history of single VTE.

In coagulation laboratories, the effect of APC is rendered as the APC-sensitivity ratio, i.e. the ratio of an aPTT value with over one without added APC (29). In APC resistance, this ratio decreases in the aPTT based method (29) and increases it in the ETP based APC resistance method (30). We prefer to express the effect of TM as the % inhibition that it causes. This is essentially again a ratio, but it has the advantage that defective function of the APC system is seen as a decreasing number.

Venous thromboembolism is a multifactorial disease, which is not only dependent on the presence of single congenital thrombophilic conditions but also on their interaction with acquired risk factors. High factor VIII levels have been seen to correlate with thrombosis (31). We did find a statistically significant correlation between high plasma factor VIII levels and the ETP results. Since normal plasma FVIII:C (50 – 150 IU/dl) has only a limited effect on the ETP (32), we doubt whether high FVIII:C

![Figure 1: TM-related inhibition rates of the ETP in patients presenting different prothrombotic risk factors and in those without established thrombophilia. Solid lines indicate the mean inhibition of the ETP.](https://www.thrombosis-online.com)
can be considered as the cause of the high ETP. Our results show that only in the presence of TM ETP and the height of the thrombin peak could distinguish patients with congenital prothrombotic risk factors. The highest ETP values were obtained in subjects with a heterozygous FII G20210A mutation (Fig. 2) who, however, showed no abnormal TM resistance (Fig. 1). Curvers et al. (9) did report moderate resistance to APC in carriers of prothrombin gene mutation, possibly explained by an inhibitory effect of prothrombin on APC dependent-FVa inactivation (33, 34). It should be noted that in our series, patients with FII mutation had plasma FII activities in the normal range (98 ± 5 IU/dl). Our data which show very high TG capacity in the carriers of G20210A allele correspond well with data previously published by Kyrlle et al. (24) who showed a very high ex-vivo thrombin generating capacity in a family which comprised heterozygous and homozygous carriers of the G20210A mutation. As expected, the highest resistance to TM inhibition was obtained in patients with PC deficiency (Figs. 1 and 2) and in patients with combined abnormalities, that are known to have a major risk of VTE (35) (Fig. 1). The overlap observed between patients and controls could be explained at least partly by the large inter-individual variability of results in normal subjects. This complicates the determination of a cut-off value. Thus, the test in its present form can not be used alone as a screening test for prothrombotic risk factors. However, it has recently been reported that the thrombin generation test could be a useful tool for identifying patients who require specific thrombophilia testing (36). Using thrombinography with added exogenous activated protein C (APC) in frozen-thawed platelet-rich plasma, Hezard et al. (36) established cut-off values for factor V Leiden (Δ lag time ≤ 1.5 min), prothrombin gene mutation (peak > 433nM) and protein S deficiency (ETP inhibition ≤ 58%). Despite a large overlap between patients without thrombophilic risk factors and thrombophilia patients, these authors concluded that thrombin generation test could be a promising tool for screening thrombophilic risk factors, especially in patients who were carriers of factor V Leiden or factor II G20210A mutations and patients with protein S deficiency; but they noticed that protein C deficiency did not exhibit a special thrombography pattern, which is not surprising as exogenous APC was added in the test medium.

Figure 2: Representative thrombin generation curves with (ooo) and without (—) TM 15 nM from patients with different types of established thrombophilia. A) normal control; B) Heterozygous F II G20210A mutation; C) Heterozygous FV Leiden mutation; D) Combined heterozygous FV Leiden and F II G20210A mutations; E) PS deficiency 54%; F) PC deficiency 60%.
We have now demonstrated that the addition of TM makes the TG test more discriminating for thrombophilia markers. TG measurement in the presence of TM probably better reflects in-vivo conditions as it also takes into account the effect of the protein C pathway on thrombin generation. We also found a relationship between high ETP values and clinical VTE event. Our results showed the highest individual risk of a first VTE event in patients with a known thrombophilia marker (congenital pro-thrombotic markers OR = 40.5 and the total markers OR = 44). In addition, our results strongly suggest that independently of the presence of a hypercoagulability marker, a high ETP value might be a significant risk factor for a first episode of VTE (OR = 5.85). Curvers et al. (9) have previously shown that an APC-boosted measurement of the ETP may be used for the assessment of overall prothrombotic phenotype in patients combined VFs Leiden and OC or pregnancy, where routine laboratory tests are not able to reflect the additional effect of different risk factors on the coagulation system, which agrees well with our findings. In addition, our data strongly suggest that a high ETP might be considered as an independent VTE risk factor in patients not presenting a thrombophilia marker.

Our results, in a relatively limited series, already show highly significant differences between groups and suggest that a high ETP measured in the presence of TM might represent a higher risk of VTE. This data justifies further studies on a larger series of patients in order to assess whether CAT ± TM, either alone or in combination with other thrombophilia tests, can predict the individual thrombotic risk in subjects with a family history of venous thrombosis as well as for the evaluation of the risk of recurrence.

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