Review of the clinical applications for thrombin generation

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• Traditional coagulation tests like prothrombin time (PT) and activated partial thromboplastin (aPTT) time are based on the rate of conversion of fibrinogen-to-fibrin that starts after as little as 5% of the whole thrombin is generated, thus leaving the remaining 95% undetected.

• Furthermore, these tests are performed in the absence of thrombomodulin.

• Therefore, they account for the reduced levels of the pro-coagulants, but are not sensitive to the parallel reduction of anticoagulants.
• In contrast, thrombin generation assays (TGA) measure thrombin generation beyond this through the use of fluorescence probes, and can be performed in PFP, platelet-poor plasma (PPP) but also platelet rich plasma (PRP) to incorporate platelet involvement.

• However, most work is still performed in PPP to allow batching of tests with frozen stored samples.

• TGA was first described for clinical use in 1953.
• However, limitations still prevent its widespread use in clinical studies.

• This includes:
  - semiautomation,
  - relatively poor standardization (including the preanalytical phase) and
  - wide intra- and interlaboratory variation, which prevent easy comparison of data across studies.
Nowadays, TGA is used to screen hypo and hypercoagulability and their determinants in many clinical settings, to study the mechanisms of action, the efficacy and the safety of hemostatic agents, anticoagulants, antiaggregants and reversal agents.
The applications of thrombin generation in clinical settings will be covered during this talk:
- Screening of hypercoagulability
- Screening of hypocoagulability
- Treatment monitoring
Screening of hypercoagulability

- In patients at risk of venous thrombosis, TGA analysis may be used to detect acquired or hereditary predisposing factors.
## Screening of hypercoagulability: Acquired factors

<table>
<thead>
<tr>
<th>factor</th>
<th>condition</th>
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<tbody>
<tr>
<td>Cancer</td>
<td>Antiphospholipid syndrome</td>
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<tr>
<td>Age</td>
<td>Hyperhomocysteinemia</td>
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<tr>
<td>Obesity</td>
<td>Surgery</td>
</tr>
<tr>
<td>Liver diseases</td>
<td>Trauma</td>
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<td>Sickle cell disease</td>
<td>Immobilization</td>
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<td>Preeclampsia</td>
<td>Pregnancy</td>
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<tr>
<td>Myeloproliferative syndromes</td>
<td>Oral contraception</td>
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<td>Elevated levels of serum 25-hydroxyvitamin D</td>
<td>Hormonotherapy</td>
</tr>
<tr>
<td>elevated factors II, VIII, IX, XI, activated protein C resistance</td>
<td>Elevated microvesicles</td>
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</tbody>
</table>
Screening of hypercoagulability: Acquired factors

Obesity

Figure 1. A, Example of a thrombin generation curve obtained by means of the CAT method in normal individuals. The parameters derived from the thrombin generation curve are: lag time (the time in which ~10 nM of thrombin is formed), peak height (the maximum amount of formed thrombin), and ETP (the area under the curve). B, Thrombin generation curves in women across categories of BMI: normal weight (BMI: 18.5 to 24.9 kg/m²), overweight (BMI: 25.0 to 29.9 kg/m²), and obese (BMI: ≥30.0 kg/m²). Those who were overweight had 0.2%, 10.4%, and 6.3% longer/higher lag time, peak height, and ETP, respectively, and those with obesity had 5.2%, 14.6%, and 8.4% longer/higher lag time, peak height, and ETP, respectively, compared with those with normal weight (P for trend, <0.04 for all).

Beijers HJ, and coll. Arterioscler Thromb Vasc Biol. 2010
The ratio of values of thrombin activity generated in the presence and absence of thrombomodulin is 0.44 in plasmas from healthy individuals and 0.70 in plasmas from patients with cirrhosis, indicating that the latter are partially resistant to the anticoagulant action mediated by thrombomodulin.

This ratio is a biomarker of the in vitro hypercoagulability.
SCD patients, independently of the administration of hydroxyurea, were marked by a significant acceleration in the propagation phase of TG which correlated with the Ed-MP/PS+ concentration.

TG was significantly attenuated in hydroxyurea-treated patients.
• Antithrombin defect,
• Protein C defect
• Protein S defect,
• Factor V Leiden,
• Prothrombin G20210A mutation,
• Non O blood group,
• Dysfibrinogenemia
• Tissue factor (TF) and tissue factor pathway inhibitor polymorphisms.
• It has been demonstrated that TG is one of the risk factors for venous thromboembolism (VTE), and can be useful as a predictive marker for evaluating thrombosis on an individual basis.

• The greatest risk association was obtained using MaxR (rate of thrombin generated and total thrombin); with a 2.6-fold increased risk at MaxR exceeding the 90th percentile.

• The odds ratio (OR) for MaxR was 3.9 in men, 2.1 in women, and 2.9 in women on OCs.
TG reflects acute hypercoagulability during AMI and partly also in the 6-month period after the acute event.
• TGA allow screening for hypocoagulability which is particularly important in hemophilia.

• The prediction of the individual bleeding risk of haemophilic patients and above of all the individual response of patients to antihaemophilic treatment (factor concentrates or rVIIa) still remains a challenge.
• Thrombin generation assay is a good candidate but prospective studies with clinical relevant endpoints are still required.

• The setup of such trials is currently limited by the lack of standardization of TGT.
Screening of hypocoagulability

- In hemophilic patients, the initiation phase (and thus aPTT and PT) is less affected than the propagation phase stressing the importance of TGA.
• In patients with bleeding disorders, ETP and peak thrombin levels have been shown to be significantly inhibited in the presence of reduced FVIII/IX:C.

• Low thrombin generation has also been observed among hemophilia A patients with inhibitors.

• Moreover, ETP measurements show a significant correlation with the one-stage FVIII:C assay, not with the two-stage clotting assay, when used to assess cases of assay discrepancy.
Ex vivo studies have shown that the capacity for thrombin generation differs among patients who have similar plasma levels of FVIII, which suggests that the TGT may be useful to individually assess bleeding risk in hemophilia patients or to define the phenotypic heterogeneity seen in these patients.

Finally, TGA may also allow studying the mechanisms of hypocoagulability following cardiac surgery and von willebrand disease.
• TGA is useful to study the mechanisms of action, the efficacy and the safety of hemostatic agents, anticoagulants and reversal agents.

• TGA may also be useful for dose-adjustment of haemophilic treatment
• In vitro, TGA may be used to study and compare the thrombin generation capacity of different therapeutic plasmas and to investigate the increase or the decrease of thrombin generation of platelet concentrates and fresh frozen plasma, respectively, during storage.

Matijevic N, and coll. Thromb Res. 2011
Treatment monitoring: Hemostatic agents

Fig. 1 Representative thrombinograms obtained with each plasma preparation (FFP, quarantine fresh frozen plasma; MB FFP, plasma treated with methylene blue; SD FFP, plasma treated with solvent/detergent; IA FFP, plasma treated with amotosalen). ETP, ETP measured after triggering with 5 μM of tissue factor; ETP₁, ETP measured after triggering with 1 μM of tissue factor; ETP₅₆TY, ETP measured after triggering with 5 μM of tissue factor in the presence of thrombomodulin.

In vivo, TGA was used to assess the treatment of perioperative dilutional coagulopathy treated with fresh frozen plasma and fibrinogen concentrate and its relationship to bleeding.
Treatment monitoring: Hemostatic agents

Fig. 3  Effects of addition of factor concentrates (FC) on thromboelastometry and thrombin generation. Pre-intervention plasma samples were reconstituted with 0·8 g/l fibrinogen concentrate and/or 0·22 U/ml prothrombin complex concentrate. Rotational thromboelastometry (ROTEM) and thrombin generation [calibrated automated thrombogram (CAT)] were measured in reconstituted platelet rich plasma (PRP) or platelet-free-plasma (PFP) with phospholipid vesicles, after triggering with 10 pm tissue factor. Means ± SEM (n = 20–21), *P < 0·05.
TGA may be used to compare the efficacy of different anticoagulants employed in clinical management of patients with ACS.

Monitoring this therapy is of particular importance since several studies have pointed out the negative prognostic impact on long-term mortality of these bleeding adverse events associated with anticoagulants and antiagregants used in ACS.
Some years ago, it was shown that the calibrated automated thrombin (CAT) generation test could be used in monitoring unfractionated heparin (UFH) and low molecular weight heparin (LMWH) and that it could also be more sensitive than the traditional tests.

### Table 2: Sensitivity to heparin treatment

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>APTT</th>
<th>APTT ratio</th>
<th>ETP</th>
<th>ETP inhibition</th>
<th>AIIa</th>
<th>AXa</th>
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<tbody>
<tr>
<td>All</td>
<td>432</td>
<td>34</td>
<td>55</td>
<td>80</td>
<td>98</td>
<td>95</td>
<td>98</td>
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<tr>
<td>R.S.</td>
<td>264</td>
<td>43</td>
<td>74</td>
<td>93</td>
<td>100</td>
<td>99</td>
<td>100</td>
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<tr>
<td>UFH</td>
<td>108</td>
<td>27</td>
<td>41</td>
<td>68</td>
<td>96</td>
<td>95</td>
<td>99</td>
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<tr>
<td>MMW</td>
<td>108</td>
<td>51</td>
<td>65</td>
<td>86</td>
<td>97</td>
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<td>100</td>
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<tr>
<td>Certoparin</td>
<td>108</td>
<td>26</td>
<td>61</td>
<td>82</td>
<td>99</td>
<td>95</td>
<td>99</td>
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<tr>
<td>Enoxaparin</td>
<td>108</td>
<td>31</td>
<td>55</td>
<td>84</td>
<td>98</td>
<td>97</td>
<td>100</td>
</tr>
</tbody>
</table>

Each figure (except N) gives the percentage of samples in which the test in the column was significantly different from the relevant normal control (see text). All, All samples except the 0 and 24-h time points; R.S. (restricted set), the previous group with the 0.5, 8 and 10-h time points omitted. Lower rows: samples after injection of the heparin indicated.
• New oral anticoagulants (rivaroxaban, apixaban, edoxaban and dabigatran) do not require monitoring nor frequent dose adjustment.

• However, searching for the optimal dose in the individual patient may be useful in some situations

• This will be discussed this afternoon…
Finally, TGA was also used to show that the inhibition of tissue factor pathway inhibitor (TFPI) by the aptamer BAX499 improves clotting of hemophiliac blood and plasma.
Treatment monitoring: anticoagulant agents

Fig. 2. BAX499 enhances ex vivo thrombin generation. The anticoagulant factor pathway inhibitor at a given BAX499 concentration-dependently enhanced thrombin generation in plasma from hemophilic patients (shaded bars) from samples collected at site A (S₁) (top panel) and site 1 (S₂) (bottom panel), and, to a lesser extent, in plasma from healthy controls (plain bars). Data are plotted as mean ± 95% confidence intervals. ETP, endogenous thrombin potential.

• Extented-release dipyridamole affects thrombin activity predominantly by a dose-dependent inhibition of endogenous thrombin potential and demonstrated a trend to delayed initiation of thrombin production.

• Statins but not aspirin reduce thrombin generation in vivo.
Overt proteinuria is a strong risk factor for VTE, owing to changes in the levels of various coagulation proteins and urinary antithrombin loss.

It has been recently shown by TGA that antiproteinuric therapy ameliorates the prothrombotic state.
• Haemophilia frequently require replacement therapy using clotting factor concentrates that increase the plasma level of the missing clotting factor.

• The classical adjustment of the therapy is mainly based on the measurement of the plasma clotting activity of the protein administered.
• However, the common clinical laboratory coagulation assays do not reflect the clinically relevant hemostatic activity of bypassing agents.

• If one considers that a certain level of thrombin generated would predict clinical efficacy, monitoring of thrombin formation might offer new possibilities to individually predict the bleeding phenotype, select the most adapted therapeutic product and tailor the dose.
Fig. 1. A representative case illustrating the use of the three-step protocol.

Dargaud Y, and coll. Haemophilia 2012
New oral anticoagulants are responsible for hemorrhagic complications.

Due to the absence of specific antidotes, the reversal of rivaroxaban and dabigatran was tested in vitro using prothrombin complex concentrate (PCC), rVIIa and FEIBA at various concentrations.

In addition, when antidotes will be available for these new oral anticoagulants, it will be important to have reliable test to confirm whether the anticoagulant effects are persisting in case of bleedings.

Nowadays, TGA is used to screen hypo and hypercoagulability and their determinants in many clinical settings, to study the mechanisms of action, the efficacy and the safety of hemostatic agents, anticoagulants, antiagregants and reversal agents.

The applicability of global assays like TGA in clinical practice will benefit from standardization of the preanalytical and analytical steps, automation and development of whole blood and point of care tests.

In addition, in the future, TGA should be combined with a test measuring plasmin generation or with thromboeleastography.
Thank you for your attention
Thank you….

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Christian Chatelain
Claire Loosen

Justine Baudar
Anne Spinewine
Gaetane Remy

Jean-Michel Dogné
Jonathan Douxfils