**CAT**

**Critically Appraised Topic**

# Title: Clinical applications of thrombin generation assays in coagulation diagnostics

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Date: 20-05-2025

## CLINICAL BOTTOM LINE

Thrombin generation assays (TGA) are global coagulation assays which, in contrast to routine coagulation assays (prothrombin time (PT), activated partial thromboplastin time (APTT)), capture the 'complete' coagulation process by means of continuously monitoring the generation and simultaneously occurring inhibition of thrombin after addition of tissue factor and phospholipids to citrated plasma. As such, they account for both procoagulant (e.g. clotting factors) and anticoagulant (antithrombin III, protein C/S, …) factors involved in secondary hemostasis. Nevertheless, thus far, they have found only limited implementation in clinical practice and are still largely unknown to the general laboratory public. In current critically appraised topic, we elaborate on the working principle behind TGA and how TGA parameters may be interpreted in light of hemostasis. Next, we further discuss their various potential clinical applications such as monitoring (the reversal) of anticoagulant drugs or the evaluation of a patient's bleeding or thrombotic risk. Moreover, as multiple automated TGA instruments are currently commercially available thereby facilitating the potential implementation of TGA in clinical laboratories, we also discuss (pre-)analytical considerations that have to be considered prior to their implementation into routine practice.

## CLINICAL/DIAGNOSTIC SCENARIO

In hemostasis, thrombin plays a crucial role given its importance in the secondary hemostasis phase where it, among other functions, is responsible for the generation of fibrin from fibrinogen thereby being a key factor in clot formation.[1-3] Given its prothrombotic nature, however, overproduction or overactivity of thrombin has to be avoided which is therefore regulated through various anticoagulatory mechanisms (e.g. antithrombin, protein C and protein S pathway, etc.).[1-3] Hence, hemostasis is a delicately balanced process in which both thrombin formation and inhibition play equally important roles. However, currently used routine coagulation assays (e.g. PT or APTT) only capture the thrombin formation process and 'disregard' the thrombin inhibition part. Moreover, the amount of thrombin required to complete a PT or APTT assay only entails 5% of all thrombin formed during the hemostatic process thereby illustrating how these routine assays fail to capture the 'full impact' of thrombin on bleeding and thrombotic risk.[4] Furthermore, they are not sensitive to the anticoagulatory effects of the protein C and protein S pathway (which mostly occur after the PT or APTT assay has already been completed).[5,6] In other words, currently used assays only 'capture' a small fraction of the complete hemostatic process and hence, ideally, hemostasis would be more fully monitored via assays which capture both the formation as well as the inhibition of thrombin. One type of 'global' coagulation assay that would be capable of tracking the whole hemostatic process are the thrombin generation assays (TGA).

TGA continuously monitor the formation and inhibition of thrombin in a patient's plasma after addition of tissue factor (TF) and phospholipids and, as such, visualize both the procoagulant as well as anticoagulatory mechanisms simultaneously involved in secondary hemostasis.[5,6] While multiple of these assays are currently commercially available, their application has mostly been limited to research settings. Moreover, despite the first concept of TGA being developed in 1953 and the potential added value over routine coagulation tests, these assays are still largely unknown by many laboratory specialists. Therefore, in current critically appraised topic, we wanted to explore via literature search what these TGA exactly entail, what their potential applications according to current research data might be and which of these applications might be of interest for clinical practice in the university hospital of Leuven (UZ Leuven). In this aspect, we also reviewed the practical aspects of implementing TGA in a routine clinical laboratory by addressing factors to be considered prior to implementation such as the currently available TGA formats/instruments, recommended pre-analytics, etc.

## QUESTION(S)

1) What are thrombin generation assays?
2) What are the potential clinical applications of thrombin generation assays?
3) What (pre-)analytical factors have to be considered before implementing thrombin generation assays in routine practice?

## SEARCH TERMS

During this literature review, the Medline (Pubmed) database was systematically searched for eligible articles concerning thrombin generation assays and their clinical applications. The following key words were used: “Thrombin generation test”, “Thrombin generation assay”, “Global coagulation assay”, “Thrombin generation AND thrombosis risk”, “Calibrated automated thrombogram”. In addition, the reference lists of the retrieved (review) articles were searched.

## APPRAISAL

### 1. The central role of thrombin in hemostasis

Hemostasis is a delicately balanced process that can be subdivided in four distinct phases: i) vascular contraction, which reduces blood flow/ blood loss; ii) primary hemostasis, during which the initial platelet plug is formed; iii) secondary hemostasis, in which the coagulation cascade results in the formation of a stable fibrin clot; and iv) fibrinolysis, during which the fibrin clot is dissolved by the action of fibrinolytic enzymes such as plasmin.[1-3] In the process of secondary hemostasis, thrombin, formed out of prothrombin (coagulation factor II), plays a pivotal central role (Figure 1).[1-3,7]

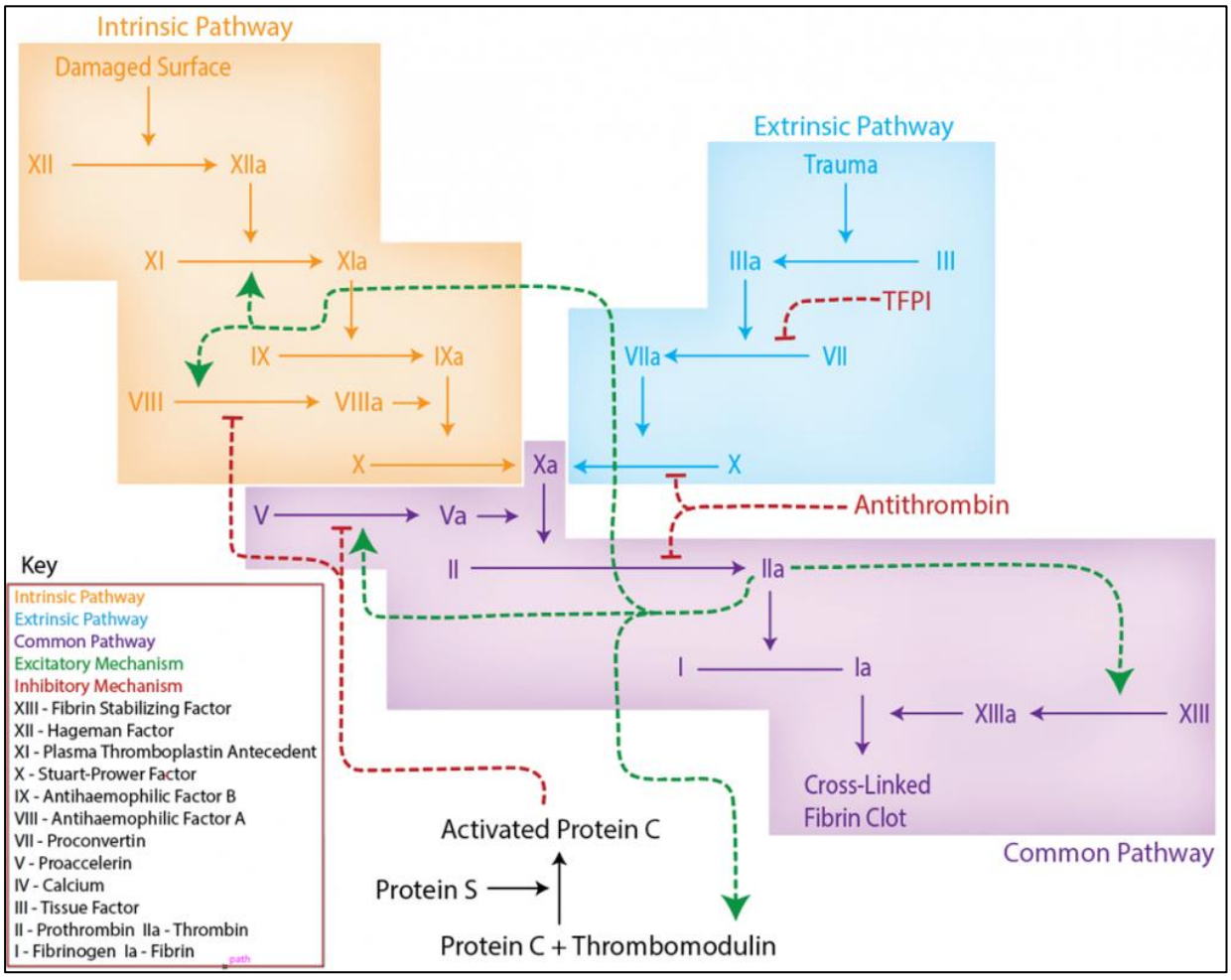


**Figure 1. The coagulation cascade[7]**

Thrombin formation in the secondary hemostasis is mediated by coagulation factor Xa and can also be divided in three distinct phases: i) the initiation phase, during which the TF-FVIIa complex of the extrinsic pathway results in small amounts of activated factor Xa which in turn leads to the formation of initial minor amounts of thrombin; ii) the amplification phase, during which positive feedback mechanisms excised by thrombin on the activation of coagulation factors V, VIII and XI (see green arrows in Figure 1) prepare to strongly enhance its own formation and iii) the propagation phase, during which, due to positive feedback mechanisms activated in the previous phase, a large burst of thrombin is formed (Figures 1 and 2).[2,3,7]

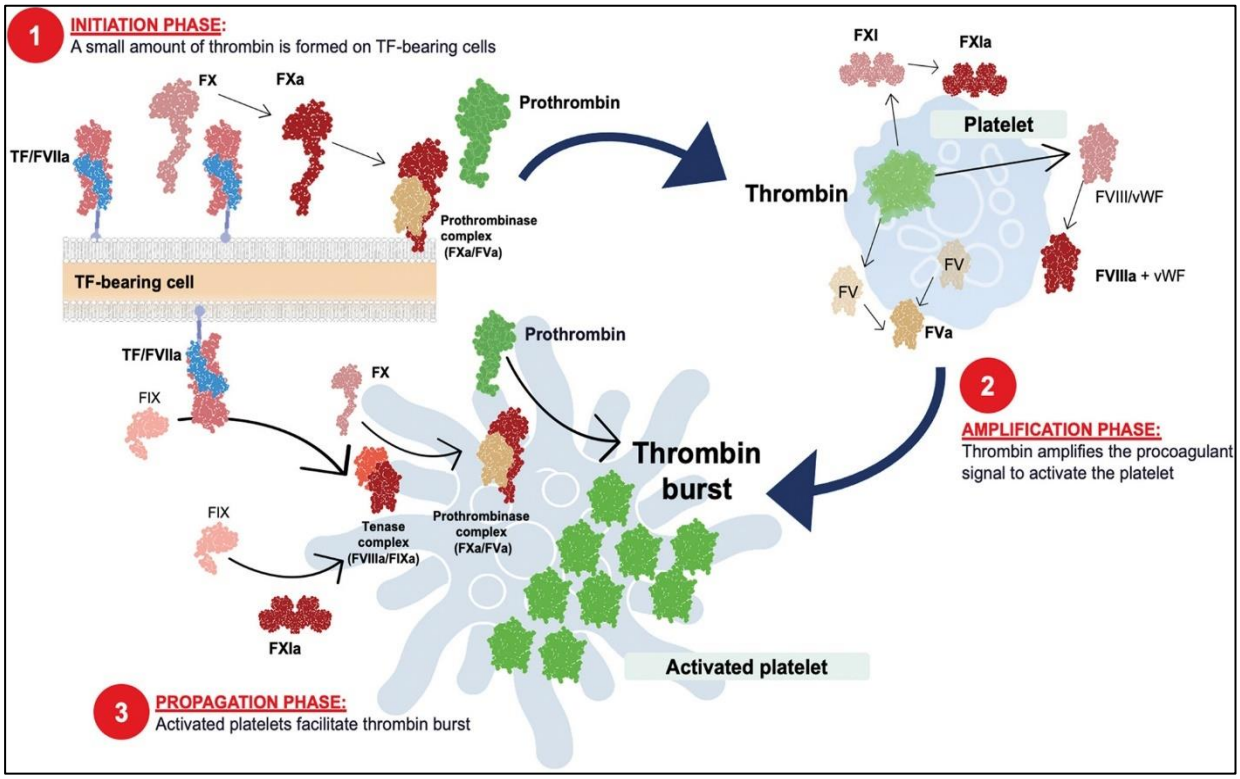


**Figure 2. The phases of thrombin formation**

Three distinct phases can be recognized in secondary hemostasis. Abbreviations: TF, tissue factor; vWF, Von Willebrand factor. Adapted from Sidonio Jr et al. *Res Pract Thromb Haemost* 2022[2]

Once produced, thrombin plays an essential role in secondary hemostasis via its most known action, namely the conversion of fibrinogen into fibrin strands which in turn form the structural basis of the fibrin clot. However, thrombin also catalyzes multiple other effects such as stimulating platelet activation/aggregation or activating clotting factors V, VIII and XI thereby mediating the aforementioned a positive feedback loop (Figure 1). Lastly, it also transforms coagulation factor XIII to activated factor XIII (FXIIIa) which mediates fibrin strand crosslinking to enhance clot stability.[2,3] These effector mechanisms of thrombin all signify its importance in hemostasis but simultaneously illustrate that an overactivity or overproduction of thrombin may easily result in excessive clot formation and hence, thrombosis.[8] As such, in order to prevent thrombin overproduction/overactivity and balance clot formation, multiple regulatory mechanisms are simultaneously also in place such as i) antithrombin and α-2 macroglobulin, which both bind and inhibit circulating thrombin; ii) the protein C and protein S

pathway, in which activated protein C (activated by thrombin-thrombomodulin complex enhanced by the endothelial protein C receptor (EPCR)) with cofactor protein S enters the clot and inactivates factors Va and VIIIa thereby dampening thrombin production; and iii) tissue factor pathway inhibitor (TFPI), which inhibits the TF-VIIa complex and thus factor X activation and associated thrombin generation in the initial phase of thrombin formation (see red arrows in Figure 1).[1-3,7] Moreover, thrombin also stimulates the release of tissue plasminogen activator which converts plasminogen to plasmin, thereby supporting the eventual breakdown of fibrin clots.[2,3] In short, the formation of a blood clot is a much more complex, delicate and balanced process than only the generation of fibrin out of fibrinogen by thrombin!

## 2. Thrombin generation assays

While routine coagulation assays such as the PT or APTT, in which the time to clot formation by thrombin after reagent addition is measured, provide valuable information to the clinician with respect to e.g. increased bleeding or thrombotic risk, these assays fail to provide a comprehensive overview of all procoagulant and anticoagulatory factors (e.g. as described above in *1. The central role of thrombin in hemostasis*) simultaneously involved in hemostasis. For example, the amount of thrombin formation required to 'complete' a PT or APTT assay, which mimic the initiation phase of thrombin formation, has been shown to be only 5% of all thrombin formed in total during the coagulation process (i.e. thrombin and fibrin generation still continues after the endpoint of these assays has been reached).[4,5] Hence, this illustrates how these routine assays may fail to pick up disorders associated with increased (thrombotic events) or decreased (bleeding risk) thrombin formation. Moreover, these assays do also not account for thrombin regulating/ anticoagulant mechanisms (e.g. antithrombin III, activated protein C and protein S activity, TFPI) although defects in these mechanisms are likewise associated with increased thrombotic risk (e.g. antithrombin III deficiency).[5,6,9] This is further corroborated by the observation that patients with e.g. hereditary antithrombin deficiency present with normal PT and APTT results although manifesting a 5- to 50-fold increased risk for venous thrombosis.[10,11] Likewise, PT and APTT results are usually not divergent in patients with a prothrombin G20210A mutation who similarly demonstrate increased thrombotic risk.[12] Similarly, routine assays utilized to screen for thrombophilia (e.g. determination of antithrombin, protein C and protein S levels/activity) fail to provide a complete picture of the hemostatic process ongoing in the patient. For example, a patient who displays normal activities for the aforementioned antithrombotic mechanisms may yet also display an increased bleeding tendency due to e.g. factor deficiencies. Hence, to correctly and fully estimate a patient's thrombotic or bleeding risk, both procoagulant and anticoagulatory mechanisms (i.e. all mechanisms involved in secondary haemostasis), should ideally be simultaneously monitored in one assay. This would potentially also yield the added value of a more simplified interpretation/diagnosis of coagulation or bleeding disorders as only one assay has to be utilized/interpreted (i.e. as opposed to having to interpret multiple independent assays which each only evaluate a fragment of the haemostatic process).[6] While certain point-of-care assays such as ROTEM (rotational thromboelastometry) can provide a more

complete picture of hemostasis in the patient, its need for a fresh blood sample (sampled ideally less than 30 minutes prior to analysis) that has to be pipetted manually into the instrument and relative insensitivity to mild hemostasis defects/abnormalities limits their use in the hemostasis laboratory.[13]

One other possible type of assay that may be used to monitor the 'global' process of secondary hemostasis and may be suited for implementation in routine clinical laboratories are the so-called thrombin generation assays (TGA). In these assays, thrombin formation is triggered in the patient's plasma (usually platelet poor plasma (see *4.2.2 Sample matrix)*) through addition of phospholipids and TF, after which both the formation and the simultaneously occurring inhibition of thrombin is monitored over time through the use of thrombin-specific substrates (fluorogenic or chromogenic) (Figure 3).[6,9,10,14] This results in a sigmoidal curve in which three distinct phases can be identified: the initiation, propagation and resolution phases (figure 4A).[6] The initiation phase mimics the initiation phase of secondary hemostasis and visualizes the initial steps of thrombin formation in which initially only a limited amount of thrombin is being formed. The time required to complete this phase corresponds to the time of initial fibrin formation (i.e. clotting time) of routine tests such as PT or APTT.[4] Next, a propagation phase occurs during which the formation of thrombin is more pronounced than its inhibition by means of positive feedback mechanisms (as described in Figure 1) which is visible on the curve as a steadily increasing signal. Lastly, the curve transits to a resolution phase in which no further increase in signal is observed and which corresponds to a stage during which thrombin formation is outperformed by the activity of thrombin inhibitors such as antithrombin, α-2 macroglobulin and activated protein C.[6] Through mathematical derivatization (first derivative) of the aforementioned sigmoidal curve and the use of a thrombin standard curve (obtained by incubating human thrombin, usually complexed with α-2 macroglobulin, with various dilutions of the thrombin-specific substrate), the so-called thrombogram can be obtained which expresses the amount of thrombin formed in nM over time (Figure 4B)**.**[6,9,10,14]

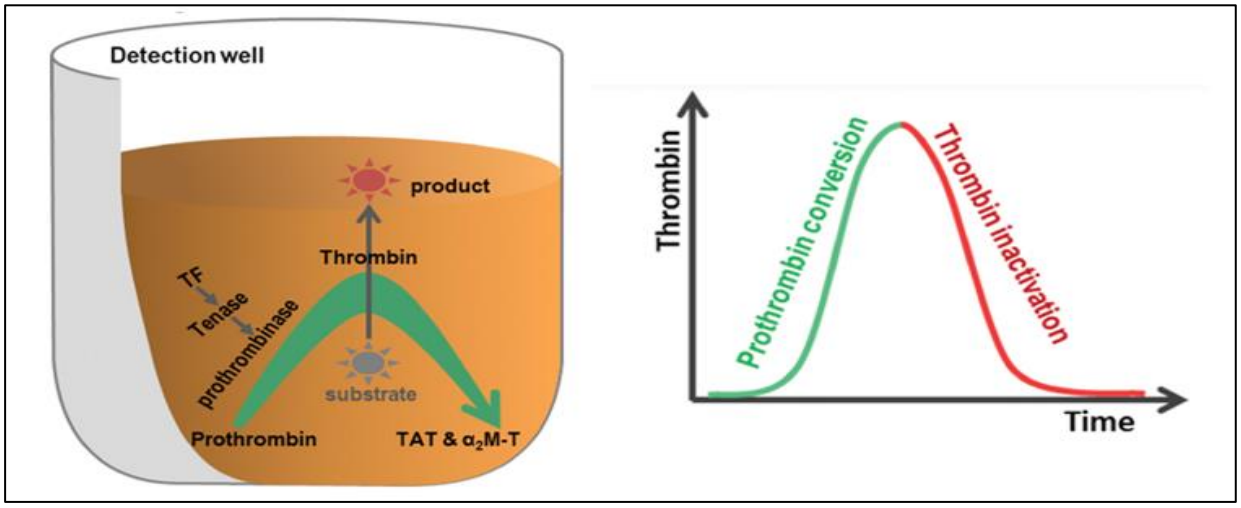


**Figure 3. Principle of thrombin generation assays**

After reagent addition (TF, phospholipids), thrombin formation and inhibition is monitored through the use of a thrombin-specific substrate. Abbreviations: $\alpha_2$M-T, $\alpha_2$-macroglobulin–thrombin; TAT, thrombin-antithrombin complex; TF, tissue factor. Adapted from Wan et al. *Thrombosis and Haemostasis* 2021[9]

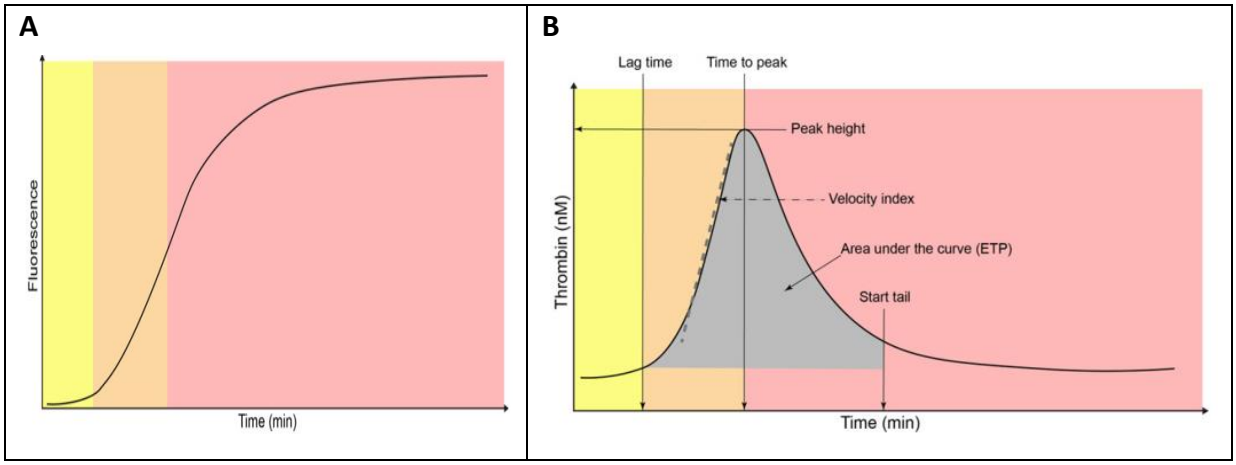


**Figure 4. Raw data and thrombogram obtained from thrombin generation assays**
A) Three distinct phases can be recognized during thrombin generation: the initiation phase (yellow), the propagation phase (orange), and the resolution phase (red). B) Thrombogram curve obtained via mathematical derivatization of the curve depicted in panel A. Various related thrombogram parameters can be obtained and are depicted on the curve. Abbreviations: ETP, endogenous thrombin potential. Adapted from Depasse F et al. *J Thromb Haemost* 2021[6]

As shown in Figure 4B, various parameters can be derived from the thrombogram: lag time, the time taken for thrombin to first reach a detectable concentration (corresponds to the clotting time of standard clotting tests); peak thrombin, the maximum amount of thrombin generated during the reaction; time to peak, the time it takes to achieve peak thrombin; the velocity index, the slope of thrombin generation between the time to peak and the lag time; start tail, the time until thrombin generation comes to an end with all further generated thrombin being inhibited; and lastly, endogenous thrombin potential (ETP), the net amount of thrombin activity during the reaction or, in other words, the total amount of thrombin the sample can generate under the action of the two opposing (pro- and anticoagulant) forces in the plasma (corresponding to AUC of the thrombogram).[6,10] These parameters visualize the activity and formation of thrombin over time and could therefore reflect hypo- or hypercoagulability. For example, in a patient with hypercoagulability due to e.g. a prothrombin G20210A mutation, a shorter lag time and time to peak with increased peak thrombin and ETP may be observed (Table 1).[10] As such, multiple of these parameters have been evaluated as predictors for bleeding or thrombotic risk (see *3. Potential clinical applications of thrombin generation assays*).

**Table 1. Significance of the thrombogram parameters**

| | Lag time | Time to peak | Peak thrombin | ETP | Velocity index |
|---|---|---|---|---|---|
| **Hypercoagulability** (e.g. F5 Leiden, F2G20210A, AT deficiency,…) | ↓ | ↓ | ↑ | ↑ | ↑ |
| **Hypocoaguliability** (e.g. hemophilia A, hemophilia B, factor deficiencies,…) | ↑ | ↑ | ↓ | ↓ | ↓ |

Abbreviations: AT, antithrombin; ETP, endogenous thrombin potential. Adapted from Tripodi A, *Clin Chem* 2016[10]

## 3. Potential clinical applications of thrombin generation assays

Since TGA, as opposed to routine tests such as PT or APTT, provide a comprehensive overview of both procoagulant and anticoagulatory mechanisms involved in secondary hemostasis (i.e. reflecting the duality of thrombin function), clinical applications of these assays extend to both hypercoagulability (i.e. thrombosis) as well as hypocoagulability (i.e. hemorrhagic risk).[6,10,15] As such, apart from their use in research to elucidate coagulation mechanisms, multiple clinical applications for TGA have also been investigated such as the monitoring of (reversal of) anticoagulant drugs, the prediction of (recurrence of) venous thrombo-embolisms (VTE), monitoring bleeding and thrombotic risk in cardiovascular disorders and monitoring replacement therapy in hemophilia. These applications will be further elaborated upon in more detail below.

### *3.1 Monitoring anticoagulant therapy and its reversal in acute settings*

While various assays are available to monitor anticoagulant therapies (e.g. APTT for unfractionated heparin, PT (INR) for vitamin K antagonists, anti-Xa testing for DOACs, LMWH and heparin), these 'regular' assays only measure the drug-specific activity but do not reflect the actual overall hemostatic status.[10] Hence, TGA may provide a more global picture and have hence been proposed for monitoring anticoagulant therapies such as direct acting oral anticoagulants (DOACs). Although DOACs do not require routine monitoring, this may nevertheless be advised in case of emergency surgery, abnormal bleeding, complications, evaluation of potential overdose, the presence of renal failure or in light of assessing therapy compliance.[16] While DOACs are typically only monitorable through dedicated assays (e.g. anti-FXa assays) and not by routine clotting assays such as PT or APTT as these do not accurately reflect the degree of anticoagulant effect, they may also be monitored through the use of TGA.[17] For example, in study populations with nonvalvular atrial fibrillation or knee and hip replacement, administration of apixaban significantly increased the thrombogram lag time while reducing the ETP and peak height compared to samples taken at through levels thereby illustrating that TGA's could monitor DOAC therapy in select patient populations.[18,19] However, given that dedicated laboratory tests for DOAC monitoring (or other anticoagulant drugs) are already available such as the diluted PT for dabigatran and anti-FXa tests for other DOACs, the potential role of TGA in this aspect is likely to be limited.

A more important observation from studies evaluating TGA in anticoagulant therapies, however, is that these assays were not only able to monitor the effect of DOACs via changes in thrombogram parameters, but that they could also assess the efficacy of non-specific haemostatic agents (NHA) in reversing the DOAC anticoagulatory effect (NHA include e.g. (activated) prothrombin complex concentrate (PCC) or rFVIIa and are drugs/measures taken in the acute setting to reverse the anticoagulant drug's effect in case of increased bleeding tendency due to e.g. drug overdose). For example, a study involving patients on dabigatran or rivaroxaban showed that administration of these drugs reduced the thrombogram's ETP and peak height, but that these changes were significantly corrected by the use of NHA.[19] Furthermore, by illustrating that reversal of the DOAC effect was more pronounced by PCC than rFVIIa

the authors showed that TGA may potentially be used to determine the most effective reversal agent thereby guiding reversal therapy (Figure 5).[19] These findings were later also replicated by multiple other studies, including clinical trials evaluating novel reversal agents.[20,21] Other, established assays such as e.g. anti-FXa assays are not capable of measuring reversal effects. As such, TGA may provide added clinical value by allowing to identify the most efficient NHA reversal strategy (i.e. as exemplified in Figure 5) and by evaluating its effect which, in turn, might influence clinical decision making (e.g. whether emergency surgery should be delayed or not).

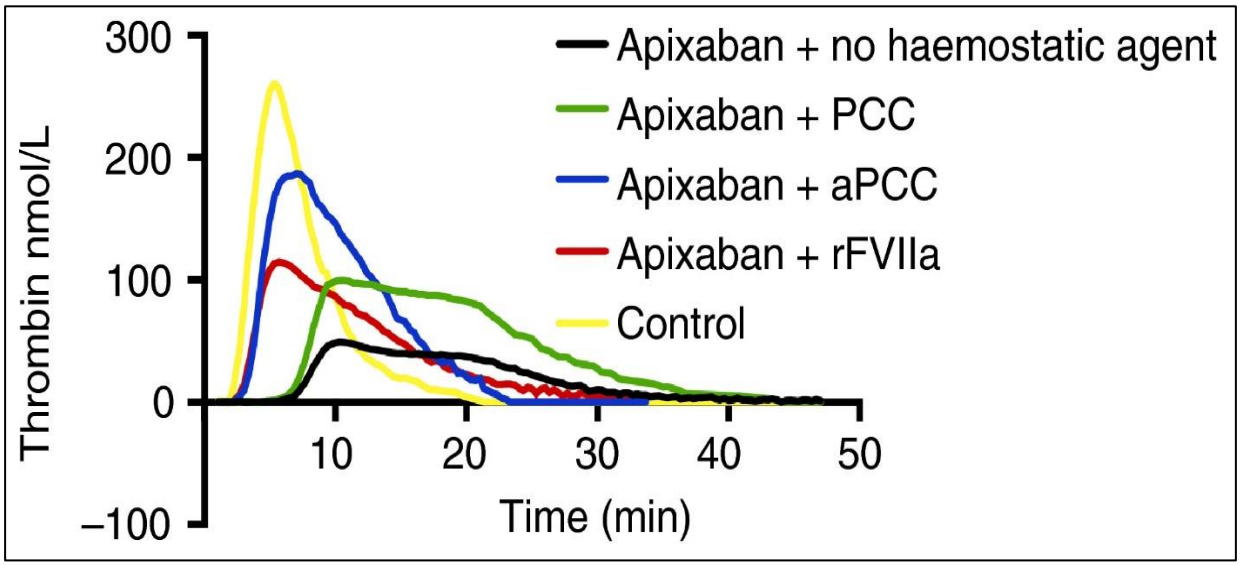


**Figure 5. TGA to monitor the effect of reversal of DOAC by non-specific haemostatic agents.**
An example of using TGA to monitor DOAC reversal strategies: as aPCC restores the thrombogram curve most efficiently to that of the control sample, this therapy choice would be indicated. Abbreviations: aPCC, activated prothrombin complex concentrate; rFVIIa, recombinant factor VIIa. Adapted from Schultz et al. *Res Pract Thromb Haemost* 2017[20]

Apart from DOACs, TGA have also been described to monitor the effects of other antithrombotic therapies such as vitamin K antagonists, heparin and fondaparinux.[22-25] Likewise, a recent study indicates that they may be used to identify patients using dual antiplatelet therapy who are at increased risk of bleeding.[26] Like for DOACs, TGA have also been shown to be able to monitor the reversal of these agents' effects.[27,28]

**Added value for UZ Leuven clinical practice**: as well-founded, dedicated assays exist to monitor DOACs and other antithrombotic therapies, the added value of TGA in this application in UZ Leuven is most likely limited. On the other hand, novel therapies are continuously being introduced and a specific assay to monitor such novel drugs may not yet be available at time of market launch. A global assay such as TGA, however, may be used to monitor coagulation independent of the individual drug's working mechanism and may thus show value in monitoring novel drugs for which no specific assay has yet been developed. Moreover, the observation that TGA may be used to monitor the reversal of anticoagulant therapies thereby guiding clinical decision making would also be of value in our hospital. However, it should be acknowledged that the applicability of this use for TGA may yet be limited as so far, only limited clinical experience has been garnered with TGA's (i.e. data on monitoring reversal mostly stem from research settings as opposed to from real-life clinical data).

*3.2 Monitoring thrombotic risk*

Venous thrombo-embolism (VTE) is a multifactorial disease with several well-established risk factors including prolonged immobility, advanced age, obesity or pregnancy and inherited thrombophilia.[8,29,30] However, most of these risk factors only show weak correlation with risk of VTE (recurrence). As VTE risk factors act by perturbing the haemostatic balance[31], which can be monitored through TGA, multiple studies have evaluated TGA parameters in light of VTE prediction. For example, one prospective study evaluating 914 idiopathic VTE patients showed that patients who demonstrated VTE recurrence over the next four years displayed increased thrombin generation (TG; corresponding to the ETP) as opposed to those patients in whom the VTE did not occur again.[32] Moreover, the authors calculated that patients with TG>400nm had a 4 year relative risk (RR) on VTE occurrence of 20% while those with TG<400 nM demonstrated a RR of only 6.5%. Similar findings have also been described in patients with cancer (which itself is a risk factor for thrombosis) as patients with an increased peak thrombin (>611 nM) displayed an increased risk (hazard ratio 2.1) on developing VTE.[33] Also in patients with inherited thrombophilia (e.g. due to defects/deficiencies in antithrombin, protein C, protein S, factor V Leiden, F2G20210A,...), TGA may provide better risk stratification of VTE recurrence as other VTE risk factors (including genetic factors) show only weak correlation with thrombosis recurrence.[15,34] Multiple other studies have described similar findings where increased peak thrombin or increased ETP was associated with increased risk on VTE or VTE recurrence.[35,36] As such, TGA may be used to identify those patients at increased risk for VTE development or recurrence, even in the absence of other risk factors, and hence, aid in selecting a more intensive prophylactic regiment in these patients (e.g. by intensifying therapy in those patients with increased prothrombotic tendencies). TGA are currently already being used in this application at Maastricht UMC, a center with extensive experience with respect to TGA (personal communications).

Apart from their use in predicting VTE (recurrence), TGA's have also been investigated as tools to hint at or exclude the presence of genetic prothrombotic factors such as Factor V Leiden, protein S deficiency or prothrombin G20210A mutations. For example, the study by Hézard et al. found that the presence of the prothrombin G20210A mutation could be excluded when peak thrombin was lower than 426 nmol/L. However, more research is likely required before TGA could steer genetic testing in routine clinical practice.[37]

TGA results have also shown to be associated with thrombotic risk in patients with lupus anticoagulant antibodies (LAA) as LAA-harbouring patients who develop thrombosis during follow-up have been shown to demonstrate higher thrombogenicity on TGA as opposed to LAA-harbouring patients in whom thrombosis did not occur. Furthermore, modified versions of the TGA have also been described to be able to detect the presence of LAA via the presence of a significantly shortened lag phase and increased TG in the presence of thrombomodulin (thrombomodulin is involved in activating protein C and hence, its addition to TGA assays allows the assay to also evaluate the activated protein C pathway).[38-41] As such, TGA could potentially provide an alternative to routine tests which are troubled by antibody heterogeneity, specificity issues and assay standardization.[38] However, as these applications require

modified TGA's (e.g. through spiking of the plasma with activated protein C) their use in detecting LAA and predicting thrombotic risk in LAA+ patients is currently limited to research settings. Moreover, a recent review paper also indicates that further prospective studies as well as assay harmonization is required in order to utilize TGA parameters as marker predictive of thrombosis in LAA+ patients.[38]

Another area in which TGA in light of assessing thrombotic risk have garnered attention is the field of cardiovascular disorders.[42,43] For example, increased TG, possibly reflecting hypercoagulability and thus increased thrombotic risk, has been reported to be present long after acute coronary syndrome (ACS).[42,44] Hence, TGA's may be used to identify those patients who have higher risk at thrombosis after ACS (i.e. those with increased TG) and guide prophylactic treatment escalation in these patients accordingly. Moreover, in patients receiving percutaneous coronary intervention with stent implantation, TGA, by monitoring the amount of TG that occurred, were able to distinguish between patients experiencing stent thrombosis and those in whom thrombosis did not occur, thereby suggesting that TGA's may again help clinicians in identifying those patients who require prolonged thromboprophylaxis.[43] TGA parameters have also shown to be associated with mortality during follow-up after ACS.[42]

**Added value for UZ Leuven clinical practice**: TGA may provide added value in VTE prediction/risk management as multiple studies have shown clear correlations between TGA parameters and the risk on VTE (recurrence). Moreover, ETP showed stronger RR than many other risk factors for VTE thereby potentially providing a strong tool in VTE prediction. As such, the TGA can provide added value to UZ Leuven's clinical practice by identifying those patients at increased risk for VTE (recurrence) and thus those patients in need of more intense anticoagulant therapy. Likewise, they would also be of value to guide (intensification of) prophylactic strategies in patients who presented with ACS. Another important factor to consider in this aspect is that TGA are currently already being used in clinical practice for these purposes (e.g. in Maastricht UMC) and, as such, clinical experience for this application is already available. Other applications of TGA in thrombotic disorders (e.g. in LAA+ patients) likely require further study prior to implementation in clinical practice.

*3.3 Monitoring bleeding risk*

Given thrombin's crucial role in clot formation, it is easily understood how reduced thrombin generation (TG) may result in impaired clot formation and hence, increased bleeding risk. For example, it has been shown that hemorrhagic diathesis develops once TG drops below 20% of normal.[45] Hence, TGA may be of use in monitoring the bleeding risk in a variety of inherited and acquired disorders. One of such conditions is hemophilia A, a genetic disorder resulting in decreased Factor VIII levels, which, in turn, results in increased bleeding tendency. However, the bleeding phenotype may differ between hemophilia A patients even when demonstrating the same factor VIII level thereby issuing a challenge to clinicians who have to estimate the individual patient's bleeding risk in light of guiding therapeutic/prophylactic regiments.[46] In these situations, TGA may be of use. For example, it has been shown that in patients with severe hemophilia A (factor VIII <2%), reduced ETP was associated with

increased frequency of spontaneous bleeding independent of factor VIII levels.[46,47] Likewise, the ETP has also been shown to correlate with hemophilia A patients' bleeding risk during surgery.[48] These findings also held up in patients exhibiting factor VIII inhibitors.[48,49] Hence, as TGA appeared to be a better predictor of bleeding tendency than factor levels in hemophilia patients, they may help to identify those patients at severe risk of bleeding who require early/ more intense prophylactic treatment.[15] Similar findings have also been reported in Von Willebrand disease, the most frequent inherited bleeding disorder, where low peak thrombin was associated with increased bleeding tendencies, thereby also allowing an individualized bleeding risk stratification in this disorder.[50]

Apart from bleeding risk stratification in hemophilia patients, TGA have also shown value in monitoring treatment effect and in aiding treatment selection in these patients.[51,52] For example, studies have shown that TGA may *ex vivo* predict the efficiency of bypassing agents (e.g. emicizumab (Hemlibra®), a bispecific antibody which takes over the function of factor VIII) in restoring coagulation in hemophilia A patients by mixing the agents with patient's plasma and subsequently evaluating their effect on restoring thrombin generation.[51,52] As such, it may aid in selecting the most effective bypassing therapy for the individual patient (i.e. personalized therapy). However, it has to be remarked that these experiments were conducted in small series of patients and involved spiking patients' plasma with various dosages of the bypassing therapy which, given its individualized and labor-intensive character, likely currently limits its applicability in routine clinical practice.

Another major clinical application for TGA with respect to monitoring bleeding risk is again situated in the field of cardiovascular diseases, where TGA parameters have been shown to be associated with blood loss during or after cardiopulmonary bypass (CBP). For example, Bosch et al. demonstrated that in patients who presented with greater blood loss (>930 mL) following CBP, preoperatively performed TGA showed significant lower ETP and peak thrombin compared to patients who show only minor blood loss.[53,54] Similar predictive capacity for TGA was also observed in samples obtained intraoperatively.[54,55] As such, TGA may be used to identify patients at increased risk of bleeding who could potentially benefit from more proactive replacement of coagulation factors. An additional advantage of TGA over routine clotting tests in this application is that the TGA can also be determined in the presence of high heparinization levels (i.e. during CBP surgery where anticoagulation is necessary in order to avoid circuit thrombosis and prevent DIC while on bypass) which is not the case for the routine PT and APTT tests.

**Added value for UZ Leuven clinical practice:** as the UZ Leuven is the national coordination center for hemophilia, any assay that would provide better monitoring of bleeding risk and that could help guide treatment choices in hemophilia patients would undoubtedly be of value for clinical practice. Hence, given that TGA allow for better risk stratification than factor levels (the current standard of practice) even in the presence of factor inhibitors, TGA implementation would present a clear benefit for clinical practice. This would also be the case in the field of cardiovascular disorders where TGA may be used to predict more extensive postoperative blood loss as such postoperative bleeding after CBP has shown clear associations with increased morbidity and mortality.[56]

## 4. What (pre-)analytical factors have to be considered before implementing thrombin generation assays in routine practice?

As discussed above, TGA may prove valuable in monitoring anticoagulant therapy and bleeding/thrombotic risk but in order to effectively implement these assays in routine clinical practice various pre-analytical and analytical modalities have to be considered. These include factors such as the available assay types, manufacturers, the sample type/matrix to use, whether specific pre-analytical precautions are required, which reagent types to use (e.g. high or low TF reagent; see below), etc. These considerations will be discussed in more detail below.

### *4.1 Available TGA assays*

TGA were first described in 1953 and although revolutionary, they entailed laborious procedures in which thrombin generation had to be manually measured thereby limiting their use to research setting. However, in 2003, Hemker et al., reported on a calibrated automated thrombogram (CAT) method in which the thrombin generation was measured (semi-)automatically, thereby laying the groundwork for future commercial automated TGA instruments.[57] These currently available instruments include the ST Genesia (Stago) and the Ceveron s100 (Technoclone), which both utilize fluorogenic thrombin-specific substrates, and instruments utilizing a chromogenic thrombin-specific substrate such as the Innovance ETP assay (Siemens Healthcare Diagnostics), the HaemoScan Thrombin Generation Assay (HemoScan), and Pefakit TDT (Pentapharm). However, as current literature suggests that chromogenic systems are possibly more prone to error, require defibrination of the sample and are less sensitive compared to instruments utilizing a fluorogenic substrate, these instruments will not be discussed further.[10]

While both the ST Genesia and Ceveron s100 utilize a fluorogenic substrate, differences exist between the two instruments with respect to e.g. sample amount required, scan time, and correction techniques (Table 2). For example, while the ST Genesia requires 500µL of plasma with a scan time of 30-60 minutes, the Ceveron s100 shows a scan time of 50-120 minutes but only requires 140µL of plasma. On the other hand, the ST Genesia corrects for multiple factors while only the inner filter effect is considered for correction in the Ceveron s100 instrument (Table 2). With respect to technical performance, a recent study showed that although within-run CV's were similar between the different instruments, the ST Genesia demonstrated a significantly lower inter-run CV (2-7.6% depending on the reagent pack compared to the Ceveron s100 (13.8-21.2%).[58] With respect to sensitivity to factor deficiencies, both the ST Genesia and Ceveron s100 demonstrated acceptable results with varying sensitivity according to which factor was lacking. However, an important finding of this and other studies was that significant differences in thrombogram curves could be observed depending on the instrument and reagent pack (see below) used.[58,59] Hence, caution is advised when comparing TGA results between laboratories.

**Table 2. Characteristics of the ST Genesia and Ceveron s100 TGA instruments.**

| | ST Genesia | Ceveron s100 |
|---|---|---|
| **Substrate** | Fluorogenic (*Z*-Gly-Gly-Arg-AMC) | |
| **Plasma volume required** | 500 µL | 140 µL |
| **Calibration material** | Human thrombin in buffer and plasma AMC in plasma | Human thrombin in buffer |
| **Scan time** | 30-60 min | 50-120 min |
| **Total turnaround time** | 300 min | 150 min |
| **Correction for** | Inner filter effect, substrate consumption, plasma color | Inner filter effect |

Abbreviations: AMC, 7-Amino-4-methylcoumarin. Adapted from Haisma et al. *Thromb Res.* 2024[58]

*4.2 Pre-analytical considerations*

4.2.1 Blood sampling and sample preparation

For TGA, blood should be collected in citrated tubes. However, various pre-analytical conditions related to sample collection and processing may affect the outcome of these assays. For example, Loeffen et al. showed that the use of a glass tube resulted in a significantly increased peak height and ETP which could be explained by collection tube-dependent activation of the intrinsic pathway.[60] Likewise, single-centrifuged samples showed significantly higher ETP and peak height compared to double-centrifuged samples, most likely due to a higher number of residual platelets in the plasma.[60] Moreover, the use of a venous catheter as opposed to direct venipuncture has also been shown to affect thrombin generation.[61] In other words, multiple pre-analytical variables have been shown to potentially affect the outcome of TGA assays. Therefore, in order to increase standardization between (research) centers in TGA testing, the International Society on Thrombosis and Haemostasis (ISTH) has published several recommendations for pre-analytical conditions for measuring thrombin generation.[61,62] These recommendations are summarized in Table 3.

4.2.2 Sample matrix

Commercial TGA can be performed on both platelet poor- (PPP) and platelet rich plasma (PRP). Each matrix has it own advantages and disadvantages as summarized by Wan et al. (Table 4).[9] However, as most studies thus far have been performed on PPP plasma and since this is also the matrix of choice for the ST Genesia and Ceveron s100 instruments (i.e. current automated TGA) it may be recommended to adhere to the use of PPP plasma for the routine setting.[6,61,62] An additional advantage of selecting PPP as the matrix of choice is equivalency with other routine coagulation assays (e.g. PT) which also utilize PPP (i.e. so that identical pre-analytical procedures can be followed which, in turn, limits the chance on (human) errors to occur).

**Table 3. Recommendations on pre-analytics by the ISTH**

| Variable | ISTH recommendations |
|---|---|
| **Blood collection system** | • Direct venipuncture using straight needle preferred over butterfly needle (may induce contact activation and hemolysis)<br>• Avoid blood sampling through venous catheters for TGA on PRP, catheter sampling may be used for TGA on PPP<br>• Avoid the use of tourniquet or limit its application time to avoid hemolysis |
| **Blood collection tube** | • Collect blood in citrate anticoagulated tube<br>• Plastic tubes preferred over glass tubes<br>• Data on advantage of adding CTI still debatable but the use of specific tubes per assay may introduce source of errors in the laboratory |
| **Transport of samples** | • Avoid the use of pneumatic tube system<br>• Hand carrier transport preferred |
| **Whole blood processing** | • Keep samples at room temperature until processing<br>• Process samples as quick as possible, ideally within 1h of collection |
| **Centrifugation** | • Double centrifugation at room temperature recommended (2x2500g, 15 min) |
| **Sample storage** | • Use plasma within 4h or freeze as soon as possible at -80°C<br>• Samples may be stored at -80°C for min. 2 years<br>• Thaw samples only once at 37°C and gently mix before use |

CTI is a contact pathway inhibitor which may minimize contact activation of hemostasis which could potentially influence TGA results. Abbreviations: CTI, corn trypsin inhibitor; PPP, platelet poor plasma; PRP, platelet rich plasma. Adapted from Depasse et al. *J Thromb Haemost* 2021, Dargaud et al. *J Thromb Haemost* 2018 and Ninivaggi et al. *J Thromb Haemost* 2021[6,61,62]

4.2.3 Anticoagulant therapy

Any anticoagulant therapy taken by the patient prior to sampling will evidently affect TGA results (e.g. as depicted in Figure 5) and hence, must be accounted for (see *4.4.1 Calculation and interpretation of results*).

**Table 4. Advantages and disadvantages of using platelet poor – or platelet rich plasma for TGA.**

| | PPP | PRP |
|---|---|---|
| **Advantages** | • Commercially available semi-automated assays and fully automated assays<br>• High throughput<br>• Good standardization | • Commercially available semi-automated assays<br>• Reflects the influence of platelet dysfunction on TG<br>• Reflects the influence of some platelet-associated factors (e.g., VWF, FXI) |
| **Disadvantages** | • Blood cells are omitted in the test, thus not possible to study cell-mediated thrombosis or bleeding<br>• Centrifugation is needed to prepare PPP from WB, therefore has a long turnaround time<br>• Influence of blood flow or endothelium are not included | • Erythrocytes, leucocytes (and cancer cells) are omitted<br>• Pre-analytical variations during PRP preparation and long turnaround time<br>• Lack of standard sample for normalization and influence of blood flow or endothelium is not included |

Abbreviations: FXI, factor XI; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TG, thrombin generation; VWF, von Willebrand factor; WB, whole blood. Adapted from Wan et al. *Thromb Haemost* 2021[9]

*4.3 Analytical considerations*

4.3.1 Choice of reagent pack

For both the ST Genesia and Ceveron s100 instruments, three dedicated reagent packs are available for the investigation of i) bleeding tendency; ii) thrombotic tendency and iii) monitoring anticoagulant use. The respective reagent packs' compositions are optimized for their respective indication and differ mostly in the amount of TF and phospholipids included which initialize the TGA reaction. For example, bleeding tendency reagent packs utilize a low TF level which enhances the sensitivity for factor deficiencies as TG in the sample will be more reliant on the positive feedback mechanisms mediated by clotting factors. This has for example been illustrated by the study of Haisma et al. who demonstrated that the bleeding tendency pack indeed displayed increased sensitivity for factor deficiency.[58] On the other hand, reagents higher in TF but with very low phospholipid concentrations are more suited for discovering thrombotic tendencies while reagents containing both increased TF and PL concentrations are more suited for determining coagulation status in patients under anticoagulant therapy.[6] Moreover, reagent packs for thrombotic tendency may also contain thrombomodulin which allows to also account for the activity of activated protein C (as protein C is activated by thrombin bound to thrombomodulin) and its impact on TG. Apart from commercial reagent packs, multiple studies have also constructed their own in-house reagents. However, in order to ensure more robust and reproducible results, the ISTH recommends the use of standardized commercial reagents for clinical applications which are to be used according to manufacturer's instructions.[62]

4.3.2 Assay temperature

According to ISTH recommendations, TG measurement should be performed according to the manufacturer's recommendations. However, as temperature has shown to have a significant effect on thrombin generation – with non-preheated (to 37°C) reaction containers resulting in up to 50% increased ETP results due to less thrombin inhibition occurring – it may nevertheless be advised to preheat all samples and substrates at 37°C for increased reproducibility.[62,63]

4.3.3 Calibration

ISTH proposals recommends that calibration should be performed regularly via the use of commercial reagents according to the instructions of the manufacturer.[62]

*4.4 Post-analytical considerations*

4.4.1 Calculation and interpretation of results

For interpretation of raw data obtained via commercial assays, the ISTH recommends to utilize the software accompanying the device.[62] However, when interpreting results, it is advised to account for several clinical factors. For example, the presence of anticoagulant therapies (heparins, coumarins, DOACs, …) in the sample evidently affects TG and thus assay results (Figure 5, Figure 6). However, it is important to acknowledge that the effect on TGA parameters may be different depending on the type of anticoagulant. For example, as shown in Figure 5, direct Xa inhibitors such as apixaban result in a flat or "camel-back" curve with prolonged kinetic parameters and suppressed peak but relatively preserved ETP while direct thrombin inhibitors (e.g. dabigatran) result in a right-shifted TGA curve due to delaying the onset of TG.[64] Heparin appears to produce curves similar to direct Xa inhibitors (i.e. right-shifted, flat).[65] Vitamin K antagonists such as warfarin, on the other hand, suppress all phases of thrombin generation and lead to comparatively larger reductions in ETP compared to DOACs.[64] Conversely, the use of e.g. oral contraceptives results in an increased prothrombotic TG profile (e.g. increased ETP) due to acquired activated protein C resistance.[66] Increasing age has also been associated with increased 'procoagulant' TGA results (e.g. increased ETP and peak thrombin).[67] In other words, when interpreting the results of the TGA assay one should always keep the complete clinical picture (i.e. age, comorbidities, medication,…) in mind.

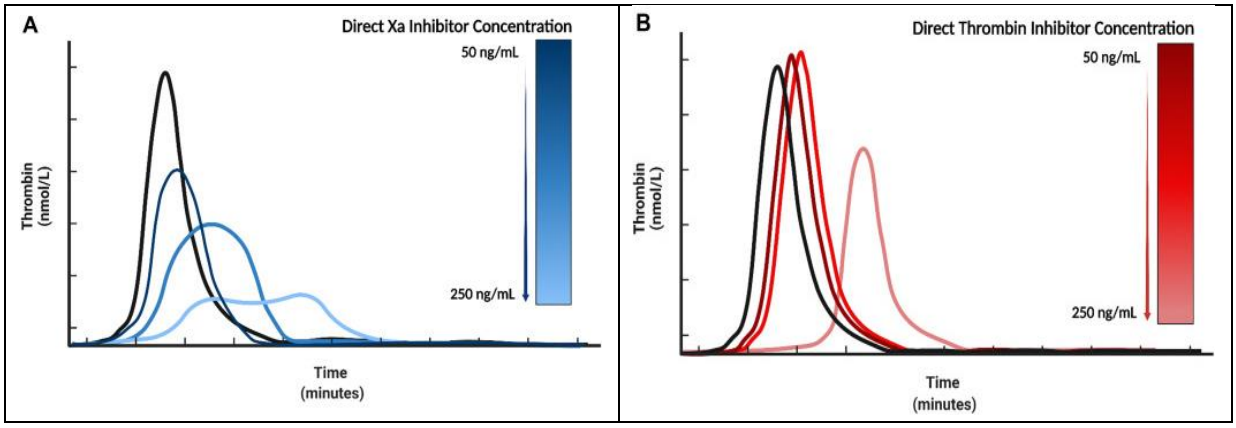


**Figure 6. The effect of anticoagulants on thrombogram profiles**.
Adapted from Shaw et al. *J Thromb Haemost* 2023[64]

4.4.2 Normalization of results

Despite the potential benefits of TGA's over routine clotting tests, several factors currently still limit their implementation in clinical practice. These limitations mainly entail the problem of standardization For example, differences exist between reagents/manufacturers which may impact the obtained results.[2] For example, TG is typically triggered by adding PL and TF to the sample, but the amount and source of these components differ between the reagent packs of different manufacturers and the exact composition is often not disclosed. Moreover, there is currently also no international reference standard for TF which impedes standardization.[2] Hence, as also advised by the IHCC 2017 guideline, it is recommended that results should be normalized in order to allow a more straightforward comparison

between platforms and laboratories.[61] As proposed by earlier studies, such normalization may be accomplished by means of a normal control plasma (e.g. commercial normal pooled plasma) measured in the same run which would allow increased inter-run, inter-platform and inter-center comparability.[58]

4.4.3 Reference ranges

Due to high variability in (in-house) TGA assays in published literature, the ISTH discourages the use of reference value from previous publications.[61,62] Instead, they advise the establishment of reagent-, age- and analyzer-specific reference values. However, several studies utilizing commercial (i.e. more standardized) reagents on commercial analyzers (mainly on the ST Genesia) have published reference values for TGA parameters which may provide initial guidance.[68,69] Nevertheless, given the reported sensitivity of TGA to (pre-)analytical variables, including laboratory-/hospital-specific ones such as e.g. transport systems, it is still advised for each laboratory to develop its own references values.

## TO DO/ACTIONS

1) Evaluate TGA in patients genetically tested for inherited thrombophilia.
2) Evaluate the practical implementation of TGA in clinical laboratory of UZ Leuven.

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