


REVIEW ARTICLE

Thrombin generation assays are versatile tools in blood coagulation analysis: A review of technical features, and applications from research to laboratory routine

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Abstract

Thrombin is the pivotal enzyme in the biochemistry of secondary hemostasis crucial to maintaining homeostasis of hemostasis. In contrast to routine coagulation tests (PT or aPTT) or procoagulant or anticoagulant factor assays (e.g. fibrinogen, factor VIII, antithrombin or protein C), the thrombin generation assay (TGA), also named thrombin generation test (TGT) is a so-called “global assay” that provides a picture of the hemostasis balance through a continuous and simultaneous measurement of thrombin formation and inhibition. First described in the early 1950s, as a manual assay, efforts have been made in order to standardize and automate the assay to offer researchers, clinical laboratories and the pharmaceutical industry a versatile tool covering a wide range of clinical and non-clinical applications. This review describes technical options offered to properly run TGA, including a review of preanalytical and analytical items, performance, interpretation, and applications in physiology research and pharmacy.

KEYWORDS

assay technology, coagulation assay, thrombin generation assay, thrombosis

1 | INTRODUCTION

Most coagulation assays used in the current hemostasis laboratory, semi-global including the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) as well as specific assays such as coagulation factor assays or antithrombin assay, specifically look at one component, either procoagulant or anticoagulant. Although

these are useful to identify patients at risk of bleeding or on the opposite at risk of thrombosis, these do not provide a global picture of the result of these two counteracting physiological systems. In contrast, global assays at the same time measure pro- and anticoagulant activities and, thus, reflect the hemostatic balance of an individual. By integrating this complex interplay, they can provide in a single measurement an accurate and clinically more relevant information

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than a panel of specific assays, the results of which may then be challenging to interpret. This paper reviews the preanalytical and analytical characteristics, performance, interpretation of thrombin generation assay, one of the most prominent global assays, as well as its application in physiology research and pharmacy. Information about the state-of-the-art of use of TGA in clinical practice will be given in another review paper (Binder NB et al., submitted 2021).

2 | BIOCHEMISTRY OF THROMBIN GENERATION ASSAYS

First descriptions of a thrombin generation test performed in human blood samples were received on the same day, October 30, 1952 from two different British teams and published in the same issue of the *Journal of Clinical Pathology* in 1953.^{1,2} During the history of development of test systems studying the formation of thrombin the terms Thrombin Generation Assay (TGA) and Thrombin Generation Test (TGT) have been used synonymously without a clear differentiation as proposed by recommendation such as in the Pharmacopoeias and other regulatory and standardization compendia. For the rest of this manuscript the acronym TGA is used. TGA evaluates the formation of thrombin resulting from both the action of procoagulant drivers and its decay resulting from the action of anticoagulant drivers, thus, assessing the balance between the two. Early papers described manual methods using surrogate markers, e.g. reaction media subsampling associated with fibrinogen clotting time,^{1,2} or direct measurement of prothrombin F1+2 fragment levels in plasma samples as an indirect indicator of thrombin generation.³ In contrast, this paper will exclusively focus on direct, continuous and simultaneous measurement of thrombin generation and inhibition.

Briefly, thrombin formation in the test plasma is triggered by small amounts of tissue factor (TF) and phospholipids, and the result of thrombin generation, counterbalanced by thrombin inhibition occurring simultaneously as soon as trace amounts of thrombin appear in the reaction media, is continuously monitored by means of a thrombin-specific chromogenic or fluorogenic substrate. Chromogenic or fluorescent signal is continuously recorded. Dedicated computer software then calculates thrombin activity in comparison with a thrombin standard, traces the thrombin generation curve, and calculates the relevant parameters thereof describing the thrombogram.⁴

Thrombin is a pivotal enzyme in the secondary hemostasis biochemistry crucial to maintaining a normal hemostatic balance. It is often referred to as a Janus-faced protein because it adopts opposing procoagulant and anticoagulant functions during the lifetime of a forming blood clot. Trace amounts of thrombin are generated at the initiation stage of in vivo coagulation via the factor Xa produced from the TF/factor VIIa complex, which is rapidly downregulated by tissue factor pathway inhibitor (TFPI). Unencumbered by the immediate action of inhibitors, the coagulation reactions achieve a burst of thrombin generation during this propagation phase, whereupon thrombin is additionally able to initiate polymerization and

Essentials

- Thrombin generation assays (TGA) are “global” assays measuring the dynamics of the blood coagulation process beyond end point assays.
- TGA have evolved from early research bench methods in the 1950s to elaborate and qualified robot assisted analytical instrument-based methods of today.
- Major advances have been made towards standardised systems for TGA building on optimized components: specific analysers with tightly controlled physical conditions, validated pipetting schemes for standardised reagents and triggers, well controlled fluorescent thrombin substrates and standards, and assay specific quality controls.
- TGA have proven as assays predicting pre-crisis changes in both thrombotic and bleeding situations, and, thus, are widely used in research and clinical practise.

stabilization of fibrin to render the forming clot hemostatically stable. Thrombin also activates more FV, FVIII, FXI and platelets. One of the main regulators of coagulation, antithrombin, does not enter the clot itself but operates as a scavenger by complexing with activated clotting factors that leak into the plasma, which would otherwise be lethal if permitted to circulate. Some of the leaked thrombin instead arrives on the vessel endothelium where it binds to thrombomodulin, a receptor anchored to the endothelial membrane. As its name suggests, thrombomodulin suppresses procoagulant abilities of thrombin, such as fibrinogen cleavage and platelet activation, but enhances >1000-fold its specificity for protein C, which is captured on the vessel endothelium by a specific endothelial protein C receptor. The consequent activated protein C is released by its receptor and enters the forming clot where, in concert with its cofactor protein S, cleaves and inactivates factor Va and factor VIIIa, thereby downregulating thrombin generation. Because of its central position, the formation of thrombin is considered one of the most important steps in coagulation.⁵

It has been shown that thrombin generation is diminished in all types of clinically significant coagulation factor deficiency and anticoagulant treatment. Conversely, thrombin generation is increased in congenital and acquired thrombophilia.⁶ In addition, TGA has been proposed as a tool to evaluate the thrombogenic activity of immunoglobulin concentrates.⁷

Ex vivo thrombin generation reflects the endogenous capacity of the hemostatic system and can be indicative of thrombotic or hemorrhagic risk, because unlike routine screening tests, it reflects contributions of procoagulant and anticoagulant elements, including the duality of thrombin function. Routine screening tests artificially section coagulation into separate compartments and thus do not fully assess enzyme interdependencies.

In contrast, TGA measures proteolytic cleavage by thrombin of a synthetic substrate which releases a chromogen or fluorophore, the output signal of which is continuously measured and is proportional to the amount of thrombin generated. Reaction kinetics comprise three stages: initiation, amplification, resolution phase (Figure 1).

Based on this initial raw optical signal (RFU) over time the first derivative is calculated to present an initial thrombin generation curve in RFU/min over time. With use of a calibration curve the optical signal can further be traced to an amount of thrombin expressed in nM over time – the thrombogram (Figure 2).

To not limit the interpretation of TGA on interpretation of curves, several parameters have been established and shown to be useful to report the results of TGA which trace back to the three stages of thrombin generation and thrombin function.

- Lag time: expressed in minutes, describes the time from starting the reaction until thrombin is first generated. It corresponds to the timepoint when the fibrin clot is formed (=clotting time) in standard clotting tests. In some assays, it can be defined as the thrombin onset time that delineates the timepoint when free thrombin is detected.
- Time to peak: expressed in minutes, corresponds to the time until the maximum amount of thrombin is formed.
- Start tail: expressed in minutes, corresponds to the time at which thrombin generation has come to an end and all the generated thrombin has been inhibited.
- Peak height: or Peak Thrombin, correlates to the maximum thrombin generation. When calibrated it is expressed in molar concentration of thrombin.

- Velocity index: the velocity index is indicative of the slope of thrombin generation between the lag – time and the time to peak and corresponds to the first derivative of this part of the curve.
- The endogenous thrombin potential (ETP) corresponds to the area under the curve (thrombogram).

3 | COMPONENTS OF TGA

There are multiple ways to measure thrombin generation described in the literature, starting with the first description of a manual method in 1953.¹ This document will focus on modern (semi) automated methods based on the specific direct measurement of thrombin activity.

3.1 | Sample and Pre-analytics

Preanalytical variables, including sample quality, will affect TGA results and adherence to recommended practice is advised.

TGA can be run on various matrixes including platelet poor plasma (PPP), platelet rich plasma (PRP)¹⁰ frozen-thawed platelet rich plasma¹¹ or whole blood.¹² Working with these different matrixes may require different reagents and instruments. This report focuses solely on PPP TGA as this is to date the most standardized and documented assay.

Loeffen et al. have conducted an extensive study comparing various procedures for blood collection and issued preanalytical recommendations summarized in Table 1 together with International Society on Thrombosis and Haemostasis (ISTH) Scientific and

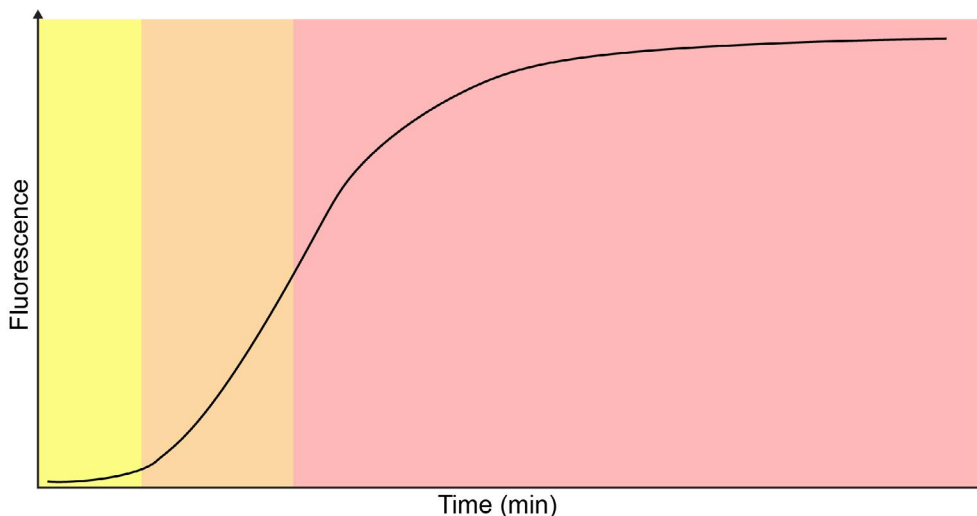


FIGURE 1 Signal recorded over time reflecting three stages of thrombin generation (yellow: initiation, orange: amplification, red: resolution). First, the trigger permits the reactions to mirror the initiation phase, which can be inhibited by TFPI. Time taken to complete this phase of TGA corresponds to the time to initial fibrin formation, or clotting time, of routine tests such as PT or aPTT; which only requires trace amounts (i.e. ~5% of total thrombin formed), and which by design does not measure or detect events beyond initial clot formation.⁸ Second, the amplification / propagation phase, where prothrombin conversion is more rapid than thrombin inhibition. Third, the resolution phase, where prothrombin conversion is outperformed by the more intensive action of various inhibitors present in the plasma sample such as activated protein C (aPC), antithrombin (AT) and alpha2-macroglobulin⁹

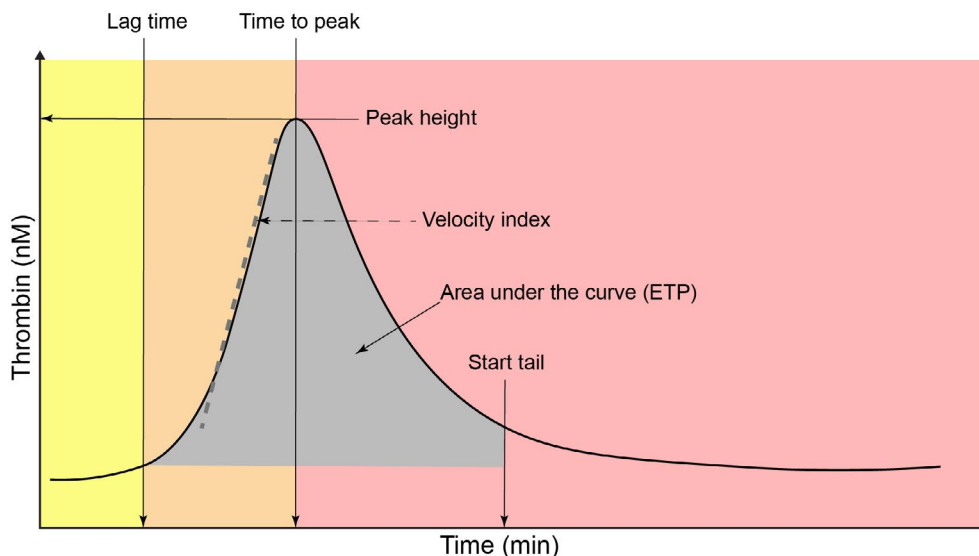


FIGURE 2 Thrombogram parameters (ETP: endogenous thrombin potential)

Standardisation Subcommittee (SSC) proposed standardized preanalytical and analytical conditions for measuring thrombin generation in the specific setting of hemophilia.^{13,14}

Of note, the use of a contact pathway inhibitor such as corn trypsin inhibitor (CTI) has been advocated to minimize contact activation that may influence TGA results, especially when low TF concentrations are used¹⁵ unless contact activation is – at least in part – of interest mechanistically. The use of CTI is however also debatable because of several limitations: first, as citrate – CTI tubes are not widely commercially available, this would require that laboratories manually prepare tubes introducing potential errors; in case of vacuum tubes, this preparation requires that vacuum is broken, possibly resulting in a blood volume error affecting the blood: anticoagulant ratio and subsequently TGA results. CTI is quite expensive, and its use may impact the cost-effectiveness of TGA. The use of a specific tube for a dedicated assay can be a source of errors for the assay and, more generally, for other assays run in the same lab. These limitations should be considered and balanced with CTI's analytical advantages before deciding whether CTI should be used or not in a specific setting.

Since a specific blood draw for TGA is not available or feasible in a routine setup, a standardized procedure such as CLSI H21-A5 is required to be implemented to minimize variability caused by preanalytical steps. As this recommendation also holds true for other coagulation parameters such as testing for lupus anticoagulant, it is recommended to use the locally established sample collection method to reduce additional preanalytical errors.

3.2 | Substrate

The groundwork for the current TGA was the continuous thrombin generation monitoring method reported by Hemker et al.¹⁶ in 1993 using a chromogenic substrate, methylmalonyl-methylanalyl-arginyl-pNA.

The thrombin-specific fluorogenic substrate, carboxybenzyl-glycyl-glycyl-arginyl-7-aminomethyl-4-coumarin was subsequently used in the fluorescence-based calibrated automated thrombogram in 2003.⁶ Over the last two decades, further improvement of this assay has resulted in availability of commercial automated thrombin generation assays.

The fluorogenic assays use the Z-Gly-Gly-Arg-AMC (ZGGR-AMC) substrate. The molecule AMC (7-Amino-4-methylcoumarin) is a fluorophore that generates a signal (390 nm excitation/460 nm emission) when released producing fluorescence. A chromogenic version of TGA using a H- β -Ala-Gly-Arg-pNA substrate (absorption at 405 nm) has been available, which required the samples to be defibrinated, or that the reagent mixture contained a fibrin polymerization inhibitor. The chromogenic system is commercially not available anymore.

The choice of the appropriate substrate is crucial to the assay principle of TGA as the substrate will always influence the coagulation in the test and both substrate binding and substrate consumption are required to be balanced. Strong substrate binding or fast substrate turnover are contra-indicated as they would not leave sufficient free thrombin for the clotting mechanisms to function normally.^{16,17} Comparability of chromogenic results with those from the more sensitive fluorogenic methods is limited. Indeed, the functional impact of thrombin inhibitors, such as alpha-2-macroglobulin, which is increased in special patient populations, in neonates and children, may not be reflected in TGA results. This can be due to the mathematical evaluation model that may not be accurate in those specific patients.¹⁸ Alternatively, some parameters such as Peak Thrombin have been shown to be less affected.¹⁹ However, this limitation can be overcome depending on the system used.²⁰ In the case of fluorogenic assays there is a significant difference in the mathematical processing of the inner filter effect, also known as fluorescence quenching. It is, however, possible using a mathematical calculation to generate an analytical plot that accounts for the inner filter effect and the substrate consumption. Finally, chromogenic and fluorogenic

TABLE 1 Preanalytical recommendations for thrombin generation test^{13,14}

Preanalytical variables	Recommendation for thrombin generation according to Loeffen et al. ¹³	ISTH SSC proposed standardized preanalytical conditions for thrombin generation in hemophilia ¹⁴
Blood collection system	Recommended: Conventional straight needle Alternative: Butterfly needles or intravenous catheters only accepted as reliable alternatives when a proper phlebotomy technique is used and samples are carefully checked for hemolysis	Direct venipuncture Blood sampling through venous catheter does not modify TG in platelet-poor plasma (PPP) Tourniquet should be applied only long enough to locate the vein, ideally 60 s or less
Blood collection tube	Recommended: Addition of CTI to the collection tube is recommended to inhibit contact activation Alternative: Without CTI addition, the Monovette [®] tube induces the least contact activation and the best alternative to the CTI tube	Data comparing different blood-drawing systems for TG test in PPP showed no significant difference between the Monovette [®] -syringe tubes (Sarstedt) and Vacutainer [®] vacuum tube (Becton Dickinson) containing sodium citrate 106 mM Need for contact pathway inhibitor (e.g. CTI) still debatable; advantage of CTI 1.45 μM (final concentration) emphasized for TG triggered by low TF concentration
Discard tube	A discard tube is required for thrombin generation measurements	No specific recommendation
Transport of blood samples to the laboratory	No specific recommendation	Pneumatic tubes should not be used for TGA blood samples
Whole blood sample processing	Direct plasma preparation is preferred to storage of whole blood for 6 h	Samples should be processed as quickly as possible, ideally within 1 h of collection
Centrifugation	Double centrifugation preferred: 2000 g, 5 min followed by 10 000 g, 10 min	Double centrifugation recommended (2 x 2500 g, 15 min)
Plasma processing	Recommended: immediate analysis after plasma thawing Alternative: plasma kept at room temperature	Plasma samples should be frozen immediately at -80°C (stable for a minimum of 2 years), then thawed at 37°C before testing Do not use plasma that has been freeze - thawed more than once
PPP quality assessment	No specific recommendation	The quality of PPP samples can be evaluated for each sample by measuring TG without any triggering agent: if plasma contains CTI, no TG should be observed in the absence of TF and phospholipids

Abbreviations: CTI, corn trypsin inhibitor; PPP, platelet poor plasma; TF, tissue factor; TG, thrombin generation; TGA, thrombin generation assay.

substrates are cleaved from the peptide at different rates and as such have different limits of detection. This would partly account for the difference in detection of thrombin generation, though the level of TF remains the main driver for the rate of thrombin generation.²¹ Altogether, fluorogenic substrates are usually preferred especially when in combination with weak activators typically use for the investigation of hemophilia and thrombophilia samples.

3.3 | Trigger/Reagent

Thrombin generation is typically triggered by addition of TF and/or phospholipids, but intrinsic pathway triggers such as celite can also be used.²² TGA results are highly dependent on the composition of the trigger reagent. Thus, a major source of inter-laboratory variation is the source and concentration of the raw material used in the trigger.

Depending on the clinical or scientific question, the appropriate trigger reagent with respective nature of activator, but also amounts of TF and phospholipids need to be selected. It had been suggested

that different TF concentrations would allow to make TGA more sensitive towards either intrinsic or extrinsic pathway reactions. In addition to pure concentration-based differences, the appropriate integration of TF into the phospholipid vesicles as well as their size is crucial as it determines the biological activity in the TGA. For further details also see the section "Establishing standardized TGA".

Reagents containing very low TF and very low phospholipid concentrations are suitable to detect hypocoagulability such as that induced by factor VIII or IX deficiencies, and to monitor hemostatic therapy in hemophilia patients.²³⁻²⁵ Other bleeding disorders may require different conditions: in factor XI deficiency, correlation between TGA and clinical bleeding risk was observed only when coagulation was triggered in samples without CTI by very low TF levels in the presence of platelets.^{14,26}

Reagents with higher TF but very low phospholipid concentrations have been able to reveal correlation of thrombin generation results with thrombotic events in patients with various conditions such as recurrent deep vein thrombosis and cancer but also Factor V Leiden and even exogenous risk factors such as smoking and contraceptives.²⁷

Reagents with both higher TF and phospholipid concentrations are suitable for determining the coagulation status of patients under anticoagulant therapy ranging from warfarin to direct oral anticoagulants.

In addition to these prototypic trigger reagents, modifications of reagents are used and described in the literature. These include the absence of TF to study the procoagulant capacity of circulating microparticles or the addition of thrombomodulin to investigate protein C function in more detail. Since in TGA activated by TF and phospholipids alone, only limited APC is generated, the investigation of protein C function is missing. To close this gap, addition of thrombomodulin to the reagent could enable the formation of APC and in turn studying protein C function. More recently contact pathway activator containing trigger reagents, and more specifically FXIa or ellagic acid, has been shown to be suitable for studying thrombin generation in the presence of non-factor replacement therapy such as emicizumab.²⁸

In summary, it is of great importance to identify the appropriate trigger for the respective analysis as using the incorrect reagent a hemophilia patient would not show reduced thrombin generation or an anticoagulated patient would exhibit no thrombin generation at all.

3.4 | Calibration

Some thrombin generation test manufacturers provide a thrombin calibrator with known thrombin activity. This calibration sample is used either in parallel with test samples or separately to allow the conversion of the optical signal (fluorogenic or chromogenic) into thrombin concentration, usually expressed as nanomolar (nM) units. Depending on the system used, the calibration can be stable over time and a single calibration per reagent lot can be sufficient provided QC sample results fall within the acceptance range.

3.5 | Instruments

Both manual, semi-automated and fully automated methods are used to measure thrombin generation. The use of fully automated analyzers has lowered imprecision including repeatability and reproducibility to currently accepted laboratory standards of performance for routine assays. Besides more accurate pipetting and higher optical resolution mostly achieved by LED systems, appropriate temperature control is required for optimal TGA results. In an international standardization study, TGA showed up to 50% higher thrombin generation when reaction containers were not pre-heated compared with one pre-heated at 37°C before TGA was performed.²⁹ The use of non-pre-heated plates might be a source of error when bleeding risk is evaluated. The main source of variability remains the difference between the temperature of the plate when it enters the fluorimeter and the temperature in the fluorimeter, which can be accounted for in an automated system.

4 | ESTABLISHING STANDARDIZED TGA

Over the last years major advances have been made towards standardized systems for TGA especially using the fluorescent substrates. These systems have, despite minor differences, major commonalities further than the theory behind TGA, covering most of the above-mentioned components:

- a specific analyzer optimized for TGA including tightly controlled temperature and light intensity
- validated pipetting schemes for the respective reagent including optimized measurement times
- well controlled substrate including traceable thrombin and fluorescence standards
- availability of specific QC controls covering the analytical measuring range
- and most importantly the use of standardized reagents or triggers

Trigger reagents based on TF and phospholipids differ in the respective concentrations for specific clinical questions. In addition to the exact concentrations of components, the preparation method, with respect to phospholipids vesicle size, phospholipid type and source and TF integration and orientations affect TGA. Needless to say, that the individual reagent concentration is also affected by the amount of plasma used (typically 30%–60% of reaction mixture) and the concentration of the concurrently active substrate. Figure 3 shows a scheme of different commercially available TF and phospholipid reagents.

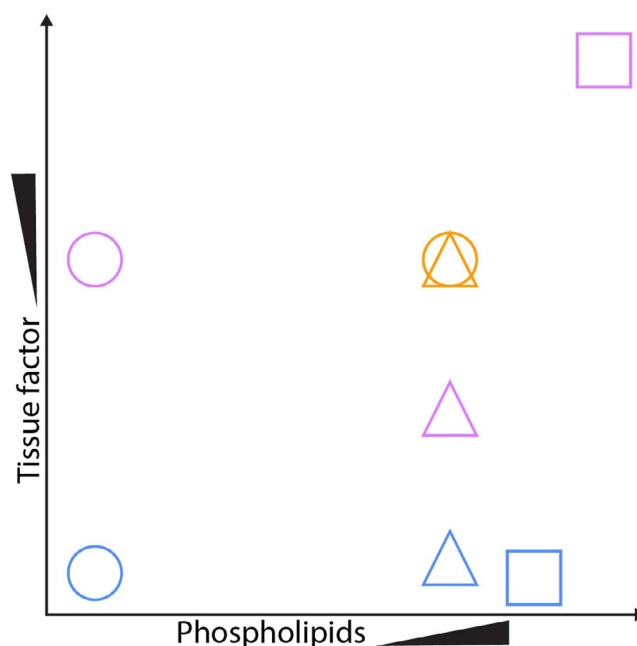


FIGURE 3 Phospholipids and tissue factor concentration of commercial reagents for different clinical applications (orange: anticoagulant, purple: thrombophilia, blue: bleeding); triangle: manufacturer 1, circle: manufacturer 2, square: manufacturer 3

To summarize currently available reagent:

- For bleeding tendencies testing on PPP, very low TF concentrations ranging from 0.4 to 2 pM are combined with phospholipid in concentration ranging from 0.4 to 4 μM.
- Trigger reagents for thrombophilia testing typically exhibit an increased TF concentration. Depending on the amount of phospholipids used, these reagents use TF between 5 and 40 pM. To exhibit the effect of APC in such a thrombophilia specific reagent, the addition of thrombomodulin has been used.
- To overcome anticoagulation, trigger reagents prepared to investigate these, exhibit higher TF and, possibly, high phospholipid concentrations typically ranging between 20 and 50 pM TF and around 4 μM phospholipids.

Of note, a major issue in summarizing trigger reagent of different systems based on their concentration characteristics is also based on the use of recombinant TF and synthetic phospholipids. Molecular mass and specific reactivity differ from the respective manufacturing procedure and the expression of the recombinant protein. Thus, most manufacturers have decided not to define the reagent composition based on antigenic concentrations since lot-to-lot activity consistency is of greater importance and is a regulatory necessity enabling an IVD use of TGA.

In addition to the above-mentioned approaches towards standardized TGA, several studies have shown the benefit of normalizing results by using a reference plasma run in the same experiment as the test sample. The various TGA parameters can then be expressed by comparison to the results obtained for the reference plasma for the same parameters. Currently available commercial automated TGA systems allow such a normalization.

5 | APPLICATIONS IN BASIC SCIENCE, NON-CLINICAL RESEARCH, AND QUALITY CONTROL OF PHARMACEUTICALS

5.1 | Basic research on the biology of blood coagulation

Global assays have advanced the understanding of the dynamics of the blood coagulation process beyond end point assays and are at the forefront of implementation in the hemostasis laboratory. With thrombin being the enzyme converting fibrinogen into fibrin by partial proteolysis and thus enabling fibrin clot formation it is almost logical to investigate mechanisms and regulation of activation and inhibition of thrombin. Availability of TGA and machine assisted TGA kits therefore essentially contributed to understanding of the blood coagulation process. Empirical TGAs and computational modelling of thrombin generation have greatly advanced the understanding of the hemostatic balance in different pathologies as described above. Implementation of these types of assays and visualization approaches in the clinical laboratory provided a basis for

the development of individualized patient care. Advances in both empirical and computational global assays have made the goal of predicting pre-crisis changes in an individual's hemostatic state one step closer.³⁰ The evolution of the TGA technology in coagulation research has been described in several reviews.^{8,12,31-37}

5.2 | Applications in toxicological studies

TGA to some extent also increased the understanding of environmental, food and drug related toxicity.³⁸⁻⁴¹ Systematic toxicological studies are usually performed in animal models where rodents are a preferred species for laboratory use. Application of TGA in such models is limited by the fact that routine TGA methods are developed and optimized for human blood and plasma samples. Thus, for analysis of blood coagulation in animals it is critical to understand and document the impact of the pre-analytical and analytical and conditions on readouts of the different TGA methods.⁴²⁻⁴⁴

5.3 | Use of TGA in drug development and elucidation of mode of action of drug substances

TGA has been applied in drug development to explore pharmacological principles and drug related side effects. TGA has for example been recently evaluated in dose finding studies of direct oral anticoagulants and to tailor therapeutic protocols with such substances.⁴⁵⁻⁴⁸

Hemophilia patients who develop inhibitory antibodies against FVIII or FIX are treated with activated prothrombin complex concentrates (APCC), with activated factor VIIa (FVIIa) or a bi-specific antibody mimicking coagulation factor VIII function by bringing together enzyme (FIXa) and substrate (FX) participating in the tenase complex. The mode of action of these so-called bypassing agents has been postulated to facilitate the impaired thrombin generation in hemophilic patients via interactions with endogenous clotting factors and platelets. TGA played a pivotal role in all these studies. For APCC it was shown that a complex of prothrombin and factor Xa induced immediate thrombin formation on the surface of activated platelets, while other pro- and anticoagulant components of APCC were necessary to achieve an optimal, not overshooting thrombin burst triggering clot formation.⁴⁹⁻⁵¹ This hypothesis established in *in vitro* models could be verified by measuring patient samples from clinical studies with TGA.⁵² Use of TGA was also essential to identify the modes of action of rFVIIa. In addition to TF bound FVIIa initiating hemostasis it was found that FVIIa at high concentration could also trigger hemostasis independent of TF.⁵³ Further data obtained by TGA studies suggested that in a cellular system high-dose FVIIa acts primarily by enhancing the rate of thrombin generation on platelet surfaces and not by overcoming inhibition of TF-dependent activation of FX by zymogen FVII. These studies also formed the basis of a wider understanding of cell-based pathways of blood coagulation beyond the activation cascades of soluble proteins in

zymogen-enzyme complexes.⁵⁴ For the bispecific antibody emicizumab TGA became a critical tool to compare hemostatic activity with classical coagulation assays.²⁸ TGA also was able to predict synergistic effects of the bispecific antibody when used concomitantly with bypassing agents ultimately resulting in thrombosis and thrombotic microangiopathies.⁵⁵

The accumulated knowledge about the mode of action of bypassing agents substantially enabled by TGA methods prompted the use of TGA as tool to monitor their pharmacokinetics and pharmacodynamics. This made possible to use biomarkers of coagulation measured by TGA for treatment monitoring of these bypassing agents which otherwise cannot be measured in the absence of suitable tests to directly determine the active ingredients.^{23,25,56}

TGA plays a specific important role in the assessment of residual prothrombotic activity in plasma protein concentrates such as intravenous immunoglobulin G (IgG) products. In recent years, IgG concentrates have been found to carry product and manufacturing related procoagulant impurities causing thromboembolic events (TEEs) *in vivo*. TGA has been found to be able to reliably predict procoagulant activities probably associated with the presence of FXIa, one of the major components responsible for TEEs, and potential thrombogenicity. Comparison of thrombin generation with product-specific acceptance criteria as well as variables from other test systems, such as amidolytic activity and molecular size, can help to monitor IgG quality and manufacturing changes with regards to thrombogenicity. As a consequence, TGA entered the routine test program of quality control procedures of IgG products.⁷

6 | DISCUSSION

The combination of accuracy, sensitivity, and versatility of TGA offers a broad spectrum of clinical and non-clinical applications. Clinical potential encompasses bleeding and thrombotic risk assessment, and on their related treatment monitoring. Regarding non-clinical applications, TGA has demonstrated a huge potential in the development of various drugs that by essence or accident impact hemostasis. This is notably the case for anticoagulants⁵⁷ and their antidotes⁵⁸ and for the variety of different established and novel hemophilia therapies.⁵⁹ Furthermore, TGA is considered a potential effective tool to detect residual procoagulant activity in drug products, thus, improving safety of pharmacotherapy by preventing iatrogenic thrombosis.⁶⁰ We therefore consider TGA as a versatile analytical test method which can be used for many different applications, when tailored by the appropriate reagent combinations and test setups for certain uses in research and clinical practice.

Global hemostasis results from the balance between procoagulant and anticoagulant antagonisms. Conventional coagulation assays selectively explore either a specific coagulation pathway, or specific procoagulant or anticoagulant physiological factors. Although a pathological result may signal a bleeding propensity as in hemophilia A, in which aPTT is usually prolonged and factor VIII level is decreased, or conversely may suggest an increased thrombotic

risk in the case of a shortened aPTT, possibly due to a high factor VIII or fibrinogen level, these assays may lack sensitivity. As such, both PT and aPTT are insensitive to physiological anticoagulant defects albeit that they may be responsible for an increased risk for thrombosis. Moreover, the aPTT prolongation observed in the presence of a lupus anticoagulant, paradoxically does not expose the patient to an increased bleeding risk in most cases but is a proven thrombotic risk when not transitory and then part of the antiphospholipid syndrome. This emphasizes the limitations of currently used laboratory tests to assess the patient's overall hemostatic status. These limitations were to some extent, with regards to the knowledge the authors had in 1952, the rationale for proposing a thrombin generation test.^{1,2}

In contrast, TGA is a global assay exploring in a continuous and simultaneous manner both thrombin formation, resulting from the activation of the extrinsic, intrinsic and common coagulation pathways, and thrombin inhibition, resulting from the counter action of inhibitors. Thus, TGA reflects the result of pro- and anticoagulant activities and as consequence the balanced or imbalanced hemostasis status of a patient. TGA is therefore a more representative model of *in vivo* physiology than conventional coagulation assays. Furthermore, in cases of disrupted homeostasis of hemostasis, TGA can be informative of a bleeding versus thrombotic tendency, which is not always the case for routine tests.

However, initial attempts at effective direct measurement of thrombin generation encountered technical difficulties. First, a thrombin-specific substrate had to be used. Chromogenic substrates were initially developed by Hemker in 1993.¹⁶ Although being a great step forward they had clear limitations, in particular PRP could not be used because the turbidity would interfere with the optical density measurements. A fluorogenic substrate then overcame this limitation, but other issues had to be solved before reliable thrombograms could be generated.⁶

A major hindrance to wide clinical uptake of TGA was the lack of reliable commercially available standardized reagents, which prevented generation of comparable results across different clinical laboratories.⁶¹ Indeed, lack of standardization is sometimes raised as a limitation of TGA, despite improvements described above. One should keep in mind that, in contrast to TGA, some very common routine assays such as aPTT which are considered to give reliable readouts equally lack standardization.⁶² Also, issues related to consistently and reliably measure coagulation factors VIII and IX have been subject to scientific debates over the last 20+ years and now more than ever are continuously discussed by scientific boards and drug regulatory agencies (e.g.⁶³⁻⁶⁵). Recent years have seen significant efforts made by manufacturers to offer standardized reagents to facilitate comparable results in various laboratories using the same system. This was achieved by both commercial availability of standardized reagents (triggers, calibrator and controls), as well as a reference plasma for result normalization. An important field study supported the actual benefits of this standardization.⁶⁶

With respect to different clinical applications, variable reagent compositions have been described. This was, in part, due to the

absence of standardization of TGA. Manufacturers have worked on developing appropriate reagents for various indications, which clearly improve result reproducibility. To achieve this, manufacturers not only had to take into account reagent components, but also the system as a whole including the instrument and the software, resulting in somewhat variable reagent composition to produce relevant results. Therefore, there is not definite reagent composition for one specific indication, making it difficult to commit on a "reference" reagent composition.

When monitoring literature, a strong uptake in publications on TGA and related testing technologies can be observed which is another indicator of the more widespread acceptance of this assay. Multiple recent publications in 2020 and 2021 on use of TGA for diagnosis and prediction of disease progression in COVID-19 brought another booster in number of published papers (Binder et al., submitted 2021). COVID-19 also triggered a deeper look into plasmin generation (PG) as enhanced PG was detected in patients with COVID-19.⁶⁷ Future developments of the global hemostasis assays, thus, suggest expansion to fibrinolytic pathways enabling to assess interplay between coagulation and fibrinolysis. One example is the so-called novel hemostasis assay (NHA) which measures thrombin and plasmin generation in a single well by a fluorimeter.⁶⁸

Finally, full automation has now made TGA a very user friendly and easily accessible tool, requiring minimal technical skill, which will promote adoption into laboratory repertoires to complement and enhance existing assays and improve clinical outcomes.

7 | CONCLUSION

A lot has been achieved since the first descriptions of a TGA and its application to the study of hemophilia in 1953. Emphasis has been placed on more reliable, more standardized and more user-friendly assays that have now reached a high level of maturity. Moreover, preanalytical conditions have been extensively studied leading to precise yet manageable recommendations, very similar to the usual preanalytical recommendations in force for routine coagulation workups. In parallel, researchers have looked at the TGA potential in various clinical and industrial settings and demonstrated the contributions of TGA in patient's diagnosis, prognosis evaluation and treatment monitoring and the benefits of TGA in non-clinical studies and industrial applications up to quality control of drugs. Altogether, this has paved the way for an imminent transition of TGA from research to routine use.

CONFLICT OF INTEREST

François Depasse, Nikolaus B. Binder, Julia Mueller, and Thomas Wissel are employed with companies developing in vitro diagnostics including thrombin generation assays. Stephan Schwerts, Matthias Germer, Björn Hermes, and Peter L. Turecek have no conflicts of interest related to contents and scope of the review manuscript.

AUTHOR CONTRIBUTION

François Depasse, Nikolaus B. Binder, and Peter L. Turecek designed and wrote the manuscript. Julia Mueller, Thomas Wissel, Stephan Schwerts, Matthias Germer, and Björn Hermes reviewed and edited the manuscript.

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