Received: 4 September 2020 | Revised: 1 February 2021 | Accepted: 7 February 2021

DOI: 10.1002/rth2.12499

rpth research & practice in thrombosis & haemostasis

ORIGINAL ARTICLE

Fluorescence artifact correction in the thrombin generation assay: Necessity for correction algorithms in procoagulant samples

William C. Chang PhD[1] | Joseph W. Jackson PhD[1] | Kellie R. Machlus PhD[2,3] | Alisa S. Wolberg PhD[2] | Mikhail V. Ovanesov PhD[1]

[1]Office of Tissues and Advanced Therapies, Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA

[2]Department of Pathology and Laboratory Medicine and UNC Blood Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[3]Vascular Biology Program, Department of Surgery, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA

Correspondence
Mikhail V. Ovanesov, Ph.D., U.S. Food and Drug Administration, 10903 New Hampshire Ave, WO52/72-4206, Silver Spring, MD 20993.
Email: mikhail.ovanesov@fda.hhs.gov

Present address
Kellie R. Machlus, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Funding information
This study was supported in part by a Postgraduate and Postbaccalaureate Research Fellowship Award to WCC and JWJ from the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration.

Abstract

Introduction: The thrombin generation (TG) test is a global hemostasis assay sensitive to procoagulant conditions. However, some TG assays may underestimate elevated TG when the thrombin fluorogenic substrate is depleted or fluorescence is attenuated by the inner filter effect (IFE).

Objectives: We sought to elucidate the extent to which procoagulant conditions require correcting for fluorogenic substrate depletion and/or IFE.

Methods: We analyzed corrections for substrate depletion and IFE and their effect on TG parameters in plasma samples with elevated blood coagulation factors in the presence or absence of thrombomodulin via commercial calibrated automated thrombogram (CAT) platform and in-house software capable of internal thrombin calibration with or without CAT-like artifact correction.

Results: Elevated thrombin peak height (TPH) and endogenous thrombin potential (ETP) were detected with 2× and 4× increases in blood coagulation factors I, V, VIII, IX, X, and XI, or prothrombin in the presence or absence of artifact correction. The effect of the CAT algorithm was evident in TG curves from both low procoagulant (thrombomodulin-supplemented) and procoagulant (factor-supplemented) plasma samples. However, in all samples, with the exception of elevated prothrombin, CAT's correction was small (<10%) and did not affect detection of procoagulant samples versus normal plasma. For elevated prothrombin samples, uncorrected TPH or ETP values were underestimated, and CAT correction produced drastically elevated TG curves.

Conclusions: Our data suggest that correction for substrate consumption and IFE, as offered by the CAT algorithm, is critical for detecting a subset of extremely procoagulant samples, such as elevated prothrombin, but is not necessary for all other conditions, including elevated factors XI and VIII.

KEYWORDS
blood coagulation factors, calibration, hemostasis, plasma, thrombin

Essentials

- Modern thrombin generation (TG) assays measure cleavage of a fluorogenic thrombin substrate.
- Substrate depletion results in underestimation of TG in plasma with elevated prothrombin levels.
- Thrombin peak height parameter fails to detect elevated TG in prothrombin-elevated samples.
- Correction algorithms are only critical for detecting prothrombin-elevated samples.

1 | INTRODUCTION

Thrombin generation (TG) is a global hemostasis assay with a potential to cover a spectrum of hemostasis abnormalities ranging from decreased TG in patients with bleeding disorders to elevated TG in patients at risk of thrombosis. The sensitivity of TG to detect elevated coagulation distinguishes it from the routine clotting time tests available in clinical laboratories, namely, prothrombin time (PT) and activated partial thromboplastin time (aPTT). PT and aPTT are reliably prolonged above the normal range in severe coagulation factor deficiencies, but they are not used for the diagnosis of procoagulant states.[1-5]

The poor reliability of clotting time assays in assessing procoagulant conditions may be related to the rapid nature of plasma clotting itself, which takes place early in the TG response, ensuring that the peak of thrombin activity and >90% of all thrombin are produced inside the clot.[6,7] In vitro, plasma forms a clot at the onset of the explosive phase of TG, potentially missing either the elevation or prolongation of subsequent TG. The formation of fibrin clots also coincides with a complete depletion of fibrinogen from liquid plasma, potentially capping these phenotypic variations in clotting times by the threshold imposed by fibrinogen consumption.[8] In contrast, substantial amounts of prothrombin remain unused inside the clot at the end of the TG reaction,[7] suggesting that the TG curve is a good estimate of hemostatic potential, reflecting a dynamic balance between the activation and inactivation of procoagulant and anticoagulant pathways of the coagulation cascade.

The clinical application of the TG assay has been suggested by multiple research studies and clinical trials.[1-7] However, commercially available TG assays remain hard to standardize and validate for routine use outside of the expert central laboratories.[8-13] Some of the challenges that complicate the use of TG assays as diagnostic tools are artifacts of the synthetic fluorogenic substrate for thrombin used by commercial TG kits.[9-11] Elevated TG may cause substrate depletion, resulting in distorted TG curve and underreported thrombin activity, particularly in highly procoagulant samples. In addition, a high concentration of fluorophore produced during substrate consumption subdues the fluorescence excitation via an inner filter effect (IFE), resulting in further underestimation of thrombin activity.[9]

Whether artifacts of substrate consumption and IFE are consequential for the diagnostic ability of the TG assay is a matter of debate. These artifacts are more pronounced near the end of the experiment, when little thrombin activity is left to measure, and whatever substrate proteolysis is measured can be driven by another substrate-related artifact, a small peptide-cleaving, biologically inert thrombin-α_2-macroglobulin complex. There are several TG kits and software packages that are commercially available or made in-house.[14] Although some TG kits, available since the early 2000s, ignore these substrate artifacts, no documented evidence is available on the underreporting of procoagulant samples by these kits. The commercial kit most notable for its substrate consumption and IFE correction algorithm is the calibrated automated thrombogram (CAT) method, supplied by Stago (Asnières-sur-Seine, France).[9,10] In this algorithm, an internal thrombin calibrator (a known concentration of thrombin-α_2-macroglobulin complex) is used to correct for the difference between the theoretical linear slope of the substrate consumption graph and the observed nonlinear rate of fluorescence growth, which starts to decrease at higher fluorescence levels as a result of substrate consumption and IFE (see Figure 1). In addition, the CAT approach also applies correction of the fluorescent signal for the internal (ie, derived from the data in the same microplate assay run) calibrator, which may also indirectly contribute to detection of procoagulant samples. CAT software does not permit the user to deactivate either of these corrections and calibrations, making it hard to compare the effects of nonlinear corrections on the TG curve.

The potential impact of substrate depletion and IFE on the clinically important ability of TG assays to separate procoagulant patients from those within the normal range has not been investigated. Chandler and Roshal[13] concluded that correction is not needed after finding essentially similar TG results in two commercial TG systems, with and without the correction algorithm, but their study was limited to normal donor samples. In a more recent report, Giesen et al[12] described that a nearly complete substrate depletion accounted for improbable or unreliable TG measurements in conditions of low antithrombin in people with hemophilia treated with fitusiran, an N-acetylgalactosamine–conjugated small interfering RNA designed to target antithrombin expression.

Since substrate consumption and IFE correction is aimed to address underreporting of high TG activity, we investigated whether these can indeed reduce the detection of known procoagulant plasma conditions. To demonstrate that the observed results are not

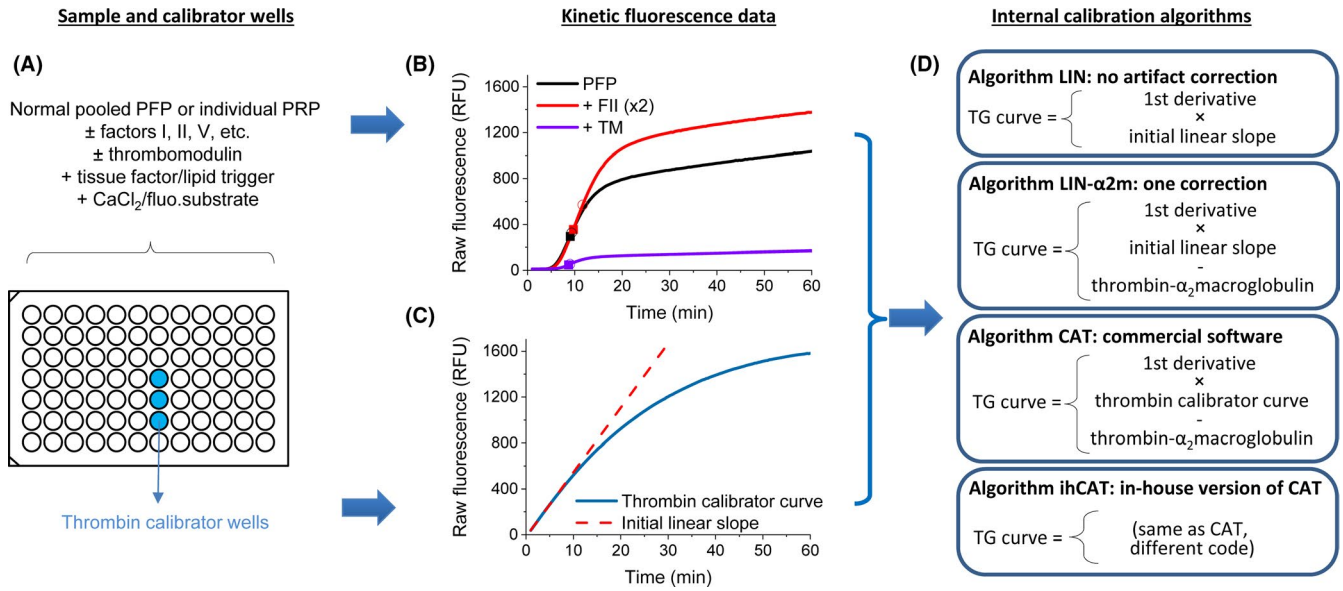

FIGURE 1 Schematic of experimental setup and algorithm analysis. Data from Machlus et al.[16] was reanalyzed by four algorithms indicated here. (A) Experimental conditions of sample and calibrator wells from which data was generated. B and C describe kinetic fluorescence data: (B) Plasma sample wells. Squares and circles indicate the time and fluorescence at peak thrombin as calculated without and with correction algorithms, respectively. (C) Thrombin calibrator wells. Solid curve indicates the fluorescence response of the calibrator wells; the dashed line models the "idealized" response in the absence of substrate consumption and IFE. (D) Internal calibration algorithms used in this study to generate TG curves. For Algorithm LIN (ie, linear calibration method without artifact correction), the calibrator concentration was divided by the slope of this dashed line, taken from the first 6 minutes of the curve or less if there were conspicuous anomalies near the end of that time frame, and this quotient was multiplied by the samples' changes in fluorescence to translate them into units of thrombin activity. Algorithm LIN-α2m was derived similarly to Algorithm LIN (ie, the first derivative was multiplied by the initial linear slope), while also subtracting for thrombin-α2-macroglobulin values. For Algorithm CAT (ie, commercial CAT algorithm), the first derivative of the fluorescence curve was multiplied by the thrombin calibration curve with thrombin-α2-macroglobulin values subtracted. Finally, Algorithm ihCAT (ie, our in-house version of commercial CAT) was calculated similarly to CAT software. CAT, calibrated automated thrombogram; IFE, inner filter effect; PFP, platelet-free plasma; PRP, platelet-rich plasma; TG, thrombin generation

affected by fluorescence artifacts such as plasma fluorescence, we previously developed an internal linear calibration software[15] that retains correction for plasma optical properties via internal calibration but does not correct for substrate consumption and IFE. We therefore applied this linear software along with commercial CAT and an in-house algorithm, similar to CAT, to analyze TG with and without correction, to a data set from procoagulant samples created by supplementing normal plasma with elevated levels of coagulation factors. The data presented here suggest that correction is needed in rare cases where an increased TG causes substantial substrate consumption, for example, in prothrombin supplemented plasma. In these samples with elevated prothrombin conversion, TG without CAT corrections was underestimated as evidenced by the high fluorescence levels consistent with nearly complete substrate depletion.

2 | MATERIALS AND METHODS

2.1 | TG experiments with elevated coagulation factors

This study is the extension of the previous computational work comparing algorithms for TG assay analysis.[15] Both studies are based on a reanalysis of the raw experimental data generated in TG experiments performed previously.[16] Briefly, normal pooled platelet-free plasma (PFP) or single-donor platelet-rich plasma (PRP) was spiked with 100% (to make the final level of 2× the normal) or 300% (4× the normal level) of blood coagulation factors V, VIII, IX, X, XI, I (fibrinogen) and II (prothrombin) to generate a range of normal to highly procoagulant samples for TG analysis described in Machlus et al.[16] Essentially, all PFP experiments used a single pool of frozen normal plasma, while PRP experiments used fresh samples from six separate healthy individuals.[16] The experiments were performed in the presence or absence of soluble thrombomodulin (TM, 5 nM), an endothelial receptor needed to activate the protein C–dependent inhibition pathway, at a concentration sufficient to lower the procoagulant potential of plasma samples. Coagulation in PFP was triggered with 4 μM procoagulant lipids (PLs) and 1 or 5 pM tissue factor (TF), via PPP-Reagent LOW or PPP-Reagent (Stago), respectively. Coagulation activation in PRP was triggered with 1 pM TF and no PL (PRP-Reagent, Stago).

2.2 | TG analysis software packages

TG parameters were calculated using both the CAT-associated Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, the Netherlands) and an in-house software based on

Origin (OriginLab, Northampton, MA, USA; the package is available from us upon request). The in-house software, which was previously described,[15,17] can use the internal calibration in two ways: without (ie, linear calibration) or with the nonlinear calibration (ie, CAT approach) as published[9,10,18,19] and detailed below. Note that our software is not a direct replica of CAT, as it calculates several steps slightly differently than the CAT's software. For example, CAT determines the end of each TG curve and calculates the endogenous thrombin potential (ETP) as an area under the curve (AUC) up to that time, whereas our software calculates the ETP as an AUC for the entire length of the read; CAT aligns replicate TG curves before averaging to match the beginnings of the lag time, whereas our software does not perform this alignment; and CAT is using a complex function for fitting of the nonlinear calibration curve, whereas our software is using a polynomial fitting.

2.3 | Internal thrombin calibration

Normal plasma was mixed with blood coagulation factors to obtain a range of elevated TG potentials in the presence and absence of TM as shown in Figure 1A. The fastest fluorescence increase was observed in the samples with 2× and 4× normal level of prothrombin, whereas the lowest was observed in TM-treated normal plasma (Figure 1B). Individual microplate experiments were internally calibrated against a well in which donor plasma was supplemented with Stago's thrombin calibrator, which served as the source of fixed substrate-cleaving activity. Other commercially available TG kits do not use internal thrombin calibrators, opting to calibrate thrombin activity in a separate experiment. To separate the effect of internal calibration from that of substrate artifact correction in this study, internal thrombin calibrator readings were used to calibrate TG experiments, regardless of whether the algorithm for correction of substrate consumption and IFE was included or not.

For substrate consumption and IFE correction, a thrombin calibrator curve-fitting approach was used in which a plot of fluorescence versus the first derivative time of change in fluorescence was fitted to a cubic polynomial; these coefficients were used to translate fluorescence changes into thrombin concentration units essentially as described in the CAT's internally calibrated TG approach (Figure 1C). Specifically,

$$Thrombin_{calibrated}(t) = \frac{Calibrator_0}{a \times RFU(t)^3 + b \times RFU(t)^2 + c \times RFU(t) + d} \times \frac{\partial RFU(t)}{\partial t}$$

where *RFU (t)* is fluorescence signal in relative fluorescence units (RFU) in the sample well as a function of time; $\frac{\partial RFU(t)}{\partial t}$ is rate of substrate consumption in [RFU/min]; *a*, *b*, *c*, and *d* are cubic polynomial fitting parameters of the graph of substrate consumption rate $\frac{\partial RFU(t)}{\partial t}$ vs. fluorescence level in the thrombin calibrator well; and $Calibrator_0$ is the concentration of thrombin calibrator in [nM] of thrombin. Variability due to irregular calibrator curves was outside the scope of our study, and calibrator wells were excluded if they deviated greatly from the two other replicates.

To apply an internal thrombin calibrator that does not account for IFE and substrate consumption, a linear slope of fluorescence over time was estimated using linear regression of the first 6 minutes of data in a calibrator well (Figure 1C), an approach that we developed specifically for this study. The following formula was used:

$$Thrombin_{calibrated}(t) = \frac{Calibrator_0}{k_{linear}} \times \frac{\partial RFU(t)}{\partial t}$$

where k_{linear} is the linear fitting coefficient of the thrombin calibrator well graph RFU (t) over the first 6 minutes.

2.4 | Algorithm nomenclature

We have thus analyzed the raw data set derived from Machlus et al,[16] with distinct algorithm methodologies labeled as follows: "Algorithm LIN" denotes internal linear calibration (ie, no corrections, described above); "Algorithm CAT" denotes commercial nonlinear and thrombin-α2-macroglobulin corrections as provided by the software supplied with the CAT instrument; and "Algorithm ihCAT" denotes our in-house implementation of CAT corrections (Figure 1D). In some analyses, we also used "Algorithm LIN-α2m"—an internal linear calibration with correction for the thrombin-α2-macroglobulin signal.

2.5 | Statistical analysis

Statistical analyses were performed in Origin Pro 2020 (OriginLab Corporation, Northampton, MA, USA). The mean and standard deviation are reported, where possible. The difference between the means was assessed with a two-sample *t* test at indicated significance levels (either 0.01 or 0.05). The results of analysis and the associated 95% confidence intervals (CIs) are summarized in the Supporting Information.

3 | RESULTS

3.1 | Effect of internal calibration on TG curve

In the simplest calibration algorithm, Algorithm LIN, which does not correct for substrate consumption and IFE, the initial slope of the calibrator's substrate consumption curve (Figure 1C) was used as a calibration coefficient to convert the rate of fluorescent signal increase (Figure 1B) into thrombin activity (nM of thrombin, Figure 2A-C). In contrast, Algorithm CAT is based on the observation that the rate of fluorescence increase is inversely proportional to the fluorescence level (Figure S1C). Therefore, CAT correction accounts for a lower substrate consumption rate for the same amount of thrombin when a fluorescent signal is high, unlike the linear calibration algorithm that assumes the same substrate consumption rate at any fluorescence level (Figure S1C, vertical dashed line). The corrections to the fluorescence levels (shaded gray area on Figure S1B)

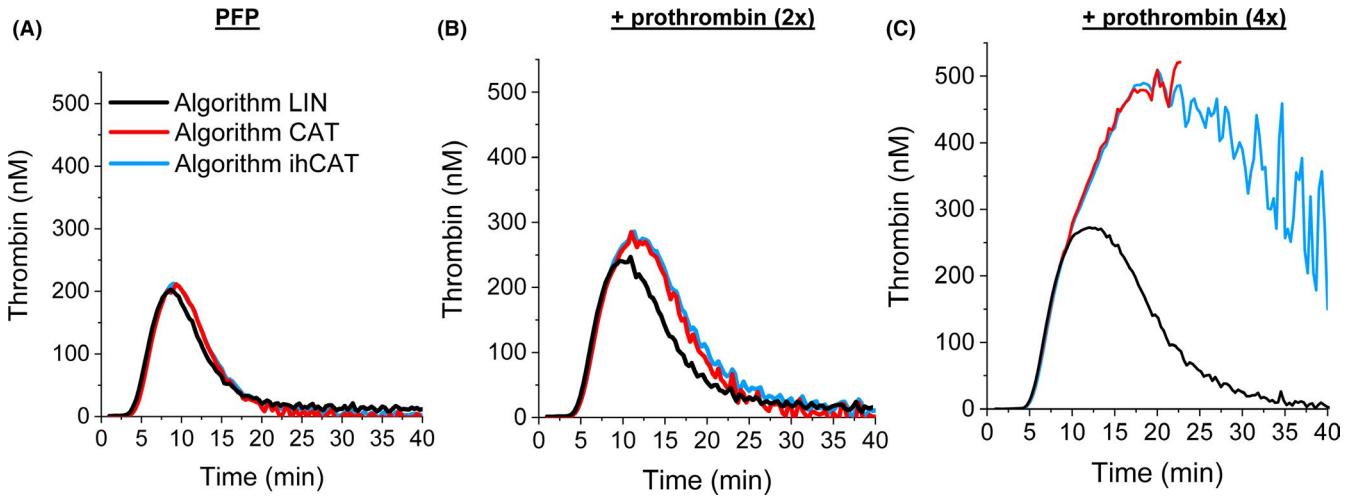

FIGURE 2 TG curves calculated by different algorithms for normal and procoagulant samples. Normal, 1 pM TF-treated PFP was treated with or without either 2× prothrombin or 4× prothrombin supplementation, and subsequently analyzed via three algorithms. (A) normal PFP (NPP), (B) procoagulant PFP (NPP +2× prothrombin), and (C) hypercoagulable PFP (NPP+4× prothrombin). Each plasma condition was subsequently analyzed via three algorithms: Algorithm LIN, Algorithm CAT, and Algorithm ihCAT. The curves shown are an average of three triplicates. A small difference in the tails of CAT and in-house curves was previously shown to be related to differences in tail averaging algorithms.[17] Under hyperprocoagulant conditions, both nonlinear correction algorithms upwardly adjust the thrombin peak in the same manner. CAT, calibrated automated thrombogram; PFP, platelet-free plasma; TG, thrombin generation

and the respective substrate consumption rates (shaded blue area in Figure S1C) are proportional to the fluorescence level (Figure S1A).

3.2 | Calibrated TG curves with and without correction algorithms

In the CAT experiments where coagulation in pooled PFP was triggered with 1 pM TF, Algorithm CAT had no visible effect on TG curves in normal plasma samples (Figure 2A; compare black and red curves). In the samples with 2× prothrombin, the TG curves were slightly taller after Algorithm CAT correction (Figure 2B, red curves). In the samples with 4× prothrombin, the most procoagulant condition, Algorithm CAT produced noticeable but also erratic, highly distorted curve tails (Figure 2C, red curves). When assessing Algorithm ihCAT, we observed TG curves similar to those generated by Algorithm CAT (compare the blue and red curves in Figure 2), confirming our implementation of this algorithm.

For the representative experiment in Figure 2, the relatively small effect of substrate consumption and IFE on TG curves was consistent with a small (<10%) difference between corrected and uncorrected curves at the fluorescence level of <600 RFU that was achieved at the peak of TG (i.e., at thrombin peak heights, which are indicated with symbols in Figure 1B, also see Figure S1).

3.3 | Effect of CAT correction on TG curve parameters in PFP

To compare TG after correction with both Algorithm CAT and Algorithm ihCAT, we used a series of 1 pM TF-triggered TG experiments in which blood coagulation factors were increased two- or fourfold in a single pool of PFP, with or without the addition of TM (n = 5-7 individual assay runs for either condition).

Only one group of samples was identified by the degree of thrombin peak height correction: prothrombin samples with uncharacteristically higher TG curves after correction (Figure 3A). All other samples were observed to have small corrections (see Tables S2 and S3). For the conditions excluding elevated prothrombin, the substrate consumption algorithm corrected the thrombin peak height up to −9.3% downward for samples with relatively low thrombin peak height (ie, samples with TM); and up to an 11% upward correction was observed for higher thrombin peak height samples (Figure 3C). Overall, the 95% CI of the mean thrombin peak height adjustment was −0.71% to 2.91%.

The area under the TG curve (ETP parameter) was affected by both Algorithm CAT and Algorithm ihCAT for all elevated prothrombin conditions (Figure 3B). Excluding elevated prothrombin conditions, the lowest AUCs were downwardly adjusted up to −53%, while the highest AUCs were adjusted slightly upward (not exceeding 7.5%) (Figure 3D). Similar results were obtained when CAT/ihCAT results were compared to the Algorithm LIN values corrected for the thrombin-α2-macroglobilin signal, that is, Algorithm LIN-α2m (Figure S2).

The prothrombin supplemented samples demonstrated slightly delayed lag time and time to thrombin peak parameters, which were extended even further by Algorithm CAT and Algorithm ihCAT (Figure S3 and Table S3).

Due to the extremely procoagulant condition of 2× and 4× prothrombin, Algorithm CAT and Algorithm ihCAT did not always compute all TG curve parameters (denoted as “failed-to-report” in Figure 3 and Figure S2 and Figure S3). For example, in 4× prothrombin

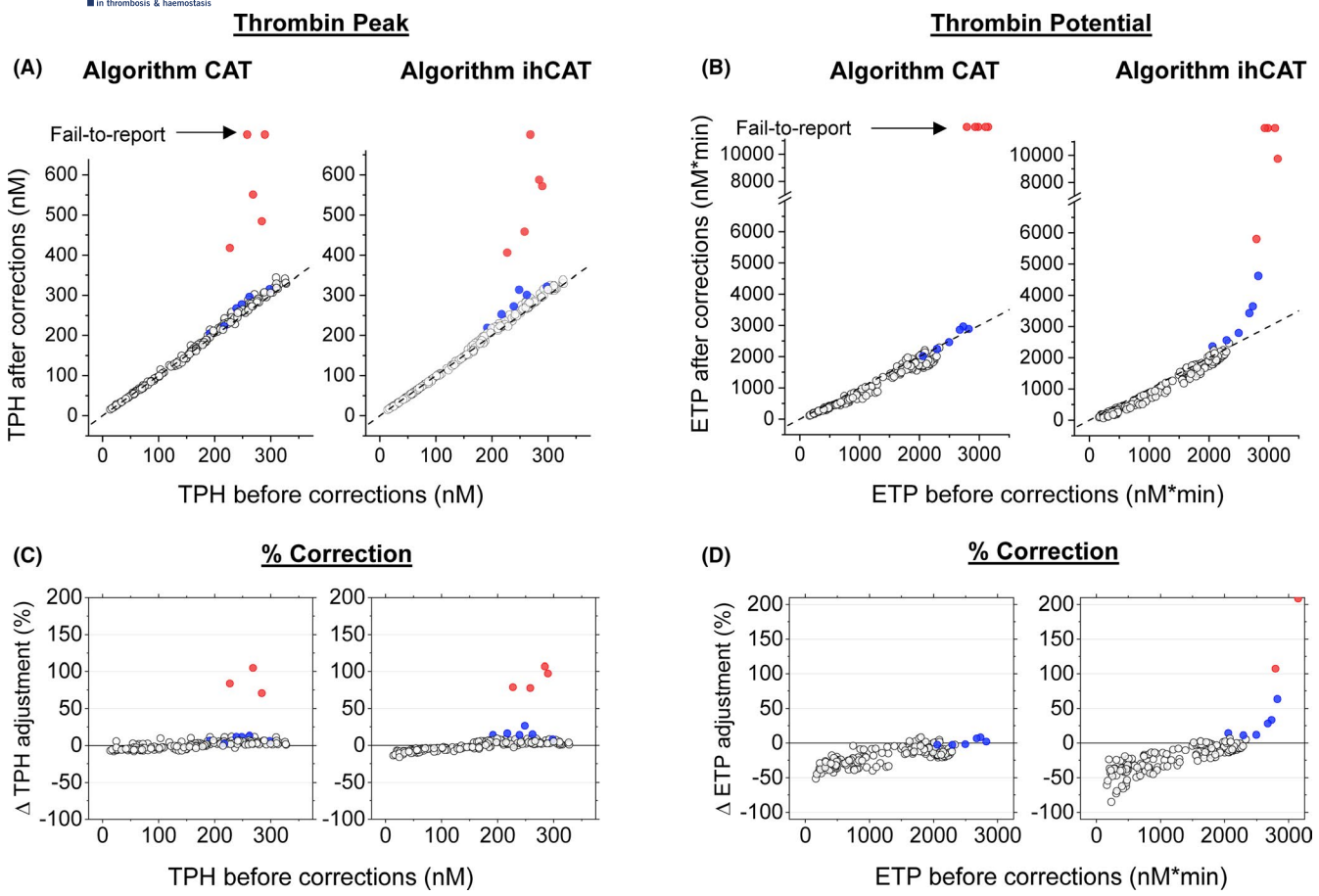

FIGURE 3 Effect of correction algorithms on thrombin peak height and ETP parameter values in PFP supplemented with coagulation factor zymogens (procoagulant condition) and/or TM (hypocoagulable condition). Each dot represents a single experimental condition (eg, NPP + 2× factor VIII + TM). Blue and red dots indicate 2× and 4× prothrombin, respectively. Each condition was repeated 6-8 times in independent experiments (n = 8) with 1 pM TF. Top row: Correlation between Algorithm LIN vs parameter values obtained by either Algorithm CAT or Algorithm ihCAT; bottom row: percent adjustment of parameter values relative to Algorithm LIN. (A and C) Thrombin peak height (TPH); (B and D) Endogenous thrombin potential (ETP). Samples where Algorithm CAT failed to return a value are plotted above the *y* axis in panels A and B. Statistical analysis (means, 95% confidence interval, and the difference between means and *P* value) of the three algorithms are tabulated in Tables S2 and S3. CAT, calibrated automated thrombogram; PFP, platelet-free plasma; TM, thrombomodulin

samples, Algorithm CAT was unable to calculate tails for all but one run of the assays performed, resulting in no ETP values calculated (Table S1).

3.4 | Effect of the correction algorithm on detecting coagulation factor elevation in PFP and PRP

Since elevated coagulation factors are expected to produce more TG, we investigated the effect of the consumption correction algorithm on TG as a function of added coagulation factor activity, separately for three assay conditions: pooled PFP triggered with 1 pM TF, individual PRP triggered with 1 pM TF, and pooled PFP triggered with 5 pM TF (see Figure 4). For all elevated coagulation factors except prothrombin, corrections resulted in downward and upward adjustments in the presence and absence of TM, respectively (Figure 4D-E). In comparing the average TG values, the thrombin peak height adjustment for the 1 pM TF condition was within 5.1% for PFP and within 8% for PRP. For PFP triggered with 5 pM TF, corrections were less than that of the 1 pM TF-triggered samples (similarly excluding samples with 2× or 4× prothrombin) (Figure 4F). Algorithm ihCAT gave similar results as Algorithm CAT in samples triggered with 1 pM TF; larger differences were observed in prothrombin samples triggered with 5 pM TF (compare blue and red squares).

In PFP and PRP samples with 2× or 4× prothrombin, the thrombin peak height was substantially upwardly adjusted by correction in the absence of TM (Figure 4; see red and blue curves). In the presence of TM, PFP samples triggered with 1 pM TF were not adjusted while PFP samples triggered with 5 pM TF and PRP samples at 1 pM TF were adjusted upwardly.

4 | DISCUSSION

Sensitivity to procoagulant conditions is an attractive feature of TG testing; however, this global hemostasis assay can theoretically be

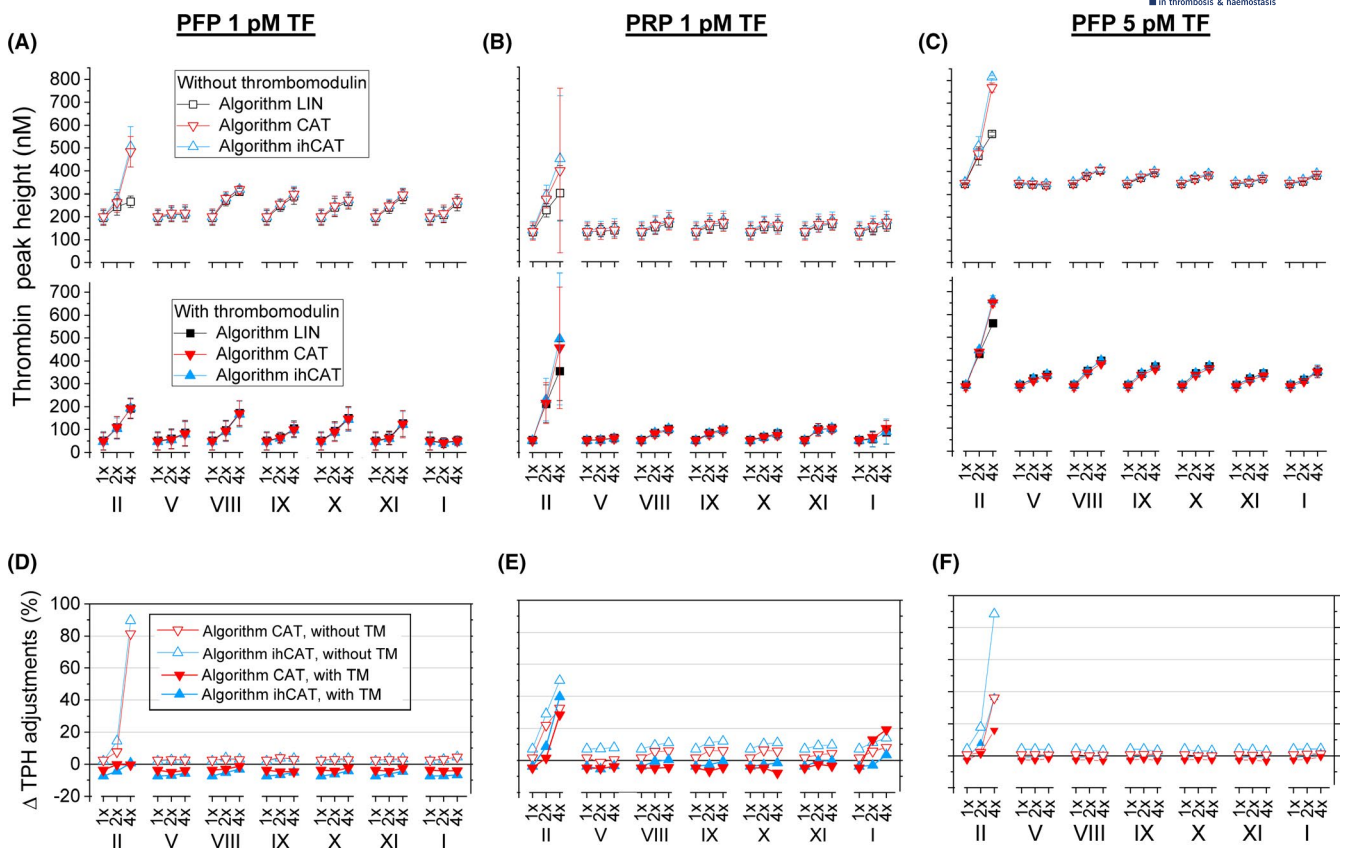

FIGURE 4 Effect of procoagulant factors II, V, VIII, IX, X, XI, and I on thrombin peak height in PFP and PRP. Black curves indicate Algorithm LIN; red curves indicate Algorithm CAT; blue curves indicate Algorithm ihCAT. Open symbols indicate the absence of added TM; filled symbols indicate the data with added TM. Factor concentrations indicated on the *x* axis are in terms of fold increase of their normal level. Top: thrombin peak heights (TPH); bottom: indicated corrections to TPH relative to Algorithm LIN correction. Left (A and D): pooled PFP triggered with 1 pM TF, where data are representative of n = 6 replicates; center (B and E): individual PRP triggered with 1 pM TF, where data are representative of n = 6 replicates; right (C and F): pooled PFP triggered with 5 pM TF, where data are representative of n = 2 replicates. CAT, calibrated automated thrombogram; IFE, inner filter effect; PFP, platelet-free plasma; PRP, platelet-rich plasma; TF, tissue factor; TM, thrombomodulin

biased by fluorogenic substrate artifacts such as substrate consumption and IFE. An elegant approach of simultaneous assay calibration and artifact correction centered around a proprietary internal thrombin calibrator reagent has been commercially available for >20 years, but, surprisingly, limited evidence has been available to demonstrate that the algorithm's corrections in procoagulant plasma samples are indeed helpful.[20-22] It is possible that such studies were hampered by the absence of convenient software tools. Commercial CAT software (denoted as Algorithm CAT in this study) does not generate calibrated TG data without applying the corrections; thus, it is not possible to compare the results with and without the substrate consumption and IFE correction algorithm. In this study, we used in-house software for the analysis of internal calibrator reagent data in two ways: (i) based on an in-house implementation of previously published CAT algorithms (denoted Algorithm ihCAT),[17] and (ii) based on a linear calibration analogous to all other commercially available and in-house TG assays (denoted Algorithm LIN). Algorithm CAT and Algorithm ihCAT showed comparable results; thus, we calculated the substrate consumption and IFE corrections as the difference between the in-house linearly calibrated and commercial CAT results. We observed that TG assays may indeed underreport thrombin activity, but only in extremely procoagulant samples with dramatic substrate consumption, for example, caused by elevated prothrombin. Although CAT analysis provided some correction of substrate consumption for less procoagulant conditions obtained via the addition of elevated factors other than prothrombin, this correction appeared small.

The unimportance of a correction algorithm for the majority of model samples in our study is explained by the minor substrate consumption at the point when TG curves reached peak heights. Corrected TG curves were within 10% of uncorrected TG curves at the moment of thrombin peak time for the majority of tested samples. Consequently, thrombin peak height was largely unaffected by correction. In contrast, ETP, which measures TG beyond the peak where substrate consumption is significant, is more upwardly adjusted by CAT correction the more procoagulant the sample is. These findings suggest that CAT correction is not needed for elevated coagulation factor VIII and XI conditions that are traditionally considered procoagulant. Similarly, Hemker and Kremers[23] previously suggested that the TG assay performance is not affected by the calculation

method but rather by the preanalytical treatment of the sample, that is, removing cell material when preparing plasma, and insufficient temperature control. However, Hemker and Kremers's conclusions about the calculation methods do not apply to severely procoagulant samples, such as those with elevated prothrombin (this work) and antithrombin deficiency.[12,15]

Here, artifacts of substrate consumption and IFE were critical for the elevated prothrombin samples. For example, thrombin peak height values in samples with 4× prothrombin overlapped with the thrombin peak height values in the remaining samples in our data set. CAT correction increased the peak height values almost two-fold. However, the ETP parameter correctly identified prothrombin samples even without the CAT correction. We ultimately found that detection of elevated prothrombin may be dependent on whether the software package is equipped with the algorithm to correct the IFE/substrate consumption, whereas other procoagulant samples in our study were forgiving to the lack of substrate artifact corrections. It should be noted that the quantitative outcome for adjusting TG curves upward by the commercial CAT software (Algorithm CAT) and our own implementation of the CAT algorithm (Algorithm ihCAT) for these hypercoagulable samples was not always quantifiable, analogous to a previous report in samples with reduced antithrombin.[12]

Our study is a limited model exercise since we used fluorescent data from normal plasma with added coagulation factors to simulate a broad range of coagulation potentials. To confirm our results on the limited corrective value of CAT algorithm in the majority of studied blood coagulation factors, a similar study should be conducted with samples from hypercoagulable patients, to establish relationships between clinical outcomes and TG corrections in a clinical laboratory. For commercial and in-house assays that do not use CAT's proprietary thrombin calibrator and algorithms, the area under the thrombin generation curve parameter rather than the thrombin peak height should be recommended as a readout.

ACKNOWLEDGMENTS

This paper is an informal communication and represents the authors' best judgment. These comments do not bind or obligate the US Food and Drug Administration.

RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

KRM and ASW provided the experimental fluorogenic TG data. WCC and MVO conducted the analysis of experimental data and compared correction algorithms. MVO wrote the analytical software. WCC and MVO wrote the manuscript with assistance from JWJ and ASW. MVO supervised the project and preparation of the manuscript.

ORCID

Joseph W. Jackson https://orcid.org/0000-0002-3826-2749
Kellie R. Machlus https://orcid.org/0000-0002-2155-1050
Alisa S. Wolberg https://orcid.org/0000-0002-2845-2303
Mikhail V. Ovanesov https://orcid.org/0000-0003-2509-1781

TWITTER

Kellie R. Machlus @theclotthickens
Alisa S. Wolberg @aswolberg

REFERENCES

1. Hillarp A, Baghaei F, Fagerberg Blixter I, et al. Effects of the oral, direct factor Xa inhibitor rivaroxaban on commonly used coagulation assays. *J Thromb Haemost*. 2011;9(1):133-139.
2. Kadauke S, Khor B, Van Cott EM. Activated protein C resistance testing for factor V Leiden. *Am J Hematol*. 2014;89(12):1147-1150.
3. Lippi G, Pasalic L, Favaloro EJ. Detection of mild inherited disorders of blood coagulation: current options and personal recommendations. Expert. *Rev Hematol*. 2015;1-16.
4. Lison S, Spannagl M. Monitoring of direct anticoagulants. *Wien Med Wochenschr*. 2011;161(3–4):58-62.
5. Qi X, Zhao Y, Li K, Fan L, Hua B. Evaluating and monitoring the efficacy of recombinant activated factor VIIa in patients with haemophilia and inhibitors. *Blood Coagul Fibrinolysis*. 2014;25(7):754-760.
6. Brummel-Ziedins KE, Branda RF, Butenas S, Mann KG. Discordant fibrin formation in hemophilia. *J Thromb Haemost*. 2009;7(5):825-832.
7. Rand MD, Lock JB, van't Veer C, Gaffney DP, Mann KG. Blood clotting in minimally altered whole blood. *Blood*. 1996;88(9):3432-3445.
8. Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost*. 2003;1(7):1504-1514.
9. Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003;33(1):4-15.
10. Hemker HC, Giesen P, AlDieri R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb*. 2002;32(5–6):249-253.
11. Ramjee MK. The use of fluorogenic substrates to monitor thrombin generation for the analysis of plasma and whole blood coagulation. *Anal Biochem*. 2000;277(1):11-18.
12. Giesen P, van Oerle R, Sehgal A, Akinc A, Spronk HMH. Measurement of Thrombin Generation in Conditions of Low Antithrombin. Abstract PB 1420. XXVI Congress of the International Society on Thrombosis and Haemostasis. *Res Pract Thromb Haemost*. 2017;1(Suppl. 1):570-571.
13. Chandler WL, Roshal M. Optimization of plasma fluorogenic thrombin-generation assays. *Am J Clin Pathol*. 2009;132(2):169-179.
14. Kintigh J, Monagle P, Ignjatovic V. A review of commercially available thrombin generation assays. *Res Pract Thromb Haemost*. 2018;2(1):42-48.
15. Chang WC, Jackson JW, Machlus KR, Wolberg AS, Ovanesov MV. Comparative analysis of thrombin calibration algorithms and correction for thrombin-alpha2macroglobulin activity. *J Clin Med*. 2020;9(10).
16. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg AS. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. *Thromb Haemost*. 2009;102(5):936-944.
17. Woodle SA, Shibeko AM, Lee TK, Ovanesov MV. Determining the impact of instrument variation and automated software algorithms on the TGT in hemophilia and normalized plasma. *Thromb Res*. 2013;132(3):374-380.
18. Hemker HC, Beguin S. Thrombin generation in plasma: its assessment via the endogenous thrombin potential. *Thromb Haemost*. 1995;74(1):134-138.
19. Hemker HC, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma

independent of thrombin decay processes. *Thromb Haemost*. 1986;56(1):9-17.

20. Castoldi E, Simioni P, Tormene D, et al. Differential effects of high prothrombin levels on thrombin generation depending on the cause of the hyperprothrombinemia. *J Thromb Haemost*. 2007;5(5):971-979.
21. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. *JAMA*. 2006;296(4):397-402.
22. Tripodi A, Martinelli I, Chantarangkul V, Battaglioli T, Clerici M, Mannucci PM. The endogenous thrombin potential and the risk of venous thromboembolism. *Thromb Res*. 2007;121(3):353-359.
23. Hemker HC, Kremers R. Data management in thrombin generation. *Thromb Res*. 2013;131(1):3-11.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Chang WC, Jackson JW, Machlus KR, Wolberg AS, Ovanesov MV. Fluorescence artifact correction in the thrombin generation assay: Necessity for correction algorithms in procoagulant samples. *Res Pract Thromb Haemost*. 2021;5:447–455. https://doi.org/10.1002/rth2.12499