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Review Article

A review of global coagulation assays — Is there a role in thrombosis risk prediction?

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ABSTRACT

Normal haemostasis requires maintenance of a careful equilibrium between the necessity to clot when bleeding and the retention of fluid phase at all other times. Disruption of this equilibrium can result in catastrophic outcomes, e.g. acute myocardial infarction and pulmonary embolism. However, despite the significant therapeutic advances in cardiovascular medicine over recent years, our ability to provide an accurate cardiovascular risk assessment remains an unmet need. Routine coagulation testing is not a useful reflection of haemostasis and cannot be reliably used to predict bleeding and thrombosis risks. Global coagulation assays such as viscoelastic testing, thrombin and fibrin generation have been proposed as better measures of the haemostatic function. These assays, particularly viscoelastic testing, have been increasingly used to assess bleeding risks and guide blood product replacement in trauma and massive transfusion settings. However, the role of these assays in thrombosis is less well-defined but given the complexities of the coagulation system, these global coagulation assays when used in combination may provide a better assessment of cardiovascular and thrombosis risk at an individual level. Hence, we explore the role of some of the currently available global coagulation assays – the viscoelastic, thrombin generation and fibrin generation tests – and provide a review of the literature of the current evidence for these assays specifically in the field of venous thromboembolism and cardiovascular diseases.

1. Introduction

Normal haemostasis requires the careful maintenance of equilibrium between the necessity to clot when bleeding and the retention of fluid phase at other times. In-vivo haemostasis is achieved by a balance between platelet adhesion and aggregation on vascular endothelium, activation of coagulation cascade and fibrinolytic as well as anticoagulant mechanisms. Disruption of this delicate equilibrium may result in the pathological states of thrombosis or bleeding [1]. The burden of this disequilibrium globally is significant; ischemic heart disease and stroke, accounted for 15.2 million deaths in 2016 and are leading causes of global morbidity, mortality and health expenditure [2]. The interplay between known cardiovascular risks (e.g. dyslipidaemia, smoking and hypertension) probably dictates the development of the pathological thrombosis but there are no readily available biomarkers that quantify the risk of thrombosis. One explanation may be that cardiovascular disease (CVD) and venous thromboembolism (VTE) often have multifactorial haemostatic contributors, hence the risk-stratification may require a combination of biomarker with the ability to globally integrate them all.

Our routine coagulation tests such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) were developed primarily to monitor anticoagulants such as warfarin and heparin respectively, and are not useful for predicting the bleeding, thrombotic or cardiovascular risk. These tests only evaluate the time to the start of clot formation; however 95% of thrombin generation occurs subsequent to

Abbreviations: CAT, calibrated automated thrombogram; CI, confidence interval; CVD, cardiovascular disease; DOAC, direct oral anticoagulants; ETP, endogenous thrombin potential; HR, hazard ratio; OCP, overall coagulation potential; OFP, overall fibrinolytic potential; OHP, overall haemostatic potential; ROTEM, rotational thromboelastometry; TEG, thromboelastography; VTE, venous thromboembolism

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this step [3]. Logically, evaluation of total clot formation and lysis, may provide a better assessment in-vivo coagulation status and hence thrombotic and bleeding risk. Therefore, a more global assessment of the coagulation system, including the final products of the coagulation cascade, such as fibrin and thrombin, is required. Global coagulation assays, such as thromboelastography and thrombin generation, have been proposed as better measures of haemostatic function.

An ideal haemostasis assessment should be reliable, reproducible, performed easily and rapidly and demonstrate correlation with risks of bleeding or thrombosis. Preceding attempts to develop global assays in the 1940s and 1950s were limited by lack of accessibility and operator reproducibility as well as being too time intensive [4]. However, recent technological advancements, particularly techniques in the automation and standardisation of global assays, have led to the resurrection of interest in these assays, with increasing acceptance in clinical applications. Thromboelastography is now increasingly being used as a point-of-care device to guide blood product replacement in bleeding and trauma [5,6]. However, the role of these assays in thrombotic situations has not been widely evaluated and may be a more holistic predictor of subsequent thrombotic risk.

Herein, we describe some of the currently available global coagulation assays: viscoelastic assessment, and quantification of thrombin using calibrated automated thrombogram (CAT) as well as fibrin generation using overall haemostatic potential (OHP), specifically in thrombotic situations such as CVD and VTE.

2. The principle of global coagulation assays

2.1. Thrombin generation using calibrated automated thrombogram (CAT)

Thrombin is one of the key components of the haemostatic system [7], contributing not only to clot formation through the conversion of fibrinogen to fibrin, but also through direct and indirect feedback on blood and the vessel wall, such as the activation of thrombomodulin, a co-factor in the activation of the protein C anticoagulant pathways [8]. The concept of total thrombin measurement was first introduced in the 1950s by MacFarlane & Biggs (in whole blood) and Pitney & Dacie (in plasma) respectively [9,10] although it was not until many years later that Hemker et al. pioneered the CAT methodology [3]. Thrombin generation via CAT uses a fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator to determine the rate and extent of thrombin generated after the addition of a tissue factor stimulus. This reduces the issue of “inner filter effect”, where fluorescent molecules absorb the light from other product molecules [3], one of the main problems faced by earlier versions of thrombin generation. In addition to correction for the inner filter effect, the calibrator also corrects for the colour of the plasma, substrate depletion and quantification of the fluorescent signal. Thrombin generation can be performed on platelet-poor plasma to detect coagulation factor deficiencies and evaluate effect of anticoagulants, or on platelet-rich plasma to investigate platelet defects and effect of antiplatelet drugs. However, there is lack of standardisation between laboratories with some generating their own regents resulting in inter-laboratory variability the tissue factor and phospholipid concentration as well as addition of thrombomodulin. Loeffen et al. had analysed different pre-analytic variables and validated the CAT method as well as highlighted the need for an international standardised protocol [11]. Of note, the International Society of Thrombosis and Haemostasis (ISTH) have recently made some recommendations for the standardisation of the measurement of thrombin generation in haemophilia using CAT [12]. There are also current efforts to provide an automated standardised method for thrombin generation with an example of this being the ST-Genesiia analyser (Diagnostica Stago, France). Fig. 1 shows the classic thrombin generation curve in which a series of parameters can be calculated.

Of note, CAT measures the capacity of plasma to form thrombin (ex-vivo thrombin generation) and is an indicator of the potential function of the haemostatic process as well as reflection of bleeding and clotting risk [13]. This is different to in-vivo thrombin generation which measures thrombin formation in the body at a particular timepoint and reflects current ongoing pathology, as measured using prothrombin fragment 1 + 2 (F1 + 2), a polypeptide released from the prothrombin during its activation to thrombin by the prothrombinase complex, as well as thrombin-antithrombin (TAT) complexes [13]. This literature review focuses on ex-vivo global coagulation assays, as the challenge clinically remains to predict future events.

2.2. Viscoelastic tests

The first viscoelastic testing, known as thromboelastography, was reported in 1948 by Hartert [14]. Thromboelastography utilises mechanical rotation to detect global dynamics of clot formation, stabilisation and dissolution, which is thought to reflect in-vivo haemostasis [15,16]. This method was originally criticised to be “too global” and cumbersome, which prevented the acceptance of this test for routine use [4]. Since then, there have been significant advancements in automation as well as the development of new trigger reagents which have led to the broadening of the clinical utility of this test.

The two most common commercial viscoelastic testing devices are thromboelastography (TEG®, Haemonetics®, United States) and rotational thromboelastometry (ROTEM®, Haemoview Diagnostics, Australia) (Fig. 2A & B). The TEG®5000 assesses whole blood clot formation via a pin suspended at 4°45′ in an oscillating cup, heated to 37 °C. As the clot forms, the fibrin strands increase the torsion around the wire, and subsequently as fibrinolysis evolves, these strands break down, thus reducing the torque on the torsion wire. These changes in torque are then transmitted through a detector which assesses the quantitative parameters of the sample to produce a graphical representation as seen in Fig. 2A. In the case of ROTEM®, it is the plastic pin rather than the cup that moves. Fig. 2B illustrates the key parameters in ROTEM®. While TEG®5000 and ROTEM® are similar in their methodology, their results are not interchangeable and ROTEM is less susceptible to movement and vibration [17–19]. The differences in the results can be attributable to the type of blood used (native whole blood vs citrated re-calcified blood) [20,21] and the different types and concentration of activators used to trigger the coagulation reaction [22]. However, the emergence of the new TEG®6s technology which measures clot viscoelasticity using a resonance method has been associated with greater ease of use with less susceptibility to vibration compared to its predecessor, however, again the transability of these results to other viscoelastic testing is unclear.

2.3. Fibrin generation using overall haemostatic potential assay (OHP)

The overall haemostatic potential assay (OHP) is based on a fibrin-aggregation curve made with the analysis of citrated plasma samples and utilises serial spectrophotometric registration to calculate fibrin generation and fibrinolysis. This is achieved via assessing two samples in parallel - one with thrombin only (to calculate the overall coagulation potential (OCP)) to drive the catalytic reaction that converts fibrinogen to fibrin, and the second with addition of tissue plasminogen activator (to calculate the OHP) to activate the plasminogen to produce plasmin, which consecutively digests the fibrin. The difference in these curves culminates in the overall fibrinolytic potential (OFP) (Fig. 3) [23].

3. Global coagulation assays in cardiovascular disease and venous thrombosis

While viscoelastic tests are increasingly used in the clinical management of bleeding patients, there are emerging potential applications of these assays in thrombotic and cardiovascular diseases. An

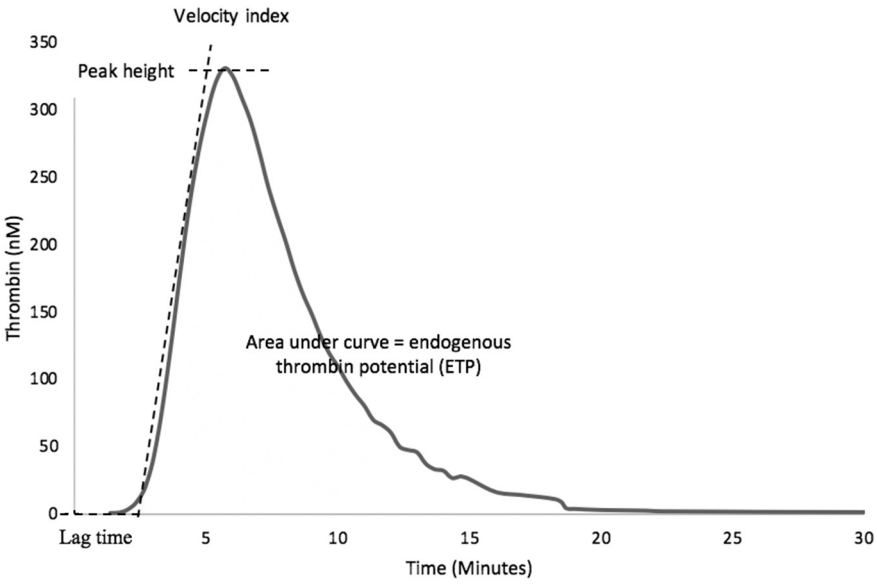

Fig. 1. An example of an automated thrombogram measured using CAT. The parameters are lag time (time required to reach 10 nM thrombin or one-sixth of the peak height), peak height, velocity index (maximum slope of the initial part of the curve) and endogenous thrombin potential (ETP, total amount of thrombin i.e. area under the curve).

interesting contradiction between bleeding and thrombotic state is in liver cirrhosis in which patients can experience thrombotic complications despite seemingly "hypocoagulable" parameters on conventional testing, particularly thrombocytopenia and prolonged prothrombin time. In contrast, thrombin generation parameters have previously been shown to be consistent with a hypercoagulable profile instead, confirming that INR or PT should not be used to assess coagulopathy in these patients [24]. This is postulated to be due to loss of anticoagulants such as antithrombin and protein C and may explain why patients with liver dysfunction can have thrombotic complications despite having a prolonged prothrombin time [25]. Similarly, a randomized controlled trial demonstrated that TEG-guided transfusion strategy in cirrhotic patients with coagulopathy led to significantly lower use of blood products compared to standard of care (transfusion guided by INR and platelet count), without an increase in bleeding complications [26].

3.1. Venous thrombosis

Thrombin generation has also been shown to not only detect prothrombotic phenotype but also predict recurrent VTE risks (Table 1) [27–29]. Some studies have demonstrated that thrombin generation measured in the presence of thrombomodulin allows for better appreciation of risk of recurrent VTE compared to measurement performed in the absence of thrombomodulin [29]. In one study, patients with first unprovoked VTE and raised thrombin generation parameters (ETP or peak) in the presence of thrombomodulin a month after discontinuation of treatment with vitamin K antagonists had greater risk of recurrence (ETP – Hazard ratio (HR) 3.41; peak – HR 4.57) [30]. Another study evaluating thrombin generation together with D-dimer 3 weeks post anticoagulation cessation, demonstrated that a raised ETP, in addition to a raised D-dimer, predicted patients with the highest risk of recurrent thrombosis (2.8 fold; 95% confidence interval (CI) 1.5–5.3) [31]. The Vienna Cancer and Thrombosis study reported that an elevated peak thrombin in their cohort of 1033 cancer patients showed increased risk of VTE (HR 2.1; 95% CI 1.3–3.3) [32]. Table 1 provides a summary of the key studies evaluating thrombin generation and viscoelastic testing in VTE.

Another important role of thrombin generation in VTE is its potential role in evaluating the therapeutic efficacy of direct oral anticoagulants (DOAC). DOAC have revolutionised anticoagulation management, primarily because of their stable pharmacokinetics that obviate the need for regular monitoring. However, whilst drug levels can be measured, there currently remains no assay that can measure in-vivo anticoagulant effect of these drugs. Previous studies have demonstrated that the anticoagulant effect of DOAC can be measured using thrombin generation [41,42] and may help to predict the balance between safe anticoagulant effect and proportional decrease of thrombotic risk. A small in-vitro study has demonstrated significant inter-individual variability despite spiking with fixed dose of DOAC, suggesting that individual responses to DOAC may be different, and could explain failure of DOAC therapy in some patients [41].

The role of thromboelastography in venous thrombosis is less clear with some studies showing no difference in thromboelastography parameters between normal controls and patients with VTE [36,37] while one study of trauma patients demonstrated that the rate of DVT doubled in patients with hypercoagulable TEG indices, despite thromboprophylaxis [38]. It has also been demonstrated that while ROTEM parameters were significantly more hypercoagulable in lung cancer patients, there were no differences seen between patients who developed venous thromboembolic complications compared to those who did not. However, it should be noted that the former group only contained six patients [39].

The OHP assay has not been widely studied in thrombosis. Curnow et al. compared 161 clinically hypercoagulable patients with arterial or VTE, pregnancy complications or autoimmune disease (including 90 patients with antiphospholipid antibodies) to 98 normal controls and found increased fibrin generation and reduced fibrinolysis in the hypercoagulable patients [43].

3.2. Cardiovascular disease and stroke (acute events)

The role of thrombin generation in pathogenesis of cardiovascular disease is less clear, with mixed data seen in both acute cardiovascular events and chronic cardiovascular diseases. While many studies have shown that thrombin generation is increased during acute coronary syndromes [44–47], the data is more variable in patients with acute ischemic strokes. A prospective cohort study of 205 stroke patients found that peak thrombin concentrations were variable among stroke patients but overall higher while another study, using similar platelet-poor plasma and 5pM tissue factor with 4 μM of phospholipids, reported that ETP and peak thrombin were not useful to define hypercoagulability in patients with acute ischemic stroke [48,49].

Viscoelastic testing has been previously also explored in cardiovascular disease and stroke in a number of small observational studies. A prospective study investigating the utility of TEG® and outcomes in ischemic strokes showed that higher maximum amplitude is associated

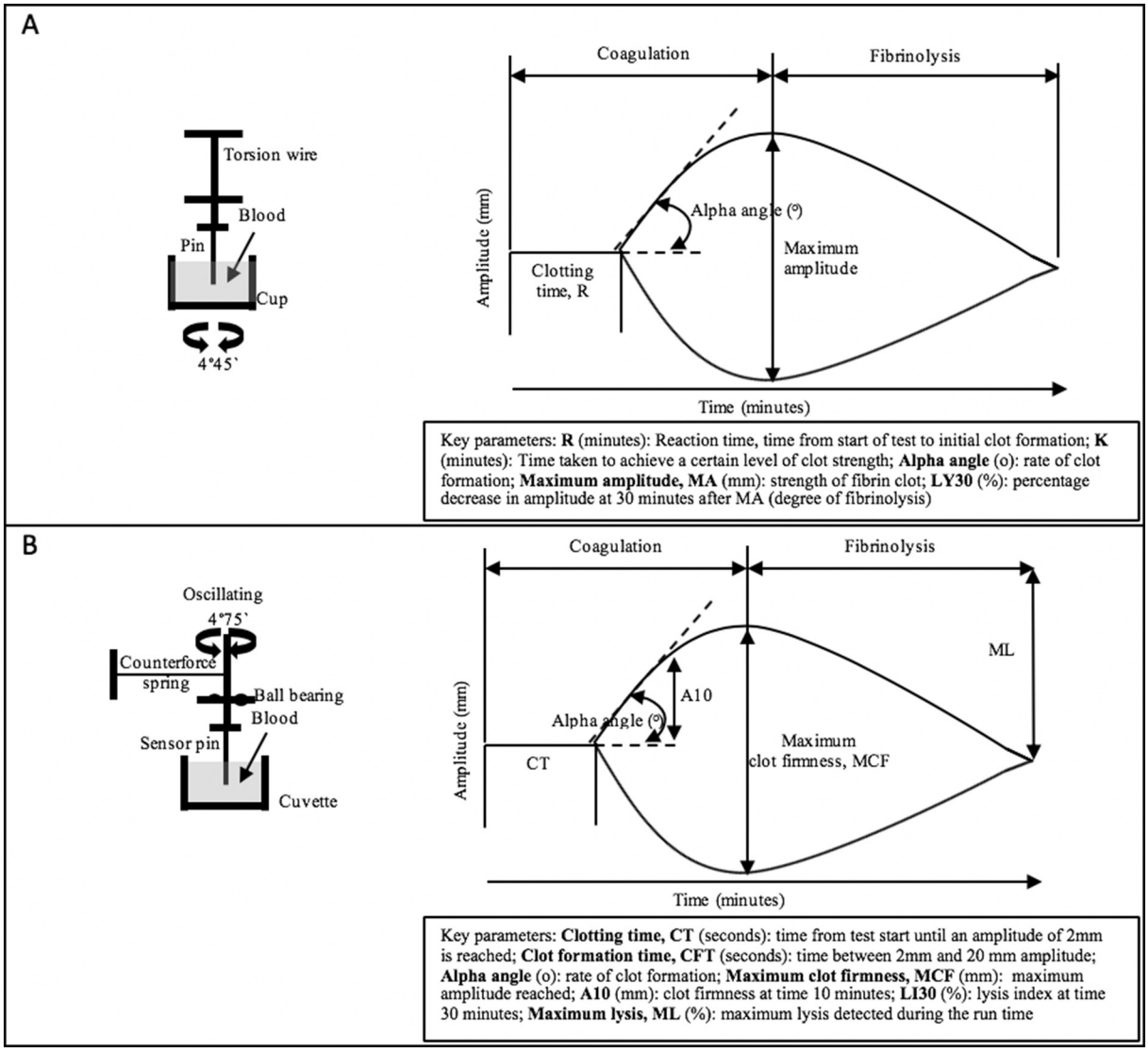

Fig. 2. The principles of TEG® and ROTEM® are as illustrated in panels A and B respectively alongside their corresponding graphical outputs. The key parameters measured by these technologies include: the time to initial fibrin formation (reaction time (TEG®); clotting time [CT] (ROTEM®)); the kinetics of fibrin formation and clot development (alpha angle (TEG® and ROTEM®)); the maximal strength and stability of the fibrin clot (maximum amplitude [MA] (TEG®); maximum clot firmness [MCF] (ROTEM®)); and the fibrinolysis phase (LY30 or percentage decrease in amplitude at 30 min post maximum amplitude (TEG®); clot lysis [CL] (ROTEM®).

with more severe strokes, longer hospital stays, and poorer one-year functional outcome post stroke (odds ratio 1.192, $p = .022$) although there was no significant difference seen in the one-year cumulative first ischemic event recurrence [50]. Another prospective observational study of 35 consecutive patients presenting to hospital with acute thrombotic complications including myocardial infarction and acute ischemic stroke showed that ROTEM may be a reliable method to detect hypercoagulable states [51], and other studies have shown that hypercoagulability identified by TEG or ROTEM prior to surgeries including cardiac surgery correlated to increased risk of postoperative arterial and/or venous thromboembolic complications [40,52]. There have been a number of small studies that have evaluated the efficacy of antiplatelet therapy in patients with acute myocardial infarction and preliminary data has shown the ability of ROTEM to identify changes in haemostasis in patients on antithrombotic therapy, although how these changes may affect outcomes remain uncertain [53].

However, the global coagulation assay results obtained during acute thrombotic events should be interpreted with caution. Acute events such as acute myocardial infarction are associated with profound systemic inflammatory response such as elevation of cytokines and chemokines as well as leucocytes and platelets in addition to local inflammation [54]. Thrombin also plays a key role in inflammatory diseases and hence the proinflammatory response following an acute thrombotic event may confound the results, making it difficult to delineate the effect of the acute cardiovascular event versus systemic inflammatory response.

Moreover, there has been recent interest in the reassessment of the mechanisms of acute coronary syndrome particularly with improvement in the management of traditional risk factors. Libby and Pasterkamp et al. have described the different characteristics between superficial plaque erosion and plaque rupture [55], of which both can contribute to acute coronary syndromes and hence, may have profound clinical implications and impact on global coagulation assays measured in the acute setting. In addition, while endothelium is essential to mediate haemostasis and prevent thrombosis, the endothelial contribution to acute thrombus formation is not covered by global coagulation assays. To add to the complexity, each vascular bed has unique structural and functional properties with site-specific properties in balancing homeostasis and focal vascular pathology [56,57], making assessment of endothelial function another whole field of study.

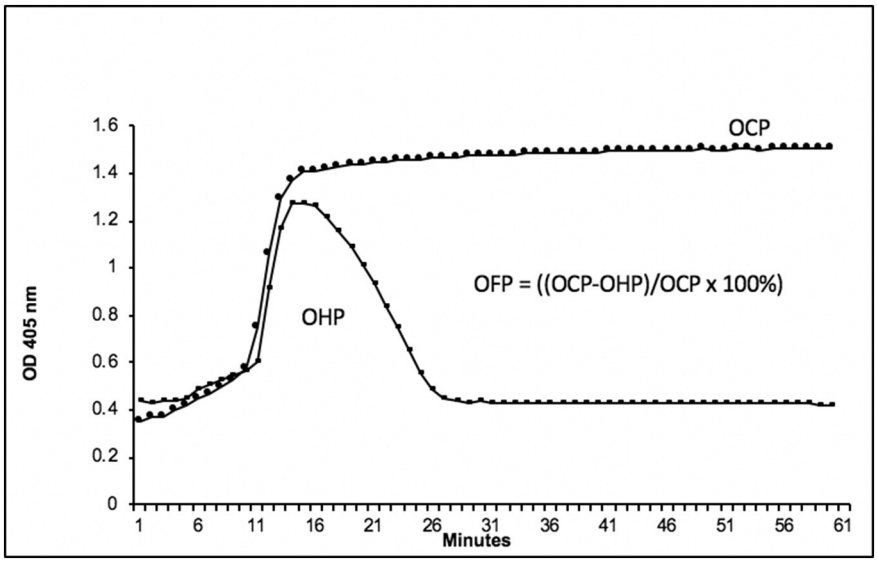

Fig. 3. The typical curves generated by the OHP assay to reflect the balance between the generation and proteolysis of fibrin. The overall haemostatic potential (OHP) curve is derived using the addition of tissue plasminogen activator (t-PA) and thrombin to plasma sample and the overall coagulation potential (OCP) curve is derived with the addition of thrombin only. The overall fibrinolytic potential (OFP) is calculated using ((OCP-OHP)/OCP x 100%).

Table 2 provides a summary of summary of the key studies evaluating thrombin generation and viscoelastic testing in acute and chronic cardiovascular disease as well as stroke.

3.3. Cardiovascular Disease (Chronic risk factors and/or prediction for future events)

Interestingly, the data for global coagulation assays collected at time of acute cardiovascular event differ from those in patients with chronic risk factors or at risk of future events. The exact relations between the levels of thrombin generation and risk of development of cardiovascular events over time remains unanswered, suggesting a more complex role of thrombin in cardiovascular disease and a possible compensatory mechanism. Plasma prothrombin fragment (F1 + 2), a measure of in-vivo thrombin generation, was measured in a population of 181 middle-aged subjects without clinically overt atherosclerotic disease [70]. F1 + 2 as measured by ELISA was found to be associated with carotid intima-media thickness, suggesting a relationship between thrombin generation and the development of atherosclerosis [70].

Previous studies have shown associations between risk of coronary heart disease and thrombin generation, albeit with mixed data. A large French prospective cohort study of over 9000 subjects aged > 65 years established a case cohort study in which they found that thrombin generation (ETP and peak height measured using CAT) positively correlated with risk of acute ischemic stroke particularly in women, but not coronary heart disease [59]. However, some studies have demonstrated a paradoxical inverse association or no relationship between thrombin generation and risk of recurrent cardiovascular events [47,58,60,62]. In the LURIC study, the event-free survival in individuals undergoing coronary angiography was lower in the individuals in the lowest ETP quartile (measured using the chromogenic INNOVANCE ETP assay) compared to those in the highest quartile ($p = .004$) [60]. In contrast, the multi-centre prospective PROSPER study (PROspective Study of Pravastatin in the Elderly at Risk) did not demonstrate association between thrombin generation (measured using CAT) and coronary heart disease although it did demonstrate that decreased thrombin generation is an independent predictor for stroke in elderly patients [61]. However, the studies mentioned, contrast other studies which found that increased thrombin generation correlated with the severity of coronary artery calcification [63–65].

Cardiovascular risk factors have also been shown to influence thrombin generation although the overall impact of risk factors on thrombin has not been fully elucidated. Obese patients have interestingly demonstrated more hypercoagulable clot formation compared to normal controls [69], an important observation to note given the role of obesity in cardiovascular disease. The Hoorn study measured thrombin generation using CAT in 586 individuals and found that total body fat, particularly central adiposity, was positively associated with higher peak height and ETP in women but not men, and the association was independent of traditional cardiovascular risk factors although attenuated by low-grade inflammation [71]. Another study measuring thrombin generation using Technothrombin-TGA before and 2 years after 36 patients underwent bariatric surgery showed significantly reduced peak thrombin (345 to 282 nM $p = .015$) and area under the curve (3962 to 3227 nM thrombin; $p < .001$) after weight loss, which may in part explain the mortality benefit of weight loss [72].

In summary, these thrombin generation studies suggest that the formation of thrombin (in-vivo) and potential to generate thrombin (ex-vivo) are altered in patients with CVD, although the risk associations remain to be determined given the variability in trend seen. Thrombin generation is dynamic, and both affects the coagulation cascade, as well as the anticoagulant pathways through triggering anti-thrombin, in addition to having a close interaction with the endovascular surface. Hence, one important question is whether a change in thrombin generation parameters may be reflecting a loss of equilibrium within the coagulation cascade, signalling an impending event.

The data for viscoelastic testing in chronic cardiovascular disease is predominantly from smaller observational studies with those having cardiovascular risk factors showing hypercoagulable viscoelastic test parameters. A study of 43 patients with stable coronary artery disease using ROTEM® showed differences in clot elasticity and formation in patients with and without diabetes mellitus [67]. This data is further affirmed by another prospective study of type 2 diabetes mellitus patients demonstrating similar hypercoagulable findings [68].

Data on OHP is less prevalent with some emerging smaller studies showing potential in this assay to detect hypercoagulability. OHP has demonstrated depressed OFP suggesting impaired fibrinolysis in patients with stable coronary artery disease [73,74]. Interestingly, the intake of omega-3 polyunsaturated fatty acid which has been inversely associated with the risk of cardiovascular events has been shown to reduce fibrin generation and peak thrombin in healthy subjects although the effect was less pronounced in those with CVD [75]. The addition of this assay to the aforementioned assays will yield a more complete assessment of the coagulation profile; both thrombin generation, as well as fibrin generation and fibrinolysis.

Table 1
Published studies investigating the utility of thrombin generation and viscoelastic testing in venous thromboembolism.

Author	Type of study	No of subjects (Case/control)	Study population	Type of thrombin generation assay	Study findings
Thrombin generation in VTE					
Dargaud et al. [27]	Observational	89/71	Patients with history of VTE	CAT	ETP in the VTE group was significantly higher than the controls and in the differences were significantly higher in the presence of thrombomodulin (TM). The VTE patients with identifiable prothrombotic risk factors had a higher ETP than those without. CAT in the presence of TM can detect prothrombotic phenotype with a sensitivity of 0.93 (95% CI 0.82–0.99).
Tripodi et al. [29]	Prospective cohort	403	Patients with and without previous VTE stratified to low, intermediate and high VTE risks	CAT	Odds ratio of relative risk of having high levels of endogenous thrombin potential (ETP) (with thrombomodulin (+TM)): - Low: 2.10 (95% CI 1.23–3.60) - Intermediate: 4.03 (2.18–7.45) - High: 4.96 (2.40–10.23)
Tripodi et al. [30]	Prospective cohort	254	Patients with first episode of unprovoked VTE	CAT	ETP (+TM) > 960 nM.min compared to < 563 nM.min➔ Hazard ratio (HR) 3.41 (95% CI 1.34–8.68). Peak (+TM) > 193 nM compared to < 115 nM ➔ HR 4.57 (95% CI 1.70–12.20).
Besser et al. [28]	Prospective cohort	188	Patients with first unprovoked/non-surgical provoked VTE	CAT	Patients with high ETP had increased rate of unprovoked recurrence (HR 2.9; 95% CI 1.3–6.6) compared to low ETP. Patients with unprovoked VTE and high ETP ➔ HR 4.0 (95% CI 1.3–11.8).
Van Hylckama et al. [33]	Case-control	360/404	Patients with previous VTE compared to controls	CAT	Individuals with ETP > 90th percentile (> 2109.0 nM.min) had 1.5-fold (95% CI 0.9–2.3) risk of first DVT. HR for recurrent thrombotic event 1.1 (95% CI 0.5–2.2).
Hron et al. [34]	Prospective cohort	917	Patients with first unprovoked VTE	Technothrombin TGA	Patients with thrombin generation < 400 nM ➔ RR 0.40 (95% CI 0.27–0.60)
Lutsey et al. [35]	Nested case-control	434/1004	Study of 2 population-based cohort studies	Technothrombin TGA	Those with thrombin generation above the median values were at 74% greater risk of VTE than the lowest quartile.
Eichinger et al. [31]	Prospective cohort	861	Patients with first episode of unprovoked VTE	Dade Behring	After adjustment for D-dimer, risk of recurrence remained high in patients with high ETP (HR 1.6; 95% CI 1.01–2.4)
Ay et al. [32]	Prospective cohort	1033	Patients with malignancies	CAT	Patient with elevated peak thrombin (75th percentile) had increased risk of VTE (HR 2.1; 1.3–3.3; $p = .002$). The cumulative probability of developing VTE after 6 months was significantly higher in patients with elevated peak thrombin than those with lower peak thrombin (11% vs 4%; p = .002).
Viscoelastic testing					
Rossetto et al. [36]	Observational	49	Patients with portal vein thrombosis (PVT) (with or without cirrhosis)	ROTEM	No significant differences in ROTEM profile (INTEM, EXTEM and NATEM assays) between PVT patients and normal controls. Maximum clot firmness (MCF) in FIBTEM was higher in non-cirrhotic PVT patients compared to healthy volunteers (19 mm vs 11 mm, $p < .05$)
Koopman et al. [37]	Observational	19	Patients with cerebral venous thrombosis (CVT)	TEG	No significant differences in TEG parameters between CVT patients and controls, neither between the subgroup of patients with a thrombophilic defect and controls.
Brill et al. [38]	Prospective cohort	983	Adult trauma patients admitted to a Level 1 trauma centre	TEG	Hypercoagulability based on admission TEG occurred in 582 (85.1%) patients. The lower-extremity DVT rate was higher in patients with hypercoagulable TEG than in those without hypercoagulable TEG (15.6% vs. 8%; $p = .039$).
Davies et al. [39]	Prospective cohort	67/72	Patients with lung cancer compared to age-matched healthy controls	ROTEM	Lung cancer patients showed increased maximum clot firmness, with strong correlation to fibrinogen. Six patients developed a VTE during follow-up and all had values for MCF at or above the upper limit of normal for EXTEM.
Hincker et al. [40]	Prospective cohort	313	Patients undergoing major noncardiac surgery	ROTEM	10 (3.2%) patients developed thromboembolic complications – these patients have increased maximum clot firmness (MCF) on preoperative ROTEM (EXTEM, INTEM) compared to those without. Odds ratio for patients showing hypercoagulable MCF (in 2 or 3 of INTEM/EXTEM/FIBTEM) developing thromboembolic events is 5.22 ($p = .01$).

Table 2
Published studies investigating the utility of thrombin generation and viscoelastic testing in cardiovascular diseases.

Author	Type of study	No of subjects (Case/control)	Study population	Type of thrombin generation assay	Study findings
Thrombin generation					
Ardissino et al. [58]	randomized, double-blinded multicenter trial	319	Patients with acute coronary syndrome (ACS) – measured at 0 (prior to discontinuation of iv heparin vs hirudin for 3–5 days), 1, 6 and 12 months	Prothrombin fragment 1 + 2 (F1 + 2)	U-shaped relationship between F1 + 2 and primary endpoint (Cardiac death or myocardial re-infarction) – intermediate levels (1.5–1.9 nM) has lowest risk. Higher (> 1.9 nM) and lower (< 1.5 nM) is associated with RR 1.56 [1.25–2.28] and 1.35 [1.11–1.86). No differences between F1 + 2 at 1,6 and 12 months.
Carcaillon et al. [59]	Prospective cohort study ➔ case-control	1177	Case = Patients with coronary heart disease and acute ischemic stroke (AIS)	CAT	High ETP and peak were associated with increased risk of AIS – HR 1.16 (95% CI 0.90–1.50) and 1.31 (1.01–1.69) for a 1 SD increase respectively
Smid et al. [47]	Case-control	171/185	Patients with first AMI – samples collected on admission, at 3 and 9 months after AMI.	CAT	Thrombin generation (TG) parameters were increased at day 0. Peak height stayed persistently increased. The lowest half of ETP values combined with upper half of D-dimer values were associated with recurrent ischemic events (Odds ratio 5.8 (1.1–30.7)).
Schneider et al. [60]	Prospective cohort	2196	Patients undergoing coronary angiography	INNOVANCE ETP F1 + 2	Highest HR for CVD in 1st ETP quartile and lowest in the 4th ETP quartile (HR 0.61 (0.44–0.84), with similar trend seen in survival analysis.
Loeffen et al. [61]	Prospective cohort	4932	Subjects (70-82y) with pre-existing vascular disease or risk factors	CAT	Baseline TG decreased in subjects with incident stroke compared to those without (normalized peak height 71.1 vs 82.3% [HR 0.71 (0.60–0.85)]; ETP 79.1 vs 87.0% [HR 0.68 (0.58–0.79)]). Baseline TG was comparable in CHD and those without; only increased normalized peak height (at 1pM, not at 5pM TF) was significantly associated with incident CHD (HR 1.17)
Loeffen et al. [44]	Prospective cohort	104/42	Patients with chest pain suspected for acute coronary syndrome (ACS)	CAT	Peak height significantly increased in ACS patients (148 vs 122 nM) but diminished ETP reduction (32% vs 41%) compared to non-ACS. Peak height on admission is predictive of recurrent cardiovascular events (OR 4.9 [1.2–20.9]).
Gremmel et al. [62]	Prospective cohort	104	Patients undergoing infrainguinal angioplasty and stenting for lower extremity artery disease	Technothrombin TGA	Low thrombin generation potential was associated with an 11.7-fold (95% CI 1.4–97.6; $P = .02$) increased risk of future atherothrombotic events
Tosi et al. [63]	Observational	775	Patients with ($n = 605$) or without ($n = 170$) angiographically proven CAD	INNOVANCE	Peak height and ETP were higher in CAD patients, than CAD-free. After adjustment for risk factors, only ETP levels remained significantly associated with CAD (highest vs lowest tertile OR 2.61 [CI 1.14–5.99]). The authors concluded that increased ETP is characteristic in patients with clinically stable CAD
Valente-Acosta et al. [64]	Observational	95	Patients having diagnostic coronary angiogram due to stable chest pain ➔ 63 patients had CAD +	TAT	CAD patients show higher TAT (40.76 μg/L vs 20.81 μg/L; $p = .002$). TAT was associated with severity of CAD (36.17 in patients with bivascular obstruction vs 31.8 in trivascular)
Orbe et al. [45]	Cross-sectional study	95/15	Patients with chronic stable CAD ($n = 35$) and patients after acute MI ($n = 60$)	Technothrombin TGA (measured between 3 and 11 months after initial diagnosis)	Acute MI vs stable CAD: increased velocity index and peak thrombin
Rooth et al. [49]	Prospective cohort	205	Patients with ischemic stroke or transient ischemic attack within 2 weeks and no atrial fibrillation	CAT	Peak thrombin was variable among stroke patients but significantly higher compared to controls ($p < .001$) and tended to be highest in patients in whom paroxysmal atrial fibrillation was subsequently documented.
Balogun et al. [48]	Observational	170/71	Patients with stroke within 48 h of symptoms onset (baseline) and in the second week	CAT	ETP and peak thrombin did not predict hypercoagulability after acute ischaemic stroke

(*continued on next page*)

Table 2 (*continued*)

Author	Type of study	No of subjects (Case/control)	Study population	Type of thrombin generation assay	Study findings
Borissoff et al. [65]	Observational	295	Patients with stable chest pain, assessed with coronary computed tomographic angiography	CAT TAT	TAT was higher in patients with CAD compared to those without (p = .004). TAT predicted the degree of coronary artery calcification: mild (odds ratio (OR) 1.56; p = .006), moderate (OR 1.56; p = .007), severe (OR 1.67; p = .002). ETP values were similar between the CAD and non-CAD group although in the former, it was significantly associated with the degree of CAD (r = 0.271, p < .001).
Viscoelastic testing					
Dimitrova-Karamfilvo et al. [51]	Prospective cohort	35/34	Patients with thrombotic complications (including 11 MI, 4 AIS) presenting to hospital	ROTEM	Increased clot formation time (CFT), a-angle, maximum clot firmness (MCF) and thrombodynamic potential index were seen in hypercoagulable patients
Kleinegris et al. [66]	Case control	40/40	Patients with peripheral arterial diseases (PAD)	ROTEM (EXTEM)	Faster clot formation (CFT) and increased clot firmness (MCF) compared to controls
Hincker et al. [40]	Observational	313	Patients undergoing major non-cardiac surgery	ROTEM	Patients with thromboembolic complications had significantly lower CFT, higher alpha angle and MCF. INTEM clot firmness at 10 min (A10) was the best predictor of thromboembolic complications.
Rafiq et al. [52]	Prospective observational	200	Patients undergoing coronary artery bypass grafting	TEG	87/200 (43.5%) of patients showed hypercoagulable TEG. There was a significant difference in 30-day combined event rate of MI, stroke and mortality (17.2 vs. 6.6%, P = .019).
Feuring et al. [67]	Case control	17/26	Patients with type 2 diabetes mellitus (DM) and non-diabetic patients with known coronary artery disease	ROTEM	Mean maximum clot elasticity (MCE) in DM patients was significantly higher using EXTEM (233.6 vs 186.7; p = .03) and INTEM (234.4 vs 190.8; p = .053)
Yurekli et al. [68]	Case control	51/40	Type 2 DM patients (27/51 patients had known diabetic vascular complications)	ROTEM	INTEM-CFT (72.2 vs 56.3 s; p = .007) and EXTEM-MCF (66.7 vs 64.3 mm; p = .029) were significantly higher in the diabetic groups compared to controls respectively.
Campello et al. [69]	Case control	80/80	Subjects with BMI ≥ 25 - > 40 kg/m^2	ROTEM	INTEM-MCF (70 vs 64 mm; p = .027) and EXTEM-MCF (69 vs 64 mm; p = .002) were significantly increased in III-degree obesity (BMI > 40) compared to normal controls.

Cardiovascular disease (Chronic risk factors and/or prediction for future events).

4. Challenges and controversies

The current available literature of global coagulation assays and thrombotic disorders is difficult to amalgamate despite the undeniable role of thrombin in thrombotic disorders. Thrombin has multiple roles in the coagulation system, including activation and amplification of coagulation, an anticoagulant role via generation of activated protein C and regulation of fibrinolysis by thrombin-activatable fibrinolysis inhibitor (TAFI). All of these aspects are difficult to capture in a single assay and may in part explain the contradictory results seen in various studies due to different methodologies of testing. In addition, the lack of correlation between in vivo and ex vivo thrombin generation measurements, absence of assay standardisation and lack of agreement on disease-specific reference ranges are among reasons hampering the use of thrombin generation in routine clinical practice. However, furthering our understanding of the multiple roles of thrombin may provide better understanding of cardiovascular disease and venous thrombosis.

Pre-analytical issues also remain a major challenge, particularly with thrombin generation. Issues include, collection process, processing and storage of platelet-poor or platelet-rich plasma, transport of samples, availability of various commercial assays, different tissue factor and phospholipid concentrations, analytical variables such as reference plasma and differing study protocols between laboratories all contribute to the poor reproducibility of the results [11]. Currently, there is no “ideal” disease-specific tissue factor concentrations nor recommendations about addition of thrombomodulin which may explain the variability in data in various disease states. Previous studies have found that addition of thrombomodulin can affect thrombin generation parameters in certain conditions such as liver disease and haemophilia [76–78] – Protein C, a naturally occurring anticoagulant must be activated by endothelial receptor thrombomodulin to exert its action and hence addition of thrombomodulin to the assay allows the assay to more closely mimic in vivo coagulation [79]. Current efforts to improve standardisation of the measurement of thrombin generation have been previously discussed. To improve the reproducibility of these assays it is recommended that future studies should adhere to the recommended practices.

In terms of viscoelastic testing, the use of fresh whole blood has contributed to difficulty in standardisation due to the lack of capacity to share samples between laboratories for external quality assurance. Previous studies have showed that the precision of the tests varied greatly and hence regular external proficiency testing is required [80]. In addition, TEG® and ROTEM results are not cross comparable further diluting the pool of knowledge of viscoelastic testing in thrombotic disorders.

Another crucial piece of the puzzle in providing a holistic assessment of cardiovascular and thrombotic risk, is the assessment of the endothelium and its function. Current global coagulation assays do not provide an assessment of this, and incorporation of other novel biomarkers such as P-Selectin, micro-RNA, lipoprotein-associated phospholipase and high- sensitivity C-reactive protein may also reflect other aspects of the development of atherosclerosis [81] and is important as we move forward in improving cardiovascular and thrombotic risk assessment.

Nonetheless, this review has highlighted the large and increasing body of evidence for the role of global coagulation assays in identifying hypercoagulable state. The next step would be to investigate in if these assays, both individually or in combination, can be correlated to the clinical occurrence of cardiovascular and thromboembolic events and hence allowing for early prophylaxis to reduce the associated morbidity and mortality.

5. Conclusion

Global coagulation assays provide a novel insight into the in-vivo coagulation profile of various diseases which are not provided by standard coagulation testing or clinical surrogate measures. While most of the evidence and clinical applications are in bleeding disorders, there is a large body of data that suggest these assays may have an important role in helping to improve risk prediction models for thrombotic disorders. The challenge now is to translate these observational data into clinical practice with larger longitudinal prospective studies to evaluate the predictive capacity of these assays for future thrombotic events.

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