Peer reviewed **REVIEW**

BLEEDING DISORDERS: FUNCTIONALITY OF GLOBAL HAEMOSTASIS ASSAYS IN ATTAINING CLINICAL OUTCOMES: A REVIEW

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SUMMARY

Many tests used to evaluate haemostasis correspond to artificially created environments. These include some of the traditional screening tests, which were established through understanding the coagulation cascade for example, the prothrombin time (PT), the activated partial thromboplastin time (aPTT) and Fibrinogen estimation. And as such, they have contributed greatly to our current knowledge of the haemostatic process. Unfortunately, these traditional laboratory tests have their limitations. Open to debate for example, is their ability to supply enough information timeously (? laboratory turnaround time) in order to diagnose and treat patients according to their phenotype.

Anarchetypical shift in haemostasis measurements using global haemostasis tests that determine the complete process in a more physiological and all-inclusive way are consequently being reevaluated.

These tests include:

- The viscoelastic tests (ROTEM/TEG) improve the treatment of acute haemorrhage, decreasing the transfusion burden, as for example in cardiac surgery and with it a more effective use of blood and blood products, thus lowering overall costs. They also provide rapid, comprehensive and accurate identification of an individual's haemostasis state, in the laboratory or in the context of near-patient testing as point-of-care instruments. This allows clinicians to drive personalised, clinically and economically sound treatment and monitoring decisions.
- Thrombin generation measurement; although expensive is elegant. And as well as having an important role in managing haemorrhage it may be sensitive to various factors that contribute to hypercoagulation. The test may also be of value in discriminating between the different phenotypes within the population of severe haemophilia A (HA) patients. It is here to stay.
- The clot waveform analysis may be less well known (i.e. presently used as a research tool). It shows promise in staging sepsis patients, having a role in the early detection of disseminated intravascular coagulation (DIC) and also in the diagnosis and treatment-monitoring of haemophiliac patients.
- Flow perfusion chambers which while they may be the 'ultimate' global assay has a considerable way to go in order to become of use clinically.

Although global haemostasis tests have been available for some time, there is a renewed awareness in their potential use for assessing both normal and abnormal haemostasis. Initial data suggests that they can provide more detailed information regarding the overall haemostatic process and its disorders. However, many challenges still remain such as with the analysers themselves. These involve their trademarked technology, their expense and finally their availability.

All four methods still need more background standardisation regarding reagents, methodologies and finally interpretation of results. Much more additional information is required involving all the parameters measured and by inference, their subsequent clinical applications. The present review describes these various global haemostasis tests giving some background information, as well as clinical outcomes and lastly some information on their limitations.

INTRODUCTION

In humans, the haemostatic processes exist as defence mechanisms against both bleeding and thrombotic tendencies. Haemostasis is a normal physiological process that maintains blood in a fluid clot-free state while inducing a haemostatic plug at the site of vessel injury. Thrombosis is the inappropriate activation of haemostatic mechanisms in uninjured vessels. Together these complex processes are dependent on a number of well-orchestrated interactions between the following: the endothelium, platelets and finally the coagulation and fibrinolytic mechanisms.

The complexity of this system is evident from the variable responses of patients when haemostasis is challenged. This can occur when disturbances in the balance between the procoagulant system (amplification) and the anticoagulant system (natural inhibitors) result in bleeding or thrombotic diseases.[1]

Haemostasis

Haemostasis when threatened, enables the individual (no matter what genus) to: seal injured blood vessels; keep the blood in a liquid condition and to remove blood clots after the repair of vascular integrity. The Greek philosopher Plato two millennia ago[2] described 'that the blood formed fibres once it left the heat of the body'. Amazingly, he was the first to perceive the term fibrin, which today refers to a significant blood clotting protein that comprise those fibre structures.[1,2] Hewson[3] in the 1770s isolated the source of these fibres to what he named the

'coagulable lymph', the straw-coloured liquid part of the blood we now call plasma .It was not until 1865, when platelets were discovered as well as their crucial function in the overall haemostatic process.[4]

In 1905, Morawitz[5] created the first coagulation model in which thromboplastin, now known as tissue factor (TF), was released by damaged vessels to convert prothrombin (II) into thrombin (IIa) in the presence of calcium (Ca^{++}).[6]

In the formation of the blood clot, thrombin (IIa) converts soluble plasma fibrinogen (I) into insoluble fibrin. This 4-clotting factor model however, could not fully explain the multifaceted process of coagulation

The overall response to a threat to the haemostastic process can now be divided into primary and secondary haemostasis.

- Primary haemostasis consists of vasoconstriction, platelet adhesion and activation, with the ultimate formation of the platelet plug.
- Secondary haemostasis involves serine protease zymogens (coagulation factors) which interact with their co-factors in a tightly regulated sequence to form cross-linked fibrin that helps in stabilising the initial unstable platelet plug. This is an exceptionally ordered process and is the result of numerous reactions.[7,8]

In the 1960s 'the coagulation cascade' was identified and described by two separate groups as the processes involved in the secondary haemostasis response.[8-10] Herein, one of the essential features of this cascade is the sequence of the number of steps required in its activation. It is an extremely complex process and is reliant on both negative and positive feedback loop mechanisms to ensure that the haemostatic response is in balance, i.e. without haemorrhage or thrombosis occurring.

Coagulation Cascade

The coagulation cascade consists of a number of clotting factors, which have been organised into extrinsic, intrinsic and common pathways,[1,11,12] and are of importance in the laboratory when assessing a defect in the haemostatic process (see Figure 1).

While it is commonly referred to as a cascade, in reality these pathways are not separate entities but are better interpreted as a network, involving numerous interlinked reactions that are closely involved in the regulation of this essential physiological process.

Abnormalities in the haemostatic process have been traditionally evaluated using plasma clotting times, such as the PT and the aPTT.[13]

These tests are dependent on the initiation of the process of fibrinogen to fibrin and not on its speed or total extent. Factor assays based on these tests have identified the different coagulation disorders including for example, haemophilia A (HA).[14,15]

Unfortunately there are a number of limitations to these tests. These include the following: all are performed under unreal physiological conditions that split the process of coagulation into artificial segments. In general, they don't evaluate the potential effect of different parts of the haemostatic framework.

Factor assay's performed utilising either the PT or aPTT are limited by their general affectability at low levels.[16] For instance, the plasma from a few patients with extreme HA can produce thrombin.[17] The accurate premise for this is not known, but it may be because of the equalisation of levels of both distinctive procoagulant and anticoagulant proteins in the blood.[18]

Global Haemostasis Assays

In routine clinical practice the PT and aPTT tests are used to evaluate a patient's haemostatic potential and prolongation of either or both of these assays is interpreted as evidence of an anticoagulated state. However, there is some debate about how useful these tests are in assessing haemostasis in chronic liver disease,[19,20] which could further extend to their usefulness in assessing haemostasis in congenital haemostatic defects.

In particular, critically ill patients who have prolonged routine coagulation screens, but do not show any overt signs of bleeding,[21] these screening tests (i.e.PT and aPTT) do not appear to reflect the actual bleeding risk.[22] In this regard other assays are required to determine a patient's haemostatic potential.

Global haemostatic tests have the ability to contribute additional information in terms of assessing bleeding,[21] particularly in

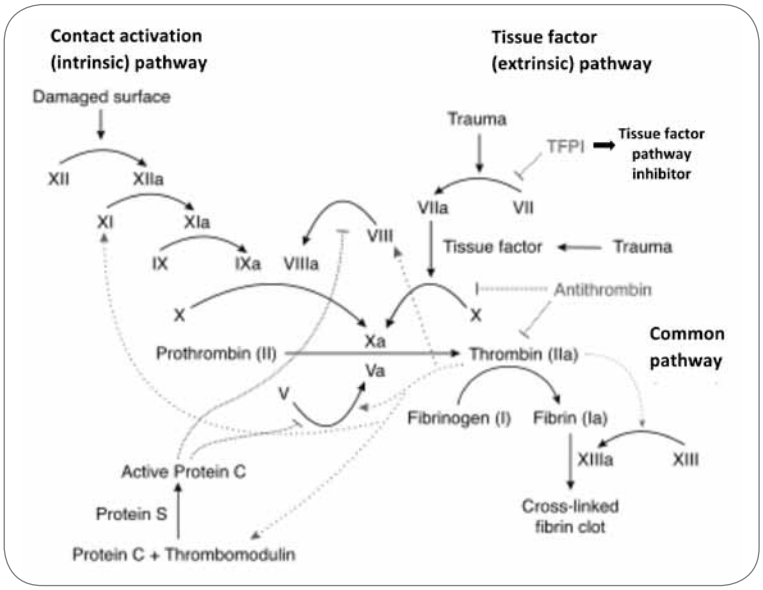

Figure 1: The coagulation cascade with arrows for positive and negative feedback loops. Adapted from.[1]

those patients known to have a poor response to a haemostatic challenge.

In vitro investigations have demonstrated the phenomenon, that during the initiation phase of coagulation, very low levels of thrombin are needed for the formation of the fibrin clot, which in fact is visible when only 3-5% of the total amount of thrombin has been produced.[23] Therefore, the conventional tests that use these fibrin-clotting endpoints are limited in their usefulness in assessing haemostasis, particularly in individuals that have impairment in their blood clotting capability as for example, HA patients.

Low concentrations of tissue factor and the rate of thrombin generation are essential in the formation of a stable fibrin clot.[24] This essential process is highly regulated through feedback mechanisms in which thrombin itself plays an important role, in controlling its own production and destruction.[25]

Model coagulation tests should be easy to perform, in that they should be able to obtain reliable and robust results quickly. Overall, all assays aligned with the haemostatic mechanism should give accurate estimates of thrombotic risk as well as the risk of bleeding.

To-date there is no widely available standardised tests that can quantitatively assess the overall haemostatic potential of the blood.

Global haemostasis assays are in vitro models of haemostasis and thrombosis that aim to mimic most important aspects of the physiological and pathological system of haemostasis, however it should be highlighted that no single test can fulfill this requirement.

These tests include the following: thrombin generation tests/assay (TGT/TGA, CAT),[26,27] thromboelastography (TEG, ROTEM),[28,29] the clot waveform analysis (CWA)[30,31] and finally flow perfusion chambers.[32]

THROMBIN GENERATION TESTS

Calibrated, automated thrombogram (CAT)

Thrombin generation is one of the best developed and tested global assays of haemostasis. Hemker et al established the conceptual methodology of the CAT method.[33,34] Using the CAT system, changes in the amount of thrombin generated in the presence of fibrin(ogen) following a given tissue factor stimulus, can be visualised continuously using a thrombin sensitive fluorogenic substrate. The definitive thrombin generation curve has a waveform form, from which a series of quantified parameters can be calculated i.e. (lag time, thrombin generation velocity, peak thrombin concentration, time-to-peak thrombin and endogenous thrombin potential (ETP),[35] Figure 2.[36])

Commercially available semi-automated analysers, can be used to perform a thrombin generation assay (TGA) that produces the definitive thrombin generation curves that may prove to be of use in screening defects in haemostasis.[37] Such analysers rely on fluorogenic or chromogenic principles. Fluorogenic assays are supplied by Stago, Kat-Medical, Florida North, South Africa (S.A.) and Technclone. Chromogenic assaysare supplied by Dade Behring and other customised tests such as the Novel Haemostasis Assay from the Radbund University Medical Centre, Nijmegen, The Netherlands.

Correlation of thrombin generation with clinical phenotype

After its initial use in research the TGA showed increased thrombin generation in thrombophilic states such as venous thrombosis due to deficiency (e.g. anti-thrombin (AT), protein C or S deficiency) as well as due to activated prothrombin complex concentration (APCC)-resistance and the antiphospholipid syndrome.[38,39]

TGAs have also been used for laboratory characterisation of a variety of bleeding disorders. HA is the best-characterised haemostatic abnormality demonstrated by thrombin generation profiles, as its rate-specific characteristics have been reported to demonstrate and reflect the clinical heterogeneity of the disease.[40-43] The TGA may describe the bleeding tendency and therefore the risk of bleeding better than the traditional tests. However, what is still a matter of debate is the feasibility of using the TGA to monitor bypassing agent therapy in haemophiliacs with inhibitors in order to improve clinical outcomes.[44-47]

Additionally, increased thrombin generation is associated with

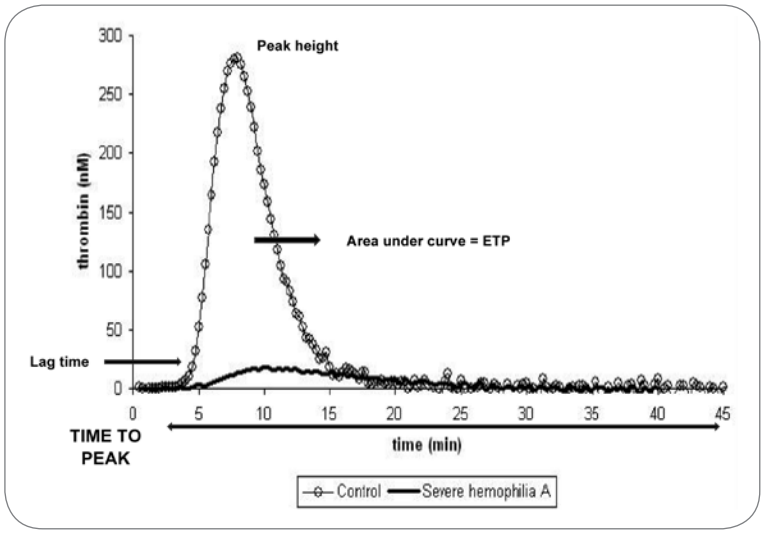

Figure 2: Normal thrombin generation curve. The major parameters (lag time, time to peak thrombin generation, peak thrombin generation and endogenous thrombin potential) of the thrombin generation curve as shown. As reference, a patient with severe HA, which shows that all three parameters are severely affected. (Adapted from[35])

arterial thrombosis such as ischaemic attack and with the acute coronary syndromes.[48,49] In these situations assessment of the TGA might be of help in guiding therapy with antithrombotics whilst trying to avoid bleeding.

Unfortunately, there are some disadvantages of the TGA assay. One of the major drawbacks is its unsuitability in emergency cases due to the duration time of the test. Another major issue of concern is in the standardisation of the TGA for broad clinical use. This lack of standardisation is due to the large variance caused by pre-analytical variables and the lack of reference ranges for specific conditions, which impedes its approval as a routine clinical tool.[50,51]

Viscoelastic assays (thromboelastography/thromboelastometry)

The most direct way to characterise clot formation is by rheometry, which is unaffected by optical phenomena and is easily applied to whole blood.

A number of rheological approaches exist, but perhaps the best studied is thromboelastography (TEG). It detects changes in the viscosity and elasticity during the clotting process of whole blood and has been used to study haemostatic abnormalities [52,53] and access whole-blood clotting dynamics. The measurements are displayed as a graph, from the beginning of clot formation to fibrinolysis. Figure 3 illustrates the association of TEG with routine screening tests of haemostasis.

The method is relatively simple and has been used extensively for evaluating the amount of fibrin polymerisation and general clotting function. As it can be performed on whole blood, it is used widely during surgical procedures where there is a high risk of bleeding.[54,55] The TEG is analogous to clotting in vivo, where cellular blood components (e.g. platelets) contribute significantly to thrombin generation at the site of bleeding.

The TEG uses a sample cuvette filled with anticoagulated whole blood to measure kinetics, strength, stability and the tensile strength of the eventual blood clot. Analysers from Haemonetics®, including the TEG 6s, are available from Barker Medical (Pty) Ltd, Broadacres, Johannesburg S.A. TEG 6s assays are performed in automatically loaded microfluidic cartridges (as opposed to a sample cup) designed for simultaneous performance of multiple TEG assays.

A pin suspended from a torsion wire is lowered into a cuvette and the cup is rotated over a set period of time. Torque from the rotating cup is transmitted from the pin to a recorder. The torque increases exponentially as the clot forms (see Figure 5).

The whole coagulation profile is then displayed as a thromboelastograph. The overall shape of the graph is determined by the blood viscoelastic property and the functional activity of the blood components. Sample TEG tracings are shown in Figure 4, which illustrates normal and abnormal results.

The method has significant disadvantages however, including instability—a freshly collected blood sample is required for analysis—poor reproducibility is also of concern.

While it is sensitive to defects in platelet aggregation and fibrinolysis, it is not sensitive in assessing platelet adhesion. There are still some trepidations concerning the standardisation of these assays. Of late a study on quality control and assurance showed a wide variation of TEG results between different institutions when compared to plasma sample analysis. Based on these results a working group was established in order to try and normalise the variability of the test.[56,57] More recently, a variation of TEG, the rotating thromboelastometry (ROTEM), has been developed.

Rotating Thromboelastometry

Rotating thromboelastometry is a whole-blood clotting test that measures the interactions of coagulation factors and their inhibitors with cellular components (i.e. red blood cells, platelets) during clotting and fibrinolysis over 60 minutes (see Figure 6). The test uses similar principles to TEG, originally developed by Hartert[58] in which the pin oscillates instead of the cup and assesses the viscoelastic properties of clot formation under low shear stress.[59] As with TEG the main drawback of the method is its unsuitability in the acute setting due to its long sample processing time.

Clot waveform analysis (CWA)

CWA is founded on one of the well-established screening tests; the aPTT assay. Braun and co-workers were the first to report this technique after assessing the aPTT and PT with light transmission.[60] A graph is created over a protracted period of time instead of a standard clotting time as for the aPTT. This graph or tracing against time should indicate the whole process of clot

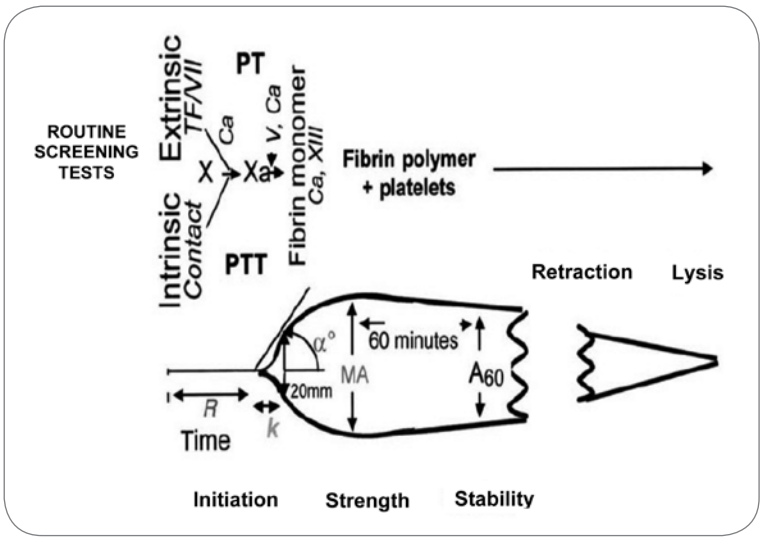

Figure 3: The relationship between a normal TEG and above the normal screening tests of haemostasis, the PT and aPTT (intrinsic-extrinsic). The PTT and aPTT only provide information on the initiation of clot formation, which is similar to that given by the R of the TEG. Adapted from.[53]

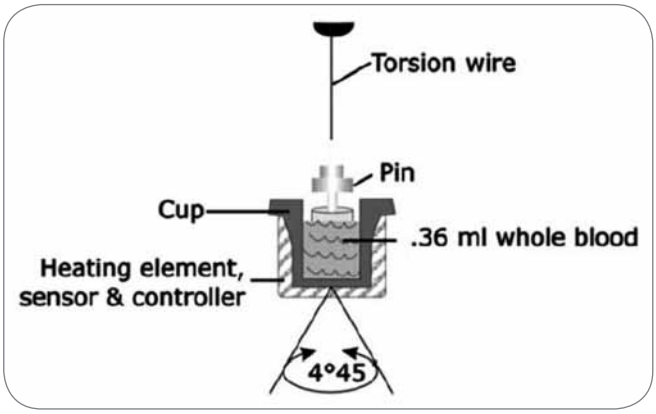

Figure 4: A representation of a TEG device where a pin suspended from a torsion wire is immersed in a cup of whole blood. Note that the cup is held in a heating block and is continually oscillated through 4-45o every 5 sec. Changes in the viscoelastic strength of the clot are directly transmitted to the torsion wire and are detected by an electromagnetic transducer.[53]

formation terminating inclot lysis (see Figure 7). The necessity of standardisation requires that some specific reagents are required that do not interfere with light transmission/absorbance.[61] Despite this need for standardisation, there are two possible clinical applications for CWA.

CWA has the potential to monitor patients with disseminated intravascular coagulation (DIC), a disease, frequently seen in critically ill patients. DIC can also be diagnosed with high specificity (97.6%) and sensitivity (98%) using CWA as a global assessment tool.[62,63] CWA may also be able to detect the DIC much earlier than when using conventional clotting assays in up to 19% of cases examined. It is therefore recommended by the guidelines for diagnosis and treatment of DIC.[64]

CWA seems sensitive to even mild deficiencies (FXII, X, IX, VIII, VII, V and II). Taking this into account the assay gives information on haemophiliacs, where it might help to distinguish between haemophilia A and B.

CWA also gives information on the clinical phenotype with regard to bleeding tendency. And as such it can be used to monitor the effectiveness of Factor VIII infusions in HA patients as well as bypassing reagents such as APCC or rFVIIa in patients demonstrating inhibitors.[65,66] CWA analysers are available from Siemens S.A.

A few authors questioned the use of CWA in assessing critically ill patients suffering from sepsis. They subsequently demonstrated that the severity and the prognosis of the disease can be predicted using this global assessment tool.[67,68]

The CWA may be relatively inexpensive and easy to perform but unfortunately there are some disadvantages inhibiting its wide use in a clinical setting.

There are only two systems currently that are able to measure the light transmittance or absorbance tracings. It should be possible however, to create the necessary graphs from analysers that use similar principles after updating their software.

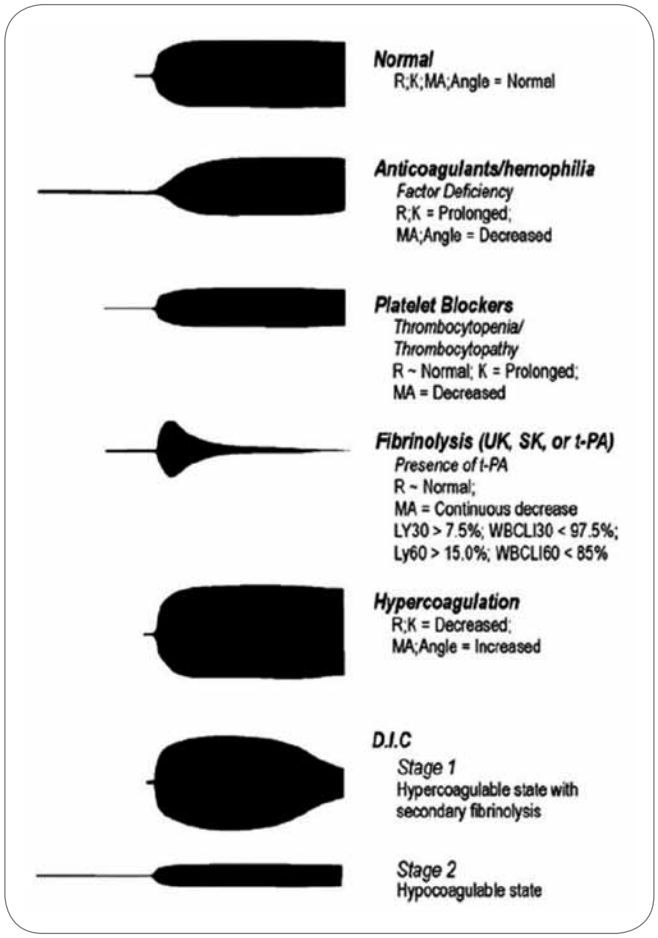

Figure 5: A representation of a normal TEG output as compared to those seen in abnormal coagulopathies.[53]

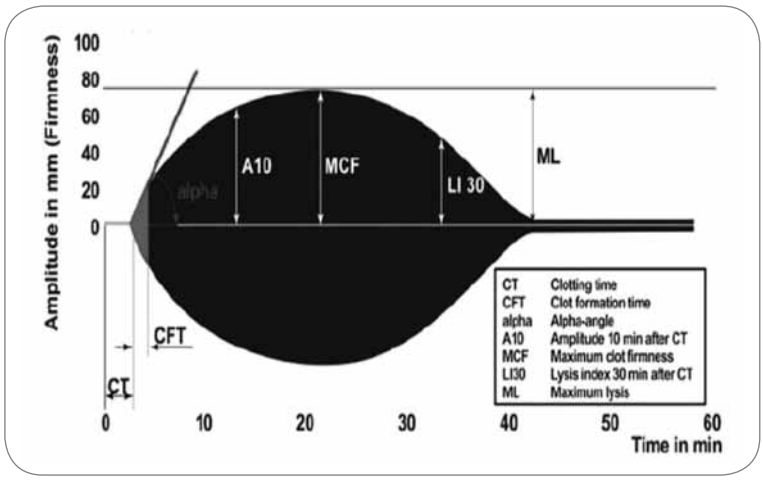

Figure 6: An illustration of a ROTEM output showing clot initiation, propagation, stabilisation and lysis.[53]

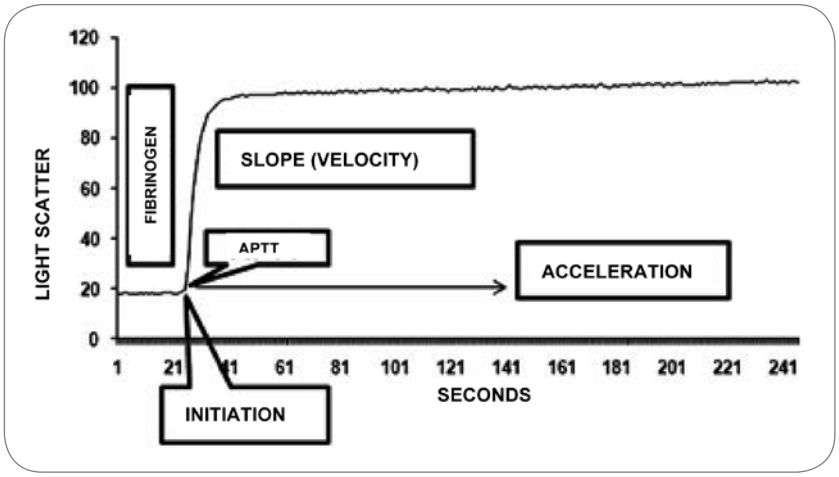

Figure 7: aPTT clot curve and its derivatives. The aPTT curve shows the changes in light scatter against time in secs. Adapted from.[61]

One of the major obstacles in using CWA is in the use of totally transparent reagents that do not interfere with the light beam.
Obviously, samples with, lipaemia and/or hyperbilirubinemia and/or haemolysis will cause interference and as such the test results will be invalid.[61]
Another area of concern is in the lack of experience with the CWA assay where the parameters are relatively unknown. Additionally, there is very little information in the literature in regard to clinical validation.

Flow or perfusion chambers

There has been a great deal of progress made over the past two decades regarding the development of flow chambers and micro capillaries to monitor and study thrombus formation in vitro. Platelets play a vital role in the occlusion of injuries to the vascularity in order to arrest and prevent profuse bleeding. Their ability to rapidly adhere to the subendothelial matrix proteins (primary adhesion response) and then to subsequently form a stable plug reinforced by the coagulation system is central to overall haemostatic function.[69,70]

Improvements in microscope technology, with high-resolution cameras and the high capacity to store digital images makes it possible to study in 'real' time the formation of platelets-and-fibrin thrombi in flow chambers. Potentially this is the 'ultimate' global assay in that it is able to determine both platelet function/s (including adhesion, aggregation and finally procoagulant activity) and blood coagulation.
As such an increasing number of laboratories are using custom-made or commercial flow chamber devices for (semi-routine) testing of impaired or increased platelet function under flow conditions to detect for example, hypercoagulation changes in blood.[71-75] Unfortunately, there are very few clinical studies and the standardisation status of these various chambers is sadly lacking.[76]

CONCLUSION

Unfortunately, the traditional coagulation tests based on clot formation do not provide all the information that clinicians may require, in order to diagnose and treat haemostatic defects effectively.
Global haemostasis tests such as TEG/ROTEM, TGA and CWA have the potential to reassess the haemostatic mechanism from new perspectives. The TEG/ROTEM tests have been shown to be meaningful in the management of acute haemorrhage. Data from the literature have demonstrated that TEG is part of the whole picture of patient blood management strategies and can effectively:

- Reduce blood products usage (FFP, RBC, platelets, cryoprecipitate).[78]
- Improve adequate use of expensive pharmaceuticals (rFVIIa, fibrinogen).
- Allow for the stratification of the risk of thrombosis.
- Reduce hospital stay related costs (ICU length of stay, re-exploration, adverse reactions to blood transfusions, blood product waste, thrombotic episodes).[79]
- There is a growing body of evidence supporting the utility of TEG in predicting bleeding.[80]
- However, the utility of TEG in guiding the management of post-operative bleeding has been the main driver of adoption.[81]
- Additionally, TEG can identify hypercoagulable states in conditions such as acute coronary syndrome, trauma, obstetrics, liver transplant, cancer and stroke.[82-84]

The TGA is of value in thrombotic risks (venous and arterial) where the sensitivity to hypercoagulation is definitely higher than that of the traditional INR and aPTT tests. Additionally it may be a significant instrument in haemostatic therapy.
CWA although less well known demonstrates increasing evidence that it may be of value in improving the diagnosis and treatment of DIC, sepsis and haemophilia.

However, there is one major problem that complicates the use of global assays in a clinical setting and that is their lack of standardisation. Results from a diversity of papers cited in this review highlight the difficulties in interpretation and reproducibility throughout the spectrum of global assays that are currently available.
Attempts at standardisation through independent groups as well as the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis are ongoing.
The present efforts at standardisation of the more established global assays such as the TEG and TGA,[50,51,77,79,83] give hope that there could be major paradigm shifts in the way we assess and evaluate haemostasis disorders in the future.

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